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Mécanisme et conséquences de la répression de DKK1 par la ténascine-C, une molécule du microenvironnement tumoral

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Mechanism and consequences of DKK1 downregulation by the tumor microenvironmental molecule tenascin-C

Background: The tumor microenvironment plays a central role in driving cancer progression. Tenascin-C is a major component of the tumor specific extracellular matrix (ECM) and its expression has been linked to tumor angiogenesis, metastasis and is a marker of bad prognosis. However, it is not well understood how TNC promotes tumor progression.

Based on a microarray approach of T98G glioblastoma cells cultured on FN or FN/TNC coated dishes the Wnt signalling inhibitor DKK1 had been identified to be strongly downregulated by TNC, suggesting DKK1/Wnt signalling as a potential mechanism for TNC induced tumor progression.

Objectives: The objective of this thesis was to elucidate the effect of DKK1 downregulation by TNC on Wnt signalling in tumor cells. Furthermore, this study was extended to the analysis of DKK1 expression and Wnt signalling activity in stromal cells in the presence of TNC. Additionally I aimed in identifying the mechanism(s) implicated in DKK1 downregulation by TNC.

Results: I demonstrated that TNC downregulates DKK1 in several tumor cells *in vitro* and that DKK1 downregulation was linked to enhanced Wnt/β-catenin signalling. In addition, stromal cells, including endothelial cells, pericytes and cancer-associated fibroblasts, exhibited reduced DKK1 levels in the presence of TNC. While TNC induced expression of the Wnt/β-catenin target gene *Axin2* in endothelial cells, *Axin2* remained unchanged in pericytes.

Second, I demonstrated that TNC reduces DKK1 promoter activity. Reduced stress fibre formation in the presence of TNC was identified as a major mechanism contributing to DKK1 downregulation. *DKK1* gene expression was inhibited upon stress fibre disruption and induced upon enforcement of stress fibres. The activity of the actin-regulated SRF co-transcription factor MKL1 was found to be reduced in the presence of TNC. My results indicate that TNC regulated MKL1 function maybe one, but not the major mechanism of DKK1 regulation by the actin status and that other factors presumably regulated by actin stress fibres are involved.

Conclusion: Enhanced Wnt signalling activity downstream of TNC-induced DKK1 downregulation might be a major mechanism by which TNC promotes tumor progression. In addition, for further studies it will be interesting to analyse whether the TNC-induced DKK1 downregulation in stromal cells impacts on β-catenin-dependent and/or β-catenin-independent pathways. A strong DKK1 downregulation by TNC in tumor and stromal cells may create an environment in tumor tissue that renders cells responsive for Wnt and, other DKK1 repressed signalling. Furthermore, this study discovered a novel mechanism of regulating the Wnt inhibitor DKK1 by the integrity of the actin cytoskeleton.

MECANISME ET CONSEQUENCES DE LA REPRESSION DE DKK1 PAR LA TENASCINE-C, UNE MOLECULE DU MICROENVIRONNEMENT TUMORAL

I. Introduction

Les interactions entre les cellules tumorales et leur microenvironnement jouent des rôles instrumentaux durant la progression tumorale. Le microenvironnement tumoral est constitué de cellules stromales associées à la tumeur, de facteurs solubles tels que des cytokines et des facteurs de croissance ainsi que de protéines de la matrice extracellulaire, celles-ci incluant la ténascine-C (TNC) (1, 2). La TNC est exprimée au cours de l'embryogenèse mais son expression est réduite voire absente dans les tissus adultes sains. Sa ré-expression se produit dans des situations pathologiques telles que le cancer. Une forte expression de la TNC est corrélée avec un pronostic péjoratif dans différents types tumoraux et son expression est par ailleurs liée à la progression tumorale, l'angiogenèse et la formation de métastases (3-5). La TNC constitue donc un acteur déterminant du stroma tumoral mais les mécanismes moléculaires sous-jacents sont toujours incompris.

Notre laboratoire a précédemment démontré que la TNC régule l'adhésion cellulaire ainsi que la dynamique du cytosquelette d'actine en bloquant le complexe intégrine α5β1/syndecan-4 (6-8) et que la TNC conduit à la sous-expression de DKK1 (8), un inhibiteur de la signalisation dépendante des ligands Wnt (9) et de l'angiogenèse (10). La participation de la TNC a la voie de signalisation Wnt a également été démontrée par d'autres (11).

Notre laboratoire a établi des modèles murins tumorigénique RipTag2 (RT2) sur-exprimant la TNC ou présentant une invalidation de la TNC. En bref, ces modèles nous ont permis de démontrer que la TNC promeut la progression tumorale en favorisant l'angiogenèse et par ailleurs que l'expression de DKK1 est corrélée de manière inverse à la TNC. De plus la surexpression de la TNC conduit à une expression accrue de l'Axine2, un gène cible de la voie de signalisation Wnt. Nous avons de plus montré en utilisant un modèle de xénogreffe que DKK1 constitue un inhibiteur majeur de l'angiogenèse et de la croissance tumorale. Ces résultats sont cohérents avec une publication récente identifiant DKK1 comme un inhibiteur puissant de l'angiogenèse suite à l'ischémie (10).

L'ensemble de ces résultats suggère que la TNC induit une activation de la voie de signalisation Wnt suite à l'inhibition de l'expression de DKK1, un inhibiteur des ligands Wnts. Ainsi, un de buts de ma thèse était d'utiliser des modèles *in vitro* afin de déterminer le niveau d'activité de la voie Wnt en fonction de la TNC dans des cellules tumorales, et également d'évaluer si la sous-expression de DKK1 induite par la TNC pouvait être la cause de l'activation de la voie Wnt. De plus je me suis intéressé à la contribution potentielle des cellules stromales présentes dans le microenvironnement tumoral dans la diminution de l'expression de DKK1 en présence de la TNC. Sur la base de données précédemment publiées par le laboratoire montrant que la TNC induit une perturbation des fibres de stress d'actine (6, 7, 12), j'ai émis l'hypothèse qu'une forte expression de DKK1 dépend de la présence d'actine filamenteuse stabilisée au sein de fibres de stress.

II. Résultats et discussion

1. Régulation de la voie de signalisation Wnt dans des cellules tumorales

J'ai montré que la TNC conduit à la sous-expression de l'inhibiteur de la voie Wnt DKK1 dans plusieurs lignées cellulaires tumorales d'origines différentes en analysant les niveaux d'expression de DKK1 au niveau de l'ARNm par RT-qPCR et au niveau protéique par Western Blot. J'ai confirmé que la TNC augmente l'activation de la voie de signalisation Wnt en utilisant un test rapporteur (TOPflash) ainsi qu'en mesurant le niveau d'expression de l'*Axin2* dans des cellules KRIB stimulées par le ligand Wnt-3A.

Il est probable que la sous-expression de DKK1 est liée de manière causale à l'induction de la voie de signalisation Wnt par la TNC. Afin de tester cette hypothèse, j'ai déterminé si l'induction de la voie Wnt par la TNC est régulée de manière dépendante de DKK1 en réalisant des expériences de surexpression ou de « knockdown » de DKK1 dans des cellules KRIB. J'ai établi des lignées cellulaires qui surexpriment mDKK1 de manière stable, ce qui a été confirmé par Western blot. La surexpression de DKK1 conduit à une répression de l'induction de la voie de signalisation Wnt induite par Wnt-3A dans des cellules cultivées sur un substrat TNC. J'ai également établi des lignées dont l'expression de DKK1 est invalidée de façon stable par des shRNAs ciblant DKK1. Après confirmation du knock-down de DKK1 par RT-qPCR et Western blot, des tests rapporteurs ont permis de mettre en évidence Que l'invalidation de DKK1 conduit à une activation accrue de la voie de signalisation Wnt. *A contrario*, sur un substrat contenant la TNC, le knockdown de DKK1 n'entraine plus d' augmentation de l'activité Wnt.

En conclusion, ces résultats suggèrent que la stimulation de la voie de signalisation Wnt médiée par la TNC dans des cellules tumorales est principalement due à la sous-expression de l'inhibiteur des ligands Wnt DKK1.

2. Régulation de la voie de signalisation Wnt dans les cellules stromales

Les cellules stromales du microenvironnement tumoral sécrètent également de la TNC et/ou sont en contact avec la TNC (13). J'ai donc analysé si, en parallèle des cellules tumorales, des cellules stromales telles que des cellules endothéliales, des péricytes et des fibroblastes associés au cancer (CAF) cultivées sur un substrat TNC en sous-exprimant l'inhibiteur de Wnt DKK1. J'ai pu montrer que les péricytes, deux lignées cellulaires de CAF et des cellules endothéliales (HUVEC) sous-expriment DKK1 en présence de TNC.

Dans les cellules endothéliales, j'ai montré que la TNC conduit également à une augmentation de l'expression du gène *Axin2*, indiquant une activation de la voie de signalisation Wnt. Il reste à déterminer si la TNC stimule la voie de signalisation Wnt dans d'autres types de cellules stromales, et si cela est lié de manière causale à la sous-expression de DKK1. Des niveaux faibles de DKK1 pourraient également exercer d'autres fonctions dans ces cellules et contribuer à l'induction de signaux Wnt non canoniques ou encore d'autres voies dans ce contexte (10, 14, 15).

3. Détermination des mécanismes impliqués dans la sous-epxression de DKK1 induite par la TNC

3.1 La TNC inhibe l'activité du promoteur de DKK1

Puisque la TNC induit une forte et rapide sous-expression de DKK1, j'ai émis l'hypothèse que l'expression du gène *DKK1* était soit régulée au niveau de l'activité de son promoteur soit au niveau de la stabilité de l'ARNm. J'ai stimulé des cellules T98G avec de l'Actinomycine D, un inhibiteur de la transcription. Ce traitement a abrogé la sous-expression de DKK1 induite par un substrat de FN (Fibronectine)/TNC comparé à un substrat de FN seul, suggérant que l'expression de DKK1 est régulée au niveau transcriptionnel. J'ai donc cloné un fragment de 3kb du promoteur de DKK1 d'origine humaine, en amont de la séquence codant pour la luciférase de luciole et analysé l'activité de ce plasmide rapporteur. J'ai observé qu'un substrat contenant de la TNC inhibe l'activité de ce promoteur synthétique de DKK1.

Comme la régulation de DKK1 par la TNC se produit de manière rapide et que les expériences précédemment décrites ont permis de montrer que cette régulation s'effectue au niveau transcriptionnel, j'ai émis l'hypothèse que la régulation de DKK1 pourrait être due à des changements du cytosquelette induits par la TNC, car ces changements représente un effet immédiat de la TNC. J'ai donc recherché un facteur de transcription qui serait directement régulé par le cytosquelette et ses changements.

3.2 La TNC régule la voie SRF/MKL1

La TNC réduit fortement l'adhésion cellulaire en inhibant la formation des fibres de stress d'actine en déstabilisant RhoA, en aval du complexe de l'intégrine α5β1/syndecan-4 (7, 16).

A partir d'une analyse précédente du laboratoire (8), je me suis rendu compte que la TNC conduit à la sous-expression d'un certain nombre de gènes cibles du facteur de transcription SRF (facteur de réponse au sérum) et de son co-facteur MKL1 (« megakaryoblastic leukemia 1 ») Le transport vers le noyau et l'activité de MKL1 est régulé par l'activité de RhoA et la dynamique de l'actine (17). Lorsqu'il est nucléaire, MKL1 agit comme un co-activateur du facteur de transcription SRF et active la transcription des gènes contenant des éléments de réponse au sérum (« boîte CARG ») dans leurs promoteurs (17, 18). Ses gènes cibles sont impliqués dans la régulation du cytosquelette et des adhésions focales. J'ai confirmé la régulation de plusieurs gènes cibles de SRF/MKL1 dans 3 lignées cellulaires cancéreuses en présence de TNC. De plus, j'ai montré que l'activation de ces gènes cibles induite par la TNC dépend bien de MKL1 en utilisant une approche de knockdown ciblant MKL1.

<u>3.3 La sous-expression de DKK1 sur un substrat de TNC est indépendante des voies RhoA et MKL1</u>

Pour déterminer si l'expression de DKK1 est contrôlée par MKL1, j'ai réalisé un knockdown de MKL1 médié par des shRNAs et montré que son expression et l'activité de son promoteur étaient inhibées. L'activation de la voie RhoA/MKL1 peut notamment s'effectuer en stimulant les cellules par un le facteur de croissance, l'acide lysophosphatidique (LPA). Un traitement par du LPA stimule l'expression de *DKK1* de manière dose-dépendante et sur un substrat de FN/TNC restaure l'expression à un niveau comparable de celui de cellules ensemencées sur un substrat de FN. Ceci s'accompagne également par une restauration de l' « étalement » des cellules. Toutefois, la surexpression d'une forme constitutionnellement activée de RhoA ou de MKL1 n'induit pas l'expression de *DKK1*, et ne restaure pas le niveau d'expression de DKK1 sur un substrat de TNC.

Ainsi, la sous-expression de DKK1 induite par la TNC est indépendante de RhoA et de MKL1. Le LPA pourrait activer une voie indépendante de la signalisation par RhoA/MKL1 et stimuler l'expression de DKK1. Le knockdown de MKL1 dans des cellules tumorales mime le blocage des fibres de stress d'actine induit par la TNC et ainsi indirectement cause une sous-expression de DKK1.

3.4 L'expression de DKK1 est régulée par la dynamique du cytosquelette d'actine

Afin de déterminer si le statut de polymérisation de l'actine régule le niveau d'expression de DKK1, j'ai analysé son expression après surexpression du syndesmos ou de la tropmyosine-1 (TPM-1), et en utilisant des drogues permettant de manipuler le statut de polymérisation de l'actine, qui déstabilisent (Latrunculine B, Cytochalasine D) ou stabilise (Jasplakinolide) la F-actine.

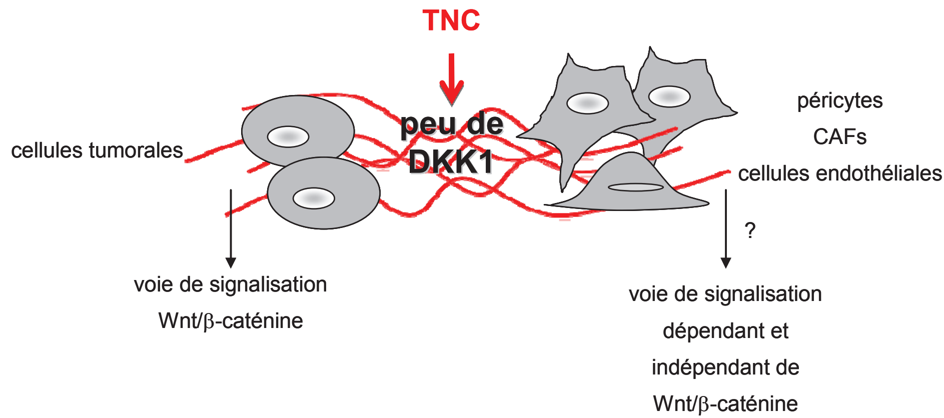
La TNC affecte notamment la stabilité des fibres de stress d'actine via la sous-expression de TPM-1 (8). La surexpresssion de TPM-1 restaure l'étalement des cellules et la formation de fibres de stress sur un substrat de de FN/TNC (8). J'ai observé que la surexpression de TPM-1 induisait fortement l'expression de DKK1 alors que le knockdown de TPM-1 conduisait à une réduction de son niveau d'expression. La TNC est en compétition avec l'activation du syndecan-4 par la Fibronectine (6). Le syndesmos permet de contourner la nécessité du syndecan-4 pour l'étalement des cellules sur un substrat de FN/TNC et de restaurer les fibres de stress d'actine et la signalisation par les adhésions focales (12). En surexprimant le syndesmos, l'expression de DKK1 était fortement augmentée. Ces résultats soutiennent l'‘hypothèse que les fibres de stress d'actine régulent la transcription de DKK1.

Le jasplakinolide induit la polymérisation d'actine et la formation d'actine filamenteuse (F-actine) stable. Mais des traitements de longue durée ou des concentrations fortes de jasplakinolide induisent une perte des fibres de stress (19, 20). Les fibres de stress sont des faisceaux de filaments inter-reliés d'actine. Le jasplakinolide réprime l'expression de DKK1, suggérant que l'expression de DKK1 est dépendante de la présence de fibres de stress.

La cytochalasine D inhibe l'élongation des polymères d'actine (22). A des concentrations fortes, la cytochalasine D réprime l'expression de DKK1. La latrunculine B se lie à la G-actine monomérique et inhibe la formation de F-actine (23). J'ai observé que la latrunculine B réduit l'expression de DKK1.

Ces données suggèrent que le niveau d'expression de DKK1 est fort dans des cellules ayant de l'actine filamenteuse stable et des fibres de stress, mais que la perturbation de ces fibres de stress et la dépolymérisation de l'actine filamenteuse conduisent à la répression de DKK1. Il sera intéressant de déterminer les mécanismes moléculaires impliqués dans cette régulation de DKK1, et en particulier d'identifier le(s) facteur(s) de transcription impliqué(s).

A



B

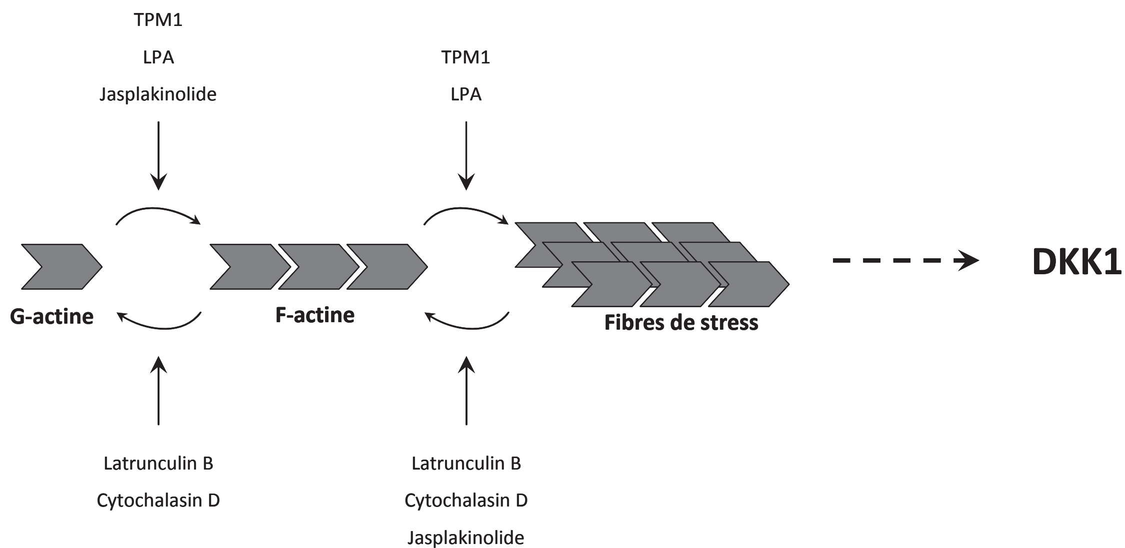


Figure : Résumé des principaux résultats concernant le mécanisme de régulation de Dikkopf-1 (DKK1) par la ténascine-C (TNC) et ses conséquences.
(A) La TNC réduit l'expression du gène *DKK1* aussi bien dans les cellules tumorales que stromales. Dans les cellules tumorales, la répression de DKK1 permet de stimuler la voie Wnt/β-catenine. Dans les cellules stromales, l'effet de la répression de DKK1 sur la voie Wnt/β-catenine doit encore être analysé plus en détail.
(B) Modèle de régulation de DKK1 par le statut de polymérisation de l'actine dans la cellule. La rupture des fibres de stress et la dépolymérisation de l'actine (Latrunculine B, Cytochalasine D) décroit l'expression du gène DKK1, tandis que l'induction de la polymérisation de l'actine ou des fibres de stress (LPA, TPM1) augmentent l'expression de DKK1. Le Jasplakinolide, qui induit la polymérisation de l'actine mais également la rupture des fibres de stress, décroit l'expression de DKK1.

IV. Conclusion

L'activation de la voie de signalisation Wnt dans les cellules tumorales contribue à la progression tumorale (24). Mes résultats soutiennent l'hypothèse selon laquelle la TNC active la voie de signalisation Wnt dans des cellules tumorales en conduisant à la répression de DKK1, ce qui pourrait contribuer à l'effet promoteur de la TNC sur la progression tumorale. La TNC conduit à la répression de DKK1 non seulement dans des cellules tumorales mais aussi dans des cellules stromales associées aux tumeurs, telles que des cellules endothéliales, des péricytes et des CAF. Il reste à déterminer si la répression de DKK1 dans des cellules stromales par la TNC est également associée à l'activation de la voie Wnt ou d'autres voies. De plus, dans un contexte tumoral, où la TNC est fortement exprimée, des niveaux faibles de DKK1 pourraient contribuer à une angiogenèse accrue.

La TNC induit des changements du cytosquelette qui semblent impliqué dans la sous-expression de DKK1 observée en présence de TNC. Mes expériences ont démontré que la restauration des fibres de stress par le LPA sur un substrat de FN/TNC, ou par la surexpression de TPM-1 ou syndesmos induit l'expression de DKK1. De plus, la restauration du niveau d'expression de DKK1 sur FN/TNC est indépendante de la voie RhoA/MKL1. Finalement j'ai montré que des facteurs/voies qui dépendent de fibres de stress stables, et probablement pas uniquement du ratio global F-actine/G-actine, pourraient être cruciaux pour l'expression de DKK1.

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Manuscript and other scientific contribution

MANUSCRIPT 1

Part of the results of this thesis contributed to a manuscript which is *accepted* in Cell Reports. My major contribution comprises the analysis of the impact of TNC on DKK1 regulation and Wnt signalling in tumor and stromal cells *in vitro*, and elucidation of the mechanism by which TNC mediates DKK1 downregulation.

Tenascin-C promotes tumor angiogenesis and progression in a neuroendocrine tumor model by downregulation of Wnt inhibitor Dickkopf-1.

Falk Saupe*, **Anja Schwenzer***, Yundan Jia*, Isabelle Gasser, Caroline Spenlé, Benoit Langlois, Martial Kammerer, Olivier Lefebvre, Ruslan Hlushchuk, Tristan Rupp, Marija Marko, Michael van der Heyden, Gérard Cremel, Christiane Arnold, Annick Klein, Patricia Simon-Assmann, Valentin Djonov, Agnès Neuville-Méchine, Irene Esposito, Julia Slotta-Huspenina, Klaus-Peter Janssen, Olivier de Wever, Gerhard Christofori, Thomas Hussenet and Gertraud Orend
(* equal contribution)

MANUSCRIPT 2, IN PREPARATION

Reduced MKL1 target gene expression by tenascin-C.

Anja Schwenzer, Annick Klein, Thomas Hussenet and Gertraud Orend

CONTRIBUTION TO A BOOK

In an exhaustive analysis, I summarized the current knowledge about the role of TNC in angiogenesis, stem cell biology and in metastasis formation. This analysis contributed to the book (section 2.3, 2.4 and 3):

The extracellular matrix and cancer: regulation of tumor cell biology by tenascin-C.

Gertraud Orend*, Falk Saupe*, **Anja Schwenzer*** and Kim Midwood*
(* equal contribution)
iConcept Press
provisionally accepted

Parts of this book have been used for the introduction of this thesis (section 1.2.3 and 1.2.4).

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Abbreviations

ADF	actin depolymerisation factor
APC	adenomatous polyposis coli
APS	ammoniumperoxodisulfate
ARP2/3	actin related protein
aSMA	alpha-smooth muscle actin
BAEC	bovine aortic endothelial cells
bFGF	basic fibroblast growth factor
CA	constitutively active
CAF	cancer-associated fibroblasts
CAMK	calcium/calmodulin-dependent protein kinase
CD	cluster of differentiation
cdc-HMEC	human dermal microvascular endothelial cells
CDM	cell-derived matrix
cDNA	complementary DNA
CM	conditioned medium
CNS	central nervous system
CRC	colorectal cancer
CTGF	connective tissue growth factor
CTR	control
DKK	dickkopf
Dll4	deltalike ligand 4
DMSO	dimethylsulfoxide
DN	dominant negative
DNA	desoxyribonucleic acid
Dvl or Dsh	dishevelled
ECM	extracellular matrix
EDNRA/B	endothelin receptor type A/B
EGF	epidermal growth factor
EMT	epithelial-mesenchymal transition
ERK	extracellular signal regulated kinase
ET-1	endothelin 1
F-actin	filamentous actin
FAK	focal adhesion kinase
FCS	foetal calf serum
FN	fibronectin
Fz or Fzd	frizzled
G-actin	globular actin
GBM	glioblastoma multiforme
GEF	guanine nucleotide exchange factor
GPCR	G-protein coupled receptor
GSK3	glycogen synthase kinase 3
h	hours
HEK	human embryonic kidney
HepII	heparin II
His	histidine
HMVEC	human dermal microvascular endothelial cells
HSPG	heparin sulfate proteoglycans
HUVEC	human umbilical vein endothelial cells
IF	immunofluorescence
IHC	immunohistochemistry
ILK	integrin linked kinase
JMY	junction mediating and regulatory protein
JNK	c-jun n-terminal kinase

kb	kilobase
kd	knockdown
KO	knockout
LB	luria broth
LIMK	LIM domain kinase
LPA	lysophosphatidic acid
LRP5/6	lipoprotein receptor-related protein
MAL	megakaryocytic acute leukaemia
MAPK	mitogen-activated protein kinase
MEF	mouse embryonic fibroblast
miR	micro RNA
MKL1	megakaryoblastic leukaemia 1
MLC	myosin light chain
MLCK	MLC kinase
MMP	matrix metalloproteinase
MRCK	myotonic dystrophy kinase-related Cdc42-binding kinase
mRNA	messenger RNA
MRTF	myocardin-related transcription factor
NFκB	nuclear factor kappa B
OD	optical density
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGF-BB	platelet-derived growth factor
PKC	protein kinase C
POSTN	periostin
qRT-PCR	quantitative real-time PCR
REF52	rat embryonic fibroblasts
RNA	ribonucleic acid
ROCK	rho-kinase
RT2	rip1-tag2
RTK	receptor tyrosine kinase
RT-PCR	reverse-transcribed PCR
SDS	sodium dodecylsulfate
sFRP	secreted frizzled related protein
shRNA	short hairpin RNA
SRF	serum response factor
TAZ	transcriptional co-activator with PDZ-binding motif
TCF	ternary complex factors
TCF/LEF	T-cell factor /lymphoid enhancer factor
TEAD	TEA domain family member
TGFβ	transforming growth factor beta
TNC	tenascin-C
TNIII	fibronectin type III-like repeats in tenascin-C
TPM	tropomyosin
VEGFA	vascular endothelial growth factor A
VEGFR2	VEGFA receptor
vWF	von Willebrand factor
WHO	world health organisation
WIF1	wnt inhibitory factor 1
wt	wild type
YAP	yes-associated protein

1 Introduction

A tumor arises from cells that transform progressively into malignant cancerous cells by activation of oncogenes or loss of function of tumor suppressor genes. During this process these cells acquire hallmark capabilities as defined by Hanahan and Weinberg (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011) allowing them to sustain proliferative signalling, evade growth suppressors, resist cell death, enable replicative immortality, induce angiogenesis and activate invasion and metastasis. A tumor can be seen as a complex and heterogeneous organ, composed of both cancer and stromal cells intermingled in a 3-dimensional extracellular matrix (ECM) that regulate cellular fates and reciprocal interactions. Both stromal cells and the tumor ECM contribute to the hallmarks of cancer, as will be discussed below.

1.1 The tumor microenvironment

The tumor microenvironment comprises stromal cells, secreted ECM molecules, and soluble signalling molecules such as cytokines, growth factors and matrix remodelling enzymes as well as blood and lymphatic vessels, nerves and inflammatory cells. The tumor microenvironment has many characteristics not found in normal tissue, including enhanced ECM deposition, increased number of fibroblasts and enhanced capillary density. Multiple crosstalks exist between the tumor cells, stromal cells and the ECM, which fuel tumor progression by supporting tumor growth, angiogenesis, invasion and metastasis (Kalluri and Zeisberg, 2006; Hanahan and Weinberg, 2011). In the following chapter I will detail which stromal cells are encompassed in the tumor microenvironment (**Figure 1**) as well as describing the importance of the matricellular components for tumor progression.

1.1.1 Cellular components

Endothelial cells (EC) line the interior surface of the tumor-associated vasculature. The growth of a tumor strongly depends on its supply of nutrients and oxygen. Along tumor progression, the angiogenic switch is considered a crucial and early event (Hanahan and Weinberg, 2011). The angiogenic switch is characterized by sprouting, new vessel formation, vessel maturation, and the recruitment of perivascular cells (Bergers and Benjamin, 2003;

Hanahan and Weinberg, 2011). The vasculature of a tumor often differs from the vasculature in healthy tissues and is characterized by abnormal vessels with a disorganized basement membrane, incomplete pericyte coverage and leakiness (De Bock *et al*., 2011).

Pericytes are a specialized mesenchymal cell type that wrap around the endothelial tubing of blood vessels. Pericytes and vascular endothelial cells are anchored to the vascular basement membrane, which is produced by both cell types. In tumors, pericytes are commonly loosely associated with the vasculature. It has been demonstrated that pericytes are crucial for the function of the tumor vasculature. Several studies suggest however that a reduced coverage with pericytes might favour cancer cell dissemination (Hanahan and Weinberg, 2011; Pietras and Ostman, 2010).

Tumor-promoting immune inflammatory cells as macrophages, mast cells, neutrophils, T and B lymphocytes have been shown to secrete tumor-promoting molecules as well as to induce and promote cancer cell proliferation, invasion, metastasis and tumor angiogenesis (Hanahan and Weinberg, 2011).

Cancer-associated fibroblasts (CAFs) are a major cell population in the stroma of most tumors. One distinguishes at least two different cell types: (1) cells that exhibit similarities to fibroblasts found in normal epithelial tissue (tissue-derived) (2) and myofibroblasts that have distinct properties and biological roles than tissue-derived fibroblasts (bone-marrow derived from mesenchymal stem cells). Those cells are also often termed “activated” fibroblasts as they secrete more ECM, proliferate more than their non-activated counterparts and have features more typical of smooth muscle cells. They are mainly characterized by the expression of α-smooth muscle actin (α-SMA), a marker of their activation. CAFs have been shown to promote cancer cell proliferation, invasion, metastasis and tumor angiogenesis (Kalluri and Zeisberg, 2006; Hanahan and Weinberg, 2011).

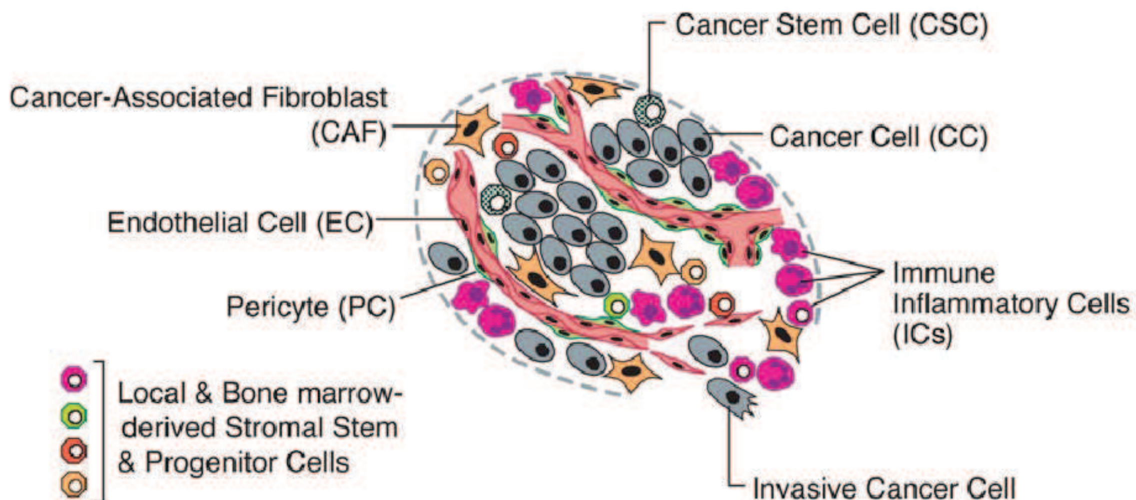


Figure 1. The cells of the tumor microenvironment (Hanahan and Weinberg, 2011). A solid tumor is constituted of distinct cell types that contribute to tumor growth and progression. ECs line the inner side of blood vessels enabling the supply of the tumor with nutrients and oxygen. Pericytes wrap around blood vessels and play an important role in stabilizing blood vessels; however they are also considered to limit dissemination of cancer cells. In addition, CAFs have been demonstrated to favour cancer cell proliferation, invasion and metastasis as well as angiogenesis. Immune inflammatory cells can both promote or inhibit tumor growth depending on their subclass.

1.1.2 Matrix components

The ECM is a complex meshwork of highly cross-linked fibrous proteins. The "core matrisome" as defined by Naba *et al.* (Naba *et al.*, 2011) is composed of collagens, ECM glycoproteins and proteoglycans, encoded by approximately 300 different genes. The complete matrisome further comprises ECM-affiliated proteins, ECM regulators and secreted factors (Naba *et al.*, 2011; approximately 1100 genes in total). Different forms of the ECM exist. The interstitial matrix contains collagens, proteoglycans and glycoproteins such as tenascin-C (TNC), periostin (POSTN) and fibronectin (FN) and is characterized by an unorganized, loose assembly of these components; it contributes to the tensile strength of tissues (Lu *et al.*, 2012). The basement membrane presents a specialized form of the ECM at the baso-lateral side of cells, which separates an epithelium or an endothelium from the adjacent tissue. It is a more compact ECM form and less porous than interstitial matrix, and contains two networks of type IV collagen and laminins that are interconnected by molecules of nidogen and perlecan (Lu *et al.*, 2012). As compared to a healthy tissue, the ECM within a tumor is markedly different in terms of composition, abundance of certain components and structure.

The ECM executes different functions (**Figure 2**). First of all, the ECM serves as a structural support for tissue architecture and integrity. Depending on the ECM structure and composition, cells can use the ECM as an anchorage site, as tracks for their migration, but the ECM can also function as migration barrier. The ECM provides mechanical cues, as cells can directly sense the mechanical properties, e.g. stiffness, of the surrounding ECM. In addition to ECM binding to integrin cell adhesion receptors, the ECM has been shown to modulate growth factor signalling. Different ECM proteins can bind growth factors and may act as reservoirs for growth factors or aid in establishing a stable gradient of growth factors; those growth factors can later be released upon ECM degradation. In addition, the ECM can function as co-receptor for growth factor binding or even intrinsic domains within ECM proteins might function as ligands for receptors, as has been shown for EGF-like repeats of laminins and TNC (reviewed in Hynes, 2009; Lu *et al*., 2012).

In summary the ECM can therefore tightly regulate cell behaviour by numerous mechanisms and is actively involved in promoting tumor growth, angiogenesis, invasion and metastasis.

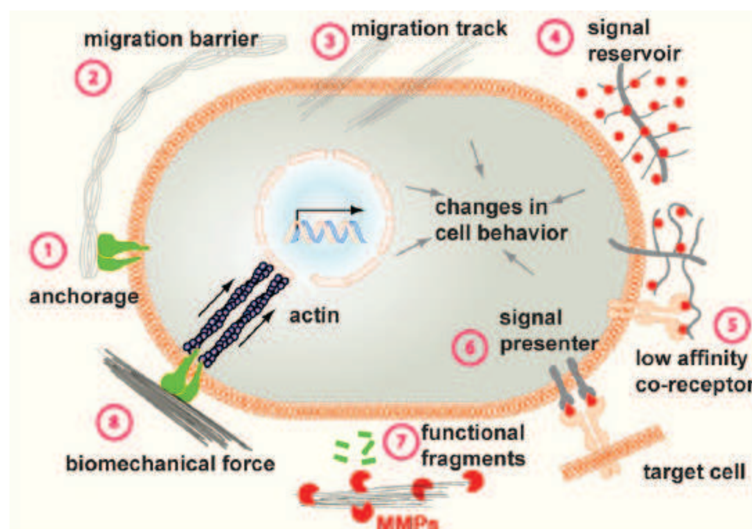


Figure 2. Role of the ECM in the tumor microenvironment (Lu *et al.,* 2012). Cells can anchor to the ECM (e.g. the basement membrane) through cell surface receptors (1). For migrating cells the ECM can function as a barrier (2) or facilitate migration by serving as migration track (3). ECM components 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).

1.2 TNC

TNC is a secreted 190 - 300 kDa glycosylated protein (**Figure 3**) and the founding member of the tenascin protein family, including TN-X, TN-W and TN-R. The N-terminal stretch of 110 residues is unique to the tenascin protein family and is followed by a short heptad repeat region and 14.5 EGF-like repeat domains. TNC contains up to 17 FN type III like domains (constitutive and alternatively spliced) and a c-terminal fibrinogen like globular domain (**Figure 3A**). TNC monomers are assembled into a hexameric form (**Figure 3B**) (Orend *et al.*, 2013).

TNC is expressed during development but nearly absent in adult tissue. However, TNC gets re-expressed under pathological conditions, such as wound healing, inflammation, fibrosis and cancer. Especially in solid tumors of the breast, brain and colon a high TNC expression has been reported. TNC expression correlates with low survival rates and promotes lung metastagenicity of breast cancer cells (Orend *et al.*, 2013).

In the following chapter I will summarize what is known about the regulation of TNC expression in tumors and which effects TNC has been shown to exert on proliferation, migration, invasion and angiogenesis. Furthermore, TNC has been described to be an adhesion-modulating ECM molecule, studies describing the possible underlying mechanisms I will summarize in chapter 1.3.5.

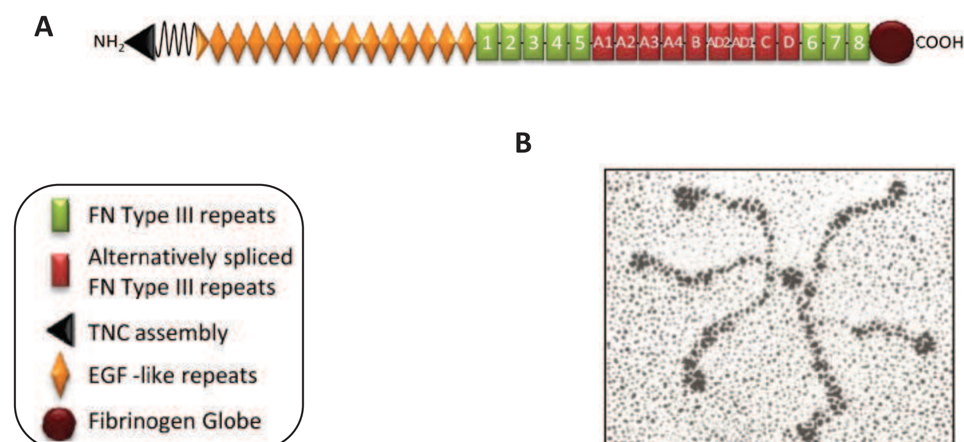


Figure 3. Domain structure of TNC. (A) Organization of TNC into different protein domains. (B) The appearance of purified TNC protein as a hexamer upon electron microscopy (Orend *et al.*, 2013; Van Obberghen-Schilling *et al.*, 2011).

1.2.1 Regulation of TNC expression

Depending on the tumor type and stage TNC can be expressed by either the tumor cells, stromal cells or both cell types, which has been addressed in several studies by immunohistochemical analysis and *in situ* hybridization.

In epithelial tumors such as from the breast and colon, TNC is mainly expressed by CAFs and forms a fibrous network that encloses unstained tumor nests (Degen *et al*., 2007; Degen *et al*., 2008; Chiquet-Ehrismann and Tucker, 2011). In contrast, in glioblastomas and melanomas the cancer cells themselves secrete TNC (Natali *et al*., 1990; Herlyn *et al*., 1991; Sivasankaran *et al*., 2009). In colorectal adenoma and carcinomas (Hanamura *et al*., 1997) as well as fibroadenomatosus tumors (Lightner *et al*., 1989) and breast ductal carcinoma (Yoshida *et al*., 1997) TNC is expressed both by stromal and cancer cells. In gliomas TNC staining overlaps with staining for desmin-positive cells, indicating that rather pericytes than endothelial cells are a source for TNC in these tumors (Martina *et al*., 2010). As analysed by a proteomic approach from human non-metastatic and metastatic melanoma cell lines xenografted in nude mice, TNC was both expressed by tumor and stromal cells in these tumors; however, in metastatic melanoma cells there was a higher contribution by stromal cells (Naba *et al*., 2011).

Oskarsson *et al.* (2011) demonstrated that breast cancer cell-derived TNC is important for breast cancer lung metastasis outgrowth, but later on the stromal compartment takes over as a source of TNC (Oskarsson *et al*., 2011). O'Connell *et al.* showed that TNC derived from S100A4-positive cells promotes metastatic colonization of breast cancer cells in the lung (O'Connell *et al*., 2011).

TNC expression can be induced by mechanical stretch, which depends on the activation of the RhoA/MKL1 pathway and TNC has been identified as a direct transcriptional target of MKL1 (Asparuhova *et al*., 2011). Furthermore, it has been shown that TNC is upregulated by hypoxia or in the presence of reactive oxygen species (Orend and Chiquet-Ehrismann, 2006). TNC expression can be induced by TGFβ, and other growth factors such as EGF, bFGF, PDGF-BB and CTGF, which are mostly secreted by stromal cells (Orend and Chiquet-Ehrismann, 2006).

Several pro-oncogenic pathways such as Ras/MAPK signalling (Maschler *et al*., 2004), Notch signalling (Sivasankaran *et al*., 2009) and Wnt signalling (Beiter *et al*., 2005; Cohen *et al*.,

2009) are involved in regulation of TNC expression. A number of regulatory transcription factor binding sites have been identified and are functional in the TNC promoter, including c-jun, NFkB, sox4, Prx1 (paired related homeobox 1) and Smad2/3. In contrast, GATA6 has been identified as a transcriptional repressor of *TNC* gene expression (reviewed in Tucker and Chiquet-Ehrismann, 2009; Chiquet-Ehrismann and Tucker, 2011). Furthermore, miR-335 can regulate TNC mRNA (Tavazoie *et al*., 2008).

1.2.2 TNC promotes proliferation and survival of tumor cells

Chiquet-Ehrismann *et al.* (1986) demonstrated that TNC increases proliferation in primary mammary tumor cells after serum depletion. In T98G and MDAMB-435 cells TNC induced proliferation by inhibition of integrin $\alpha5\beta1$/Syndecan-4-dependent cell adhesion (Huang *et al*., 2001). However, TNC inhibited DNA replication in normal human fibroblasts (MRC-5), immortal rat embryonic fibroblasts (REF52) and swiss 3T3 fibroblasts (NIH3T3) (Orend *et al*., 2003).

When grown in reconstituted basement membrane MCF-10A cells form three-dimensional polarized, growth-attenuated, multicellular acini, enveloped by a basement membrane. Addition of exogenous TNC in this setting promoted cell proliferation and luminal filling by increasing the expression and downstream signalling of c-met (Taraseviciute *et al*., 2010).

In breast cancer-derived lung metastasis TNC did not affect proliferation but promoted survival by decreasing apoptosis of metastatic breast tumor cells (Oskarsson *et al*., 2011). Also upon intravenous injection of 4T1 cells into mice, the metastatic burden (area) was significantly reduced in TNC knockout (TNC KO) mice compared with control mice, supporting the notion that TNC expressed by stromal cells plays a role in stimulating survival of metastasizing tumor cells or protecting them from apoptosis (O'Connell *et al*., 2011).

1.2.3 TNC promotes cancer cell migration, invasion and epithelial-mesenchymal transition (EMT)

It has been demonstrated that TNC increases migration and invasion using several *in vitro* tumor cellular models (breast, colon cancer, glioma, chondrosarcoma, squamous cell carcinoma) (for summary see table 7 in Orend *et al*., 2013).

Tumors derived from GBM cells knocked down for TNC and implanted in the striatum of nude mice consisted of less infiltrating tumor cells and less tumor cell clusters in the surrounding brain tissue, despite the fact that no difference in tumor growth and proliferation were observed (Hirata *et al*., 2009).

TNC also appears to play a role in the intricate cross-talk between myofibroblasts and tumor cells. Human primary myofibroblasts derived from colorectal tumor tissue stimulated the invasive behaviour of colorectal cancer (CRC) cells in a TNC-dependent manner (De Wever *et al*., 2004). CRC cells co-cultured with myofibroblasts rapidly invaded a collagen gel, which could be blocked by treatment with an antibody directed against the TNC EGFR repeats, suggesting that deposition of TNC by the myofibroblasts in the gel drives the invasion of the cancer cells. The authors linked this TNC-stimulated pro-invasive behaviour of carcinoma cells to the down-regulation of RhoA signalling (De Wever *et al*., 2004). Similarly Gaggioli and colleagues (2007) observed that squamous carcinoma cells invade an organotypic matrix upon co-culture with stromal fibroblasts that were derived from oral or vulval squamous cell carcinoma. Stromal fibroblasts invaded and strongly modified the matrix by promoting degradation and deposition of matrix including TNC and FN. The collagen matrix within these tracks was organized into thick bundles (Gaggioli *et al*., 2007). The authors demonstrated that those tracks promoted carcinoma cell invasion. However, TNC and FN seemed not necessary for tumor cell invasion, as a either single or combined knockdown of these molecules in fibroblasts did not change the number of invading carcinoma cells (Gaggioli *et al*., 2007).

An over-representation of specific TNC domains mostly encompassing the alternatively spliced TNIII repeats but not the EGF-like repeats had been demonstrated in several cancers. In particular in breast cancer the expression of TNC isoforms, containing TNIII D, TNIII BD or TNIII BAD1D domains, were associated with increased tumor cell invasion (Adams *et al*., 2002; Guttery *et al*., 2010; Hancox *et al*., 2009). The exposition of certain domains within TNC could arise from cleavage of the molecule by matrix metalloproteinases (MMPs) and other proteases. Cleavage of TNC by meprin-beta1 was shown to abrogate the syndecan-4 inhibitory activity of TNC on a mixed FN/TNC substratum (Ambort *et al*., 2010).

TNC itself might also increase the expression and activity of MMPs stimulating the invasive behaviour of cancer cells. Ilunga *et al.* (2004) showed that MDAMB231 invasion into matrigel

was stimulated by the addition of both TNC and TGF-β1, which could be blocked by a MMP-inhibitor (Ilunga *et al*., 2004). Similarly, TNC also stimulated the invasion of glioblastoma cells by increasing protein kinase C δ activity and MMP12 expression. Migration was repressed by MMP-inhibitors or an antibody specific for MMP12 (Sarkar *et al*., 2006; Sarkar and Yong, 2010).

In breast carcinoma TNC expression correlates with the expression of the mesenchymal marker vimentin and, in several cancer cell lines TNC and vimentin are found to be co-expressed (Dandachi *et al*., 2001). Moreover, TNC is highly expressed at the invasive front of colorectal tumors at sites with nuclear β-catenin in tumor cells (Beiter *et al*., 2005). Nuclear β-catenin has been demonstrated to be an EMT marker (Kim *et al*., 2002). Knock-down of β-catenin in CRC cell lines indeed results in reduced TNC expression and the authors provided evidences that β-catenin is directly regulating the activity of the TNC promoter (Beiter *et al*., 2005). Nagaharu and colleagues (2011) demonstrated that upon a combined treatment with TNC and TGFβ-1 MCF7 and T47-D breast cancer cells acquire an EMT-like phenotype, characterized by loss of membranous E-cadherin and β-catenin, which was linked to increased cell migration (Nagaharu *et al*., 2011). Furthermore, TNC seemed to be involved in regulating the EMT process in the mouse lense epithelium upon injury (Tanaka *et al*., 2010). In another study TNC promoted a partial EMT in MCF7 cells that changed their cobblestone epithelial morphology into a fibroblastoid phenotype upon growth on a TNC substratum. This was linked to an altered expression of the adaptor protein 14.3.3tau (Martin *et al*., 2003). These observations suggest that TNC promotes EMT but may also be regulated by EMT, as it was shown to be a β-catenin target gene. Thus highly expressed TNC at the tumor invasion front may enhance cancer cell migration and invasion.

1.2.4 Role of TNC in regulation of tumor angiogenesis

1.2.4.1 TNC as a marker for tumorigenic blood vessels

In several studies TNC has been found to be preferentially localized around tumor blood vessels. Analysis of 86 gliomas, where TNC expression increased along tumor progression, revealed that TNC was strongly expressed around tumor blood vessels in gliomas of WHO grade IV (glioblastoma multiforme - GBM). Although this was different in grade II and III gliomas with an overall reduced frequency of TNC lined blood vessels, perivascular TNC

staining significantly correlated with a shorter disease-free time in these grade II and III glioma (Herold-Mende *et al*., 2002). Behrem *et al.* (2005) observed that GBM with strong perivascular TNC staining contained more newly formed blood vessels than tumors with moderate or weak TNC expression, as assessed by staining of CD105 positive microvessels (Behrem *et al*., 2005). CD105 is a cell membrane glycoprotein overexpressed on tumor-associated vascular endothelium (Fonsatti *et al*., 2003). Furthermore, in tissue samples from 63 patients with non-small cell lung cancer, Ishiwata *et al*. (Ishiwata *et al*., 2005) found a correlation between TNC concentration in the serum and intra-tumoral vessel density. In juvenile nasopharyngeal angiofibroma TNC expression was also found around blood vessels and its expression correlated with vessel density, tumor stage and endothelial c-kit expression (Renkonen *et al*., 2012).

Berndt *et al*. (2010) showed that in CD31-positive blood vessels of clear cell renal cell carcinoma and atypical carcinoids of the lung TNC is localized on the extra-luminal side of the basement membrane (Berndt *et al*., 2010). By *in situ* hybridization TNC mRNA was detected in hyperplastic capillaries of astrocytoma tumor tissues. Staining was observed lining the vascular lumen, indicating the presence of TNC in endothelial cells. But other cells could also be a source of TNC, as additional staining was observed in the walls of the vascular structures. Immunostaining confirmed TNC expression within and around the walls of hyperplastic blood vessels and staining was also detected adjacent to vascular sprouts (Zagzag *et al*., 1996). Indeed, Martina *et al.* (2010) showed that TNC is expressed by pericytes in GBM but not by endothelial cells (Martina *et al*., 2010).

Recently three studies using proteomic approaches have identified TNC as a marker preferentially expressed in the vasculature of tumors or at the metastatic site. Borgia *et al*. (2009) used *in vivo* perfusion of biotin for labelling of vascular proteins. Four different TNC isoforms (containing TNIII domains A1, A2, A4, B) have been identified as molecules which were expressed in the vasculature of liver metastases in a syngeneic heterotopic model of colon cancer (Borgia *et al*., 2009). Hill *et al.* (2011) used laser capture micro-dissection of microvessels of invasive ductal carcinoma and identified TNC as one of the proteins overexpressed in tumor vessels in comparison to vessels from adjacent healthy tissue (Hill *et al*., 2011). Similarly, by using laser capture micro-dissection and protein expression profiling TNC was found to be exclusively expressed in tumor vessels of GBM but not in tissue with physiological angiogenesis (Mustafa *et al*., 2012).

In summary, these data show that in a number of tumor types TNC specifically marks tumor blood vessels. TNC expression often correlates with higher tumor stage and increased blood vessel density which argues for a role of TNC in the tumor vasculature.

1.2.4.2 TNC impacts on endothelial cell behaviour *in vitro*

TNC supported the switch from a non-angiogenic (resting) cobblestone phenotype to an angiogenic sprouting cord-forming phenotype in bovine aortic endothelial cells (BAEC) (Canfield and Schor, 1995). Schenk *et al*. (1999) showed that TNC is exclusively expressed in the sprouts and cords of the sprouting but not in the resting BAEC. For the induction of sprouting by TNC the basic fibroblast growth factor (bFGF) seems to be required. Furthermore, the authors showed that the fibrinogen globe domain of TNC was responsible for the BAEC sprouting (Schenk *et al*., 1999). The authors argued that the sprout-supporting effect of TNC might be explained with the anti-adhesive effect of TNC.

Moreover, TNC increases sprouting of HUVEC spheroids embedded in collagen gels (Martina *et al.,* 2010). When seeded on a TNC-coated basement membrane or when TNC was added to the culture medium endothelial cells had enhanced tube formation ability (Castellon *et al.,* 2002).

An anti-adhesive effect of TNC on endothelial cells of different origins was shown (Ballard *et al*., 2006; Sriramarao *et al*., 1993), however, others reported that TNC significantly stimulated endothelial cell adhesion (Delaney *et al*., 2006; Zagzag *et al*., 2002).

The anti-adhesive TNC effect reported has been linked to a reduction in focal adhesions in endothelial cells (Murphy-Ullrich *et al*., 1991). These authors showed that the TNIII A-D domain mediates the anti-adhesive effect, which can be reversed by blocking cell surface annexin II, a TNC receptor (Chung *et al*., 1996).

Despite its anti-adhesive and anti-spreading effect early upon cell plating, most endothelial cells eventually do attach and spread on TNC after culture for longer periods of time. This cell attachment can be blocked with an RGD peptide. The RGD peptide is the binding motif of integrin ligands and therefore inhibits integrin-ligand interaction. Therefore cell attachment to TNC might be integrin-dependent (Bourdon and Ruoslahti, 1989). Endothelial cell attachment and spreading on TNC is mediated by different TNC cell surface receptors

including annexin II (Chung and Erickson, 1994), $\alpha2\beta1$ and $\alpha v\beta3$ integrins (Delaney *et al*., 2006; Joshi *et al*., 1993; Sriramarao and Bourdon, 1993). But in longer term assays endothelial cells secrete other ECM molecules such as FN, and adhesion to FN would be blocked with RGD peptides. Thus the described observation may be due to inhibiting adhesion to FN with the RGD peptide rather than blocking the interaction with TNC. Altogether these studies show that through different cell surface receptors adhesion and spreading of endothelial cells can be modulated by TNC, which could be crucial in tumor angiogenesis.

TNC was shown to enhance endothelial cell proliferation (Castellon *et al*., 2002; Chung *et al*., 1996; Delaney *et al*., 2006) and migration (Ballard *et al*., 2006; Castellon *et al*., 2002; Chung *et al*., 1996; Ishiwata *et al*., 2005; Martina *et al*., 2010; Zagzag *et al*., 2002). This was demonstrated using different *in vitro* assays and with endothelial cells of different origins.

1.2.4.3 TNC supports angiogenesis

Several studies provide evidence for a correlation between TNC expression and expression of the vascular endothelial growth factor A (VEGFA). The initial study by Tanaka *et al*. (2004) suggests that TNC supports melanoma angiogenesis by regulating the expression of VEGFA. After injection of melanoma cells in immune compromised wild type (wt) and TNC knockout (KO) mice tumors in KO mice were smaller and were less vascularized. Measuring the VEGFA content of the tumors by ELISA the authors observed a lower VEGFA content in tumors from the KO mice than those grown in wt mice. Also in co-cultures with melanoma cells and the mesenchyme derived from either wt or TNC KO mice the authors measured a higher VEGFA level when the mice expressed TNC (Tanaka *et al*., 2004). Other studies support the author's observation that TNC expression correlates with VEGFA levels as e.g. in GBM (Behrem *et al*., 2005). In sera from non-small cell lung cancer patients TNC levels correlated with the VEGFA levels (Ishiwata *et al*., 2005). Moreover Sumioka *et al*. (2011) showed that occular fibroblasts derived from TNC KO mice expressed less VEGFA than wt fibroblasts which was associated with less neovascularisation in TNC KO mice in the corneal stroma (Sumioka *et al*., 2011). But how TNC impacts on the expression of VEGFA is unknown.

In summary these studies show that TNC is strongly associated with tumor blood vessels. Moreover, TNC appears to be able to promote the formation of tumor-associated blood

vessels. However, there remains a need to better understand the mechanisms and signalling by which TNC is involved.

1.3 The cell cytoskeleton

1.3.1 Extracellular signals regulating actin dynamics

The adhesion of cells to the ECM is mediated by integrin transmembrane receptors. Integrins function as heterodimers that consist of a non-covalently linked α- and β-subunit, forming binding sites for specific ECM components. Integrins are the main structural organizing components of focal adhesions, which link the ECM on the outside of the cell to cytosolic signalling proteins and the actin cytoskeleton inside the cell. Therefore, focal adhesions not only support the adhesion of the cell to the ECM but also transmit extracellular signals to the cell by mediating intracellular signalling.

Upon adhesion integrins form clusters and different proteins are recruited to the intracellular domain of the integrins, including structural and signalling proteins, which then altogether form focal adhesions (Carragher and Frame, 2004). One distinguishes several groups of proteins within focal adhesions (Geiger *et al*., 2001):

- Membrane-bound, non-integrin components of focal adhesions (e.g. the integrin $\alpha5\beta1$ co-receptor syndecan-4, the hyaluronan-binding protein layilin)
- Integrin-associated multi-molecular domains in the cytoplasm (e.g. talin, tensin), which link integrins directly to actin
- Integrin-associated molecules that do not bind directly to actin, such as the signalling molecules FAK (focal adhesion kinase) and ILK (integrin-linked kinase), caveolin and paxillin. Paxillin anchors proteins, including FAK, to the membrane.
- Actin-binding proteins that do not directly interact with integrins, such as vinculin and ERM (ezrin, radixin and moesin) proteins
- Adapter proteins, which interact with actin-bound and integrin-bound components, such as zyxin, syndesmos and the enzyme SRC

Integrin signalling directly impacts on actin dynamics by regulating the activity of FAK, ILK and SRC (Huveneers and Danen, 2009). Besides integrins, several other types of cell surface

receptors can regulate actin polymerisation, such as G protein-coupled receptors (e.g. LPA binding LPA-R), tyrosine kinase receptors (PDGF binding PDGF-R) and Ser/Thr kinase receptors (activated by the TGF-β-family of polypeptides) (Olson and Nordheim, 2010).

1.3.2 Actin polymerisation and stress fibre formation

Microfilaments, which are polymers of actin, are an important component of the cell cytoskeleton and are organized in the cytosol, providing strength and force to the cell body. Microfilaments form the cell cortex, an actin-rich layer in the cytosol beneath the plasma membrane.

Actin filaments (**F-actin**) are formed by polymerisation of monomeric, globular ATP-bound actin (**G-actin**). Actin monomers itself have a limited affinity to form dimers, therefore cells use **actin nucleation factors** to catalyze the assembly of F-actin from monomeric G-actin and **elongation factors** to control the rate, extent and shape of newly formed filaments (Pollard, 2007). In addition, the pool of assembly-ready G-actin is controlled by several mechanisms, including **sequestration** (e.g. by thymosin-β4), binding to **profilin** (inhibiting nucleation, but allows filament growth), and **capping proteins** (blocking the growing end of the filament) (Pollard, 2007) (**Figure 4**).

Actin polymerisation is regulated by the **family of Rho GTPases** through selective **Rho guanine nucleotide exchange factors** (GEFs), which are activated upon extracellular stimuli that are sensed by above-described receptors (Olson and Nordheim, 2010). The Rho GTPase family comprises the Rho (RhoA, RhoB, RhoC), Rac (Rac1, Rac2, Rac3, RhoG) and Cdc42 (cdc42, RhoQ, RhoJ) subfamilies (Boureux *et al.*, 2007).

Rho GTPases activate **ROCK** (Rho-kinase) which further activates **LIMK** (LIM domain kinase) that inhibits **cofilin** activity, a stimulator of actin depolymerisation (Lappalainen and Drubin, 1997). In addition, Rho GTPases can directly regulate the function of different actin **nucleation factors**. The actin related protein 2/3 (**ARP2/3)** complex both nucleates filaments and organizes them into branched networks, as e.g. found in lamellipodia. **Formins** (e.g. mDia) nucleate filaments and stimulate filament elongation (Campello and Welch, 2010) (**Figure 5**).

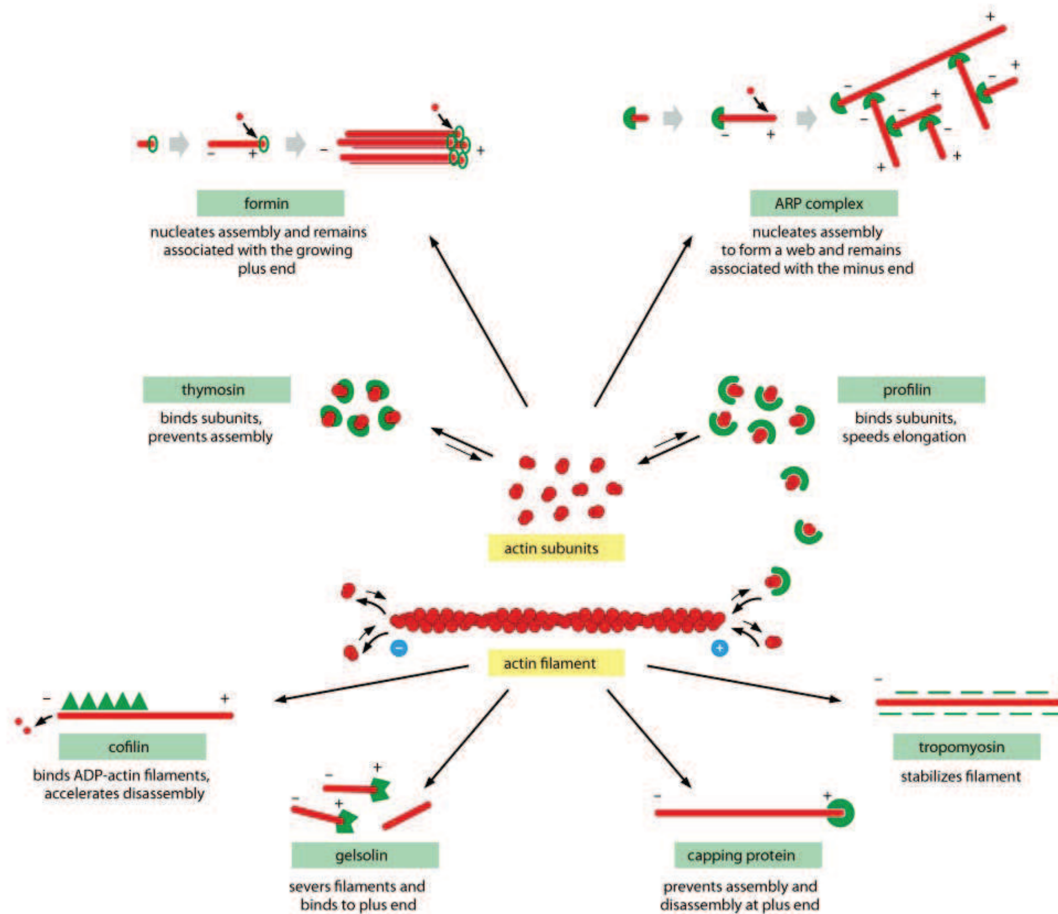


Figure 4. A selection of proteins binding actin and their function (Alberts *et al*., 2008). ACTIN FILAMENTS are formed by polymerisation of ACTIN SUBUNITS (monomeric actin/G-actin). Monomeric actin can be sequestered by actin binding proteins: THYMOSIN prevents actin polymerisation, while PROFILIN speeds elongation of actin filaments. FORMINS nucleate the assembly of actin filaments and stimulate formation of unbranched filaments and actin bundles. The ARP2/3 complex stimulates the assembly of branched filaments. Filament binding proteins: COFILIN enhances disassembly of filaments while GELSOLIN severs filaments. CAPPING PROTEINS prevent both elongation and disassembly of the filament. TROPOMYOSINS stabilize filaments.

There are different structures of polymerised actin. **Filopodia** are finger-like cellular extensions composed of unbranched actin filaments. **Lamellipodia** are sheet-like cellular protrusions that contain a dynamic Y-branched and cross-linked actin filaments meshwork. Filopodia and lamellipodia membrane extensions are essential for the forward motility of the cell (Campellone and Welsh, 2010).

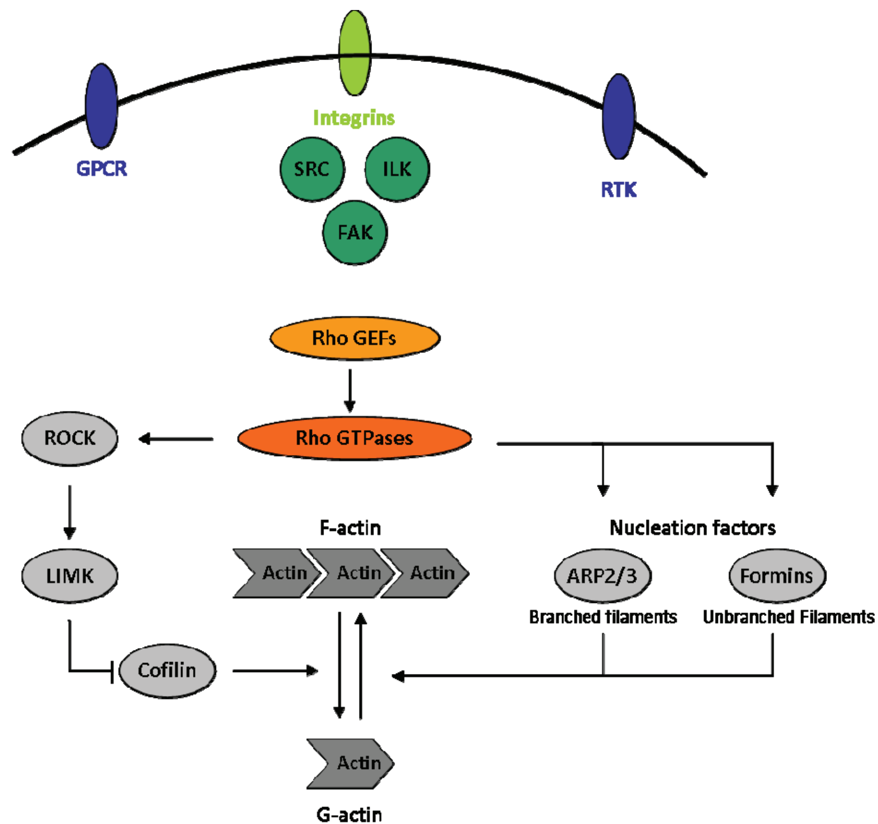


Figure 5. Regulation of actin dynamics by Rho GTPases (adapted from Olson and Nordheim, 2010). Actin polymerisation can be induced by signalling through different receptors as GPCR, Integrins and RTK. Integrins modulate the activity of several kinases, including SRC, ILK and FAK. Finally Rho GEFs activate Rho GTPases. RhoGTPases induce actin polymerisaton by two independent mechanisms. Activation of ROCK activates LIMK which inhibits cofilin-mediated actin depolymerisation (left). By modulating the activity of nucleation factors Rho GTPases can also induce actin polymerisation. Arp2/3 induces formation of branched filaments, while Formins induce the formation of unbranched filaments.

Stress fibres are the major contractile structures in the cell and are composed of bundles of 10 to 30 **actin filaments and myosin II**. Stress fibres are often anchored to focal adhesions (Pellegrin and Mellor, 2007; Tojkander *et al*., 2012). In detail, one distinguishes between three types of stress fibres. Myosin II containing **ventral stress fibres**, are attached to focal adhesions at both ends and constitute the major contractile type of stress fibres in the cell. In contrast, **dorsal stress fibres** are only anchored at their distal end to focal adhesions and do not contain myosin II, therefore are not contractile. **Transverse arcs** contain myosin II but are not attached to focal adhesions; they convey contractile force through their connection to dorsal stress fibres (Tojkander *et al*., 2012). In order to form stress fibres both ROCK and mDia activities are essential. mDia is necessary to form parallel actin filaments and ROCK for producing contractile stress fibres (Pellegrin and Mellor, 2007).

Stress fibre contractility emerges from the interaction between myosin II and actin filaments (**Figure 6**). Contractility is produced by the ATP-driven movement of the myosin II motor

domain along actin filaments. Stress fibre contractility is regulated by phosphorylation of the myosin light chain (MLC) of myosin II that increases the assembly of non-muscle myosin II filaments and the actin-activated ATPase activity of the myosin motor domain (Tojkander *et al.*, 2012). Phosphorylation of MLC is regulated by different mechanism: (i) ROCK either phosphorylates MLC directly or activates several myosin-regulating proteins (MRP) (Pellegrin and Mellor, 2007). (ii) Cdc42 activates MRCK that phosphorylates MLC (Leung *et al.*, 1998; Wilkinson *et al.*, 2005). (iii) Phosphorylation of MLC can be mediated by MLC kinase (MLCK) that is regulated by the Ca^{2+}/calmodulin pathway (Tojkander *et al.*, 2012).

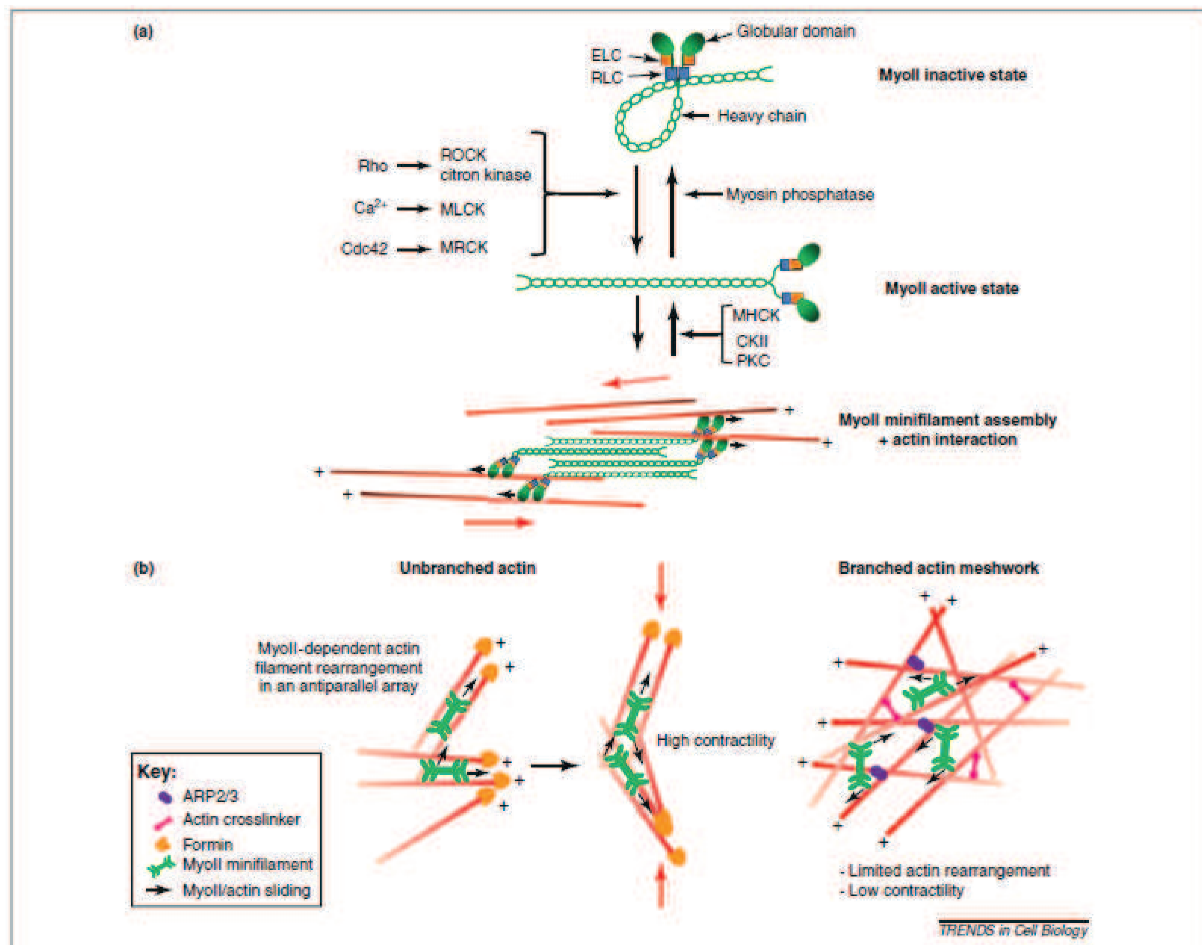


Figure 6. Regulation of stress fibre contractility (from Levayer and Lecuit, 2011). Myosin II is composed of a heavy chain, light chains (ELC and RLC, also called myosin light chain - MLC) and a globular domain. Activity of myosin II is regulated through phosphorylation of MLC by ROCK, MLCK or MRCK. In addition, myosin phosphatase decreases activity of myosin II by dephosphorylation of MLC. Myosin II interacts with actin filaments to form contractile stress fibres. Parallel arrangement of actin filaments is mediated by formins resulting in high contractility. Branched filament networks are induced by ARP2/3 and exhibit low contractility.

Stress fibres are stabilized by different actin-binding proteins. **Alpha-actinin** is an actin-cross-linking protein and is complementary localized to myosin II. **Tropomyosins** (TPM) are localized along actin filaments. They participate in the regulation of stress fibre contraction

and re-organization. They prevent filament depolymerisation at pointed ends and inhibit the actin filament disassembly that is mediated by factors severing actin filaments (ADF-actin depolymerisation factor/cofilin). Together with caldesmos TPMs promote actin stress fibre stabilization by promoting cross-linking of myosin bundles to actin filaments (Tojkander *et al.*, 2012).

1.3.3 The cytoskeleton as a regulator of transcription

Cells communicate their status of the actin filament compartment to the nucleus and induce changes in transcriptional regulation by various mechanisms/pathways. In the following section I will give a short summary about the major pathways that are regulated by actin dynamics. For a detailed overview about the topic see the review by Olson and Nordheim (2010).

1.3.3.1 NF-κB signalling

Thrombin-mediated activation of nuclear NF-κB is regulated by RhoA-ROCK signalling. This depends on dynamic alterations of the cytoskeleton, as both, treatment with the F-actin stabilizing drug Jasplakinolide as well as with the actin-disrupting drug Latrunculin B inhibited thrombin induced NF-κB activation (Fazal *et al.*, 2009). Also upon inhibition of cell-cell contacts Protein kinase D activated downstream of RhoA-ROCK signalling mediates the activation of nuclear NF-κB (Cowell *et al.*, 2009). In addition, it has been shown that activation of NF-κB by fluid shear stress requires focal adhesion kinase (Young *et al.*, 2010).

1.3.3.2 SRF/MKL1

The Myocardin-related transcription factors MKL1 and MKL2 (also termed MRTF-A/B or MAL/MAL16) are regulated by extracellular mitogenic signals or mechanical stimulations. Their activity is controlled by Rho-family GTPases mediated actin-polymerisation (Miralles *et al.*, 2003). MKL1/2 are sequestered in the cytosol upon binding of G-actin. When G-actin is polymerised into F-actin MKL1/2 are released and can enter to the nucleus (Pawlowski *et al.*, 2010), where they act as co-activators of the transcription factor serum response factor (SRF) to activate the transcription of genes with serum response elements (CArGboxes)-containing promoters (Cen *et al.*, 2003; Miralles *et al.*, 2003).

The SRF transcription factor can also interact with the family of ternary complex factors (TCFs), stimulated by the Ras-extracellular regulated kinase (ERK) signalling pathway, mediating SRF/TCF specific target gene expression, e.g. of *c-fos*. Of note, recruitment of either MKL1 or TCF to SRF seems to be mutually exclusive as both co-transcription factors contact the same surface on SRF, and multiple inhibitory crosstalks between the SRF/MKL1 and SRF/TCF pathways exist (Posern and Treisman, 2006; Descot *et al*., 2009).

1.3.3.3 YAP/TAZ

YAP (Yes-associated protein) and TAZ (transcriptional co-activator with PDZ-binding motif) were recently added to the list of co-transcription factors regulated by actin cytoskeleton dynamics. If localized nuclear they bind to the transcription factor TEA domain family member (TEAD) and mediate target gene expression (Vassilev *et al*., 2001; Mahoney *et al*., 2005; Zhao *et al*., 2008). YAP and TAZ are known downstream effectors of the Hippo pathway, which plays a role in controlling organ size and stem cell function (Zhao *et al*., 2011). The activated Hippo pathway mediates the phosphorylation of YAP/TAZ leading to their cytoplasmic re-localization or degradation (Bao *et al*., 2011).

Recently, it has been shown that YAP/TAZ are also regulated by cytoskeletal dynamics. YAP/TAZ activation is reduced when F-actin is disrupted by Latrunculin B or Cytochalasin D or when the RhoA pathway is inhibited (Dupont *et al*., 2011; Wada *et al*., 2011; Sansores-Garcia *et al*., 2011; Zhao *et al*., 2012). Stabilization of F-actin by over-expression of F-actin nucleator mDia increases YAP/TAZ activity (Dupont *et al*., 2011; Sansores-Garcia *et al*., 2011). Inhibition of ROCK, MLCK or myosin, which regulate the stress fibre contractility, results in reduced YAP/TAZ activity (Dupont *et al*., 2011; Wada *et al*., 2011). In contrast to MKL1, YAP/TAZ are not regulated by the ratio of G-actin versus F-actin, as expression of non-polymerisable G-actin has no effect on YAP/TAZ activity (Dupont *et al*., 2011). YAP/TAZ are probably rather regulated by cell spreading or a certain F-actin structure, e.g. contractile stress fibres, but the exact mechanism is not known so far (Halder *et al*., 2012).

1.3.4 Actin binding drugs

In order to study the role of the actin cytoskeleton on signalling pathways several actin modifying components can be used. I will shortly describe their effect on the actin cytoskeleton and, if known, the underlying mechanism.

Jasplakinolide both induces actin polymerisation and inhibits actin depolymerisation. Inhibition of actin depolymerisation is achieved by inhibiting the phosphorylation of Cofilin resulting in a decreased number of uncapped filament ends. Actin polymerisation is enhanced through spontaneous induction of nucleation sites. Jasplakinolide decreases the cellular pool of both free and sequestered monomeric G-actin. As Jasplakinolide induces rather the formation of "disordered" polymeric F-actin this treatment also leads to loss of stress fibres due to insufficient polymerisation-competent G-actin, which is necessary to maintain stress fibres during normal turnover (Bubb *et al*., 1994; Bubb *et al*., 2000, Visegrady *et al*., 2004). **Latrunculin B** binds G-actin monomers and therefore inhibits formation of filamentous actin (Spector *et al*., 1983). Like Latrunculin B, **Cytochalasin D** represses actin polymerisation, but by a different mechanism. Cytochalasin D inhibits elongation at both ends of the filament, but more strongly at the barbed end than at the pointed end. In addition, Cytochalasin D sequesters monomeric G-actin and hydrolysis the bound ATP to ADP (Sampath and Pollard, 1991). Cytochalasin D can be used at low concentration (0.2 µM) as it has high affinity for the barbed filament ends. At low concentrations Cytochalasin D inhibits membrane ruffling presumably by disrupting stress fibre anchorage to focal adhesions, while higher concentrations (2 – 20 µM) are necessary to remove stress fibres (Cooper, 1987; Wakatsuki *et al*., 2001).

1.3.5 TNC regulates actin dynamics

While FN promotes cell adhesion, which involves activation of integrin $\alpha5\beta1$ and integrin $\alpha v\beta3$ signalling, assembly of focal adhesions and stabilization of actin filaments, TNC specifically blocks the adhesion of cells to FN (Chiquet-Ehrismann *et al*., 1988; Spring, 1989 *et al*.; Murphy-Ullrich *et al*., 1991), and interferes with cell spreading, focal contact formation and actin stress fibre formation (Wenk *et al*., 2000; Huang *et al*., 2001; Orend *et al*., 2003) (**Figure 7**).

NIH-3T3 fibroblasts cultured in a three-dimensional fibrin matrix containing FN lack stress fibres and form actin-rich filopodia upon addition of TNC to the matrix. This is accompanied by reduced RhoA activity, but no difference in cdc42 activity is observed. Upon activation of RhoA by LPA, spreading and stress fibres can be restored in the presence of TNC. The same effect is observed upon overexpression of constitutively active RhoA (RhoA G14V) in Rat1 fibroblasts. If RhoA activity is inhibited by C3 transferase upon treatment with LPA no stress fibres can be formed in the presence of TNC, suggesting that TNC blocks cell spreading and stress fibre formation by inhibition of RhoA activity (Wenk *et al*., 2000). Furthermore, TNC reduces activity of FAK and focal adhesion assembly (Midwood and Schwarzbauer, 2002). TNC also inhibited contraction of the fibrin-FN matrix. The effect of reduced matrix contraction could be reversed by either stimulation with LPA or overexpression of constitutively active RhoA (G14V). Matrix contraction could be fully restored by synergistic treatment with LPA and phosphatase inhibitors preventing FAK dephosphorylation by TNC (Midwood and Schwarzbauer, 2002).

Syndecan-4 is essential for cell spreading on FN (Tumova *et al*., 2000; Woods and Couchman, 1994). REF52 overexpressing Syndecan-4, cultured on dishes coated with FN and TNC together, or grown in a TNC containing fibrin-FN matrix do eventually spread, form stress fibres and focal adhesions (Orend *et al*., 2003; Midwood, 2004). Furthermore, the spreading of Syndecan-4-null fibroblasts was unaffected if TNC was incorporated in the fibrin-FN matrix, while upon overexpression of Syndecan-4 cells behave the same way as wt fibroblasts that fail to spread and form stress fibres in the presence of TNC (Midwood *et al*., 2004).

Also in tumor cells, including T98G, KRIB, MDA-MB 435 and J82, TNC interferes with cell spreading, focal adhesion and actin stress fibre formation when cells are seeded on dishes coated together with FN and TNC in comparison to cells seeded on FN alone. Cell adhesion on a FN/TNC substratum was also blocked in CHO cells, irrespectively if they do not express $\alpha5\beta1$-integrin or overexpress $\alpha5\beta1$-integrin, suggesting that $\alpha5\beta1$-integrin is probably not a direct TNC target. However, cell spreading, focal adhesions and stress fibre formation can be restored by overexpression of Syndecan-4, but not Syndecan-1 or Syndecan-2. In addition, upon coating with a peptide (FNIII13) comprising the heparin binding site II (HepII) of FN, which is known to bind Syndecan-4, TNC-induced cell rounding was counteracted (Huang *et al*., 2001).

Downstream of integrin α5β1/syndecan-4 signalling TNC leads to reduced activation of FAK, reduced RhoA protein stability as well as reduced TPM1 RNA levels and protein stability (Ruiz *et al*., 2004; Lange *et al*., 2007; Lange *et al*., 2008). The FNIII13 domain of FN is able to restore levels of phosphorylated FAK (p-FAK), RhoA and TPM1 (Lange *et al*., 2008). Also upon overexpression of TPM1 cell spreading, focal adhesions and actin stress fibre formation can be restored (Ruiz *et al*., 2004).

Interestingly TNC induces cell rounding by a second mechanism, besides blocking of FN binding to Syndecan-4. TNC induces expression of EDNRA, an Endothelin-1 (ET-1) receptor. Upon blocking of EDNRA with the specific inhibitor BQ123, cell spreading can be restored, as well as p-FAK, RhoA and TPM1 protein levels (Lange *et al*., 2007). In contrast, activation of a second type of ET-1 receptor - EDNRB by ET-1 stimulation, abrogates cell rounding on FN/TNC and restores p-FAK, paxillin, RhoA and TPM1 expression. Inhibition of EDNRB by BQ788 restored cell rounding on a FN/TNC substratum (Lange *et al*., 2007).

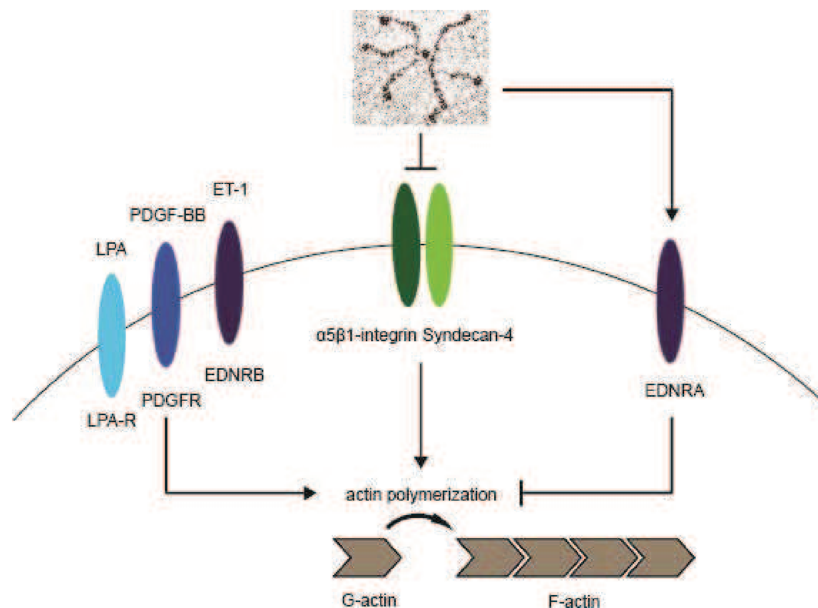


Figure 7. Regulation of actin depolymerisation by TNC. TNC binds FN and therefore prevents FN binding to the integrin α5β1 co-receptor Syndecan-4. FAK and RhoA signalling are blocked in the presence of TNC, preventing polymerisation of actin and stress fibre formation (middle). Cell spreading and stress fibre formation on a FN/TNC substratum can be restored by treating cells with growth factors LPA, PDGF and ET-1 (left). In addition, TNC induces expression of ENDRA. If ENDRA is inhibited by BQ123 cell spreading and stress fibre formation on FN/TNC is restored (right).

In addition, treatments with growth factors as LPA and PDGF-BB can restore cell spreading on FN/TNC by restoring p-FAK, p-Paxillin, RhoA and TPM1 protein levels; this mechanism is

independent of Syndecan-4, as cell spreading can still be induced in MEF (mouse embryonic fibroblasts) KO for Syndecan-4. Spreading on a FN/TNC substratum induced by LPA/PDGF-BB is dependent on Paxillin, as siRNA-mediated knockdown of Paxillin hampered cell spreading. Also overexpression of Syndesmos can restore cell spreading, which is dependent on Paxillin (Lange *et al*., 2008).

1.4 Wnt signalling

1.4.1 Canonical and non-canonical Wnt signalling

Canonical Wnt/β-catenin signalling plays a well-described role in embryonic development and tissue homeostasis (Logan and Nusse, 2004), but due to mutations of pathway members an over-activation of the pathway has been also implicated in tumor establishment and progression (Clevers, 2006; MacDonald *et al*., 2009).

In the absence of Wnt ligands the “destruction complex”, composed of Axin, Glycogen synthase kinase 3 (GSK3) and adenomatous polyposis coli gene product (APC) binds and phosphorylates the transcriptional co-factor β-catenin, hereby mediating β-catenin ubiquitination and proteosomal degradation (**Figure 8**).

Wnt signalling is activated by binding of Wnt ligands to frizzled (Fz or Fzd), a 7 transmembrane helix receptor, and the lipoprotein receptor-related protein 5/6 (LRP5/6) co-receptor. The formation of this ligand-receptor complex and the recruitment of Dishevelled (Dvl or Dsh) results in the recruitment of the destruction complex to the membrane. The phosphorylation of β-catenin is inhibited and therefore β-catenin is stabilized. β-catenin can travel to the nucleus, where it forms complexes with the transcription factor T-cell factor /lymphoid enhancer factor (TCF/LEF) family of proteins and activates the transcription of β-catenin/TCF/LEF target genes (McDonald *et al*., 2009) (**Figure 8**).

Besides activation of the canonical Wnt/β-catenin pathway, some Wnt ligands are able to activate non-canonical (β-catenin-independent) pathways (**Figure 8**). The Wnt/JNK (also referred to as Wnt/PCP - planar cell polarity) pathway is mediated by Fz and Dvl and downstream activation of small G-proteins (e.g. Rac, Rho, cdc42), leading to the activation of kinases such as Jun N-terminal kinase (JNK) and Rho-associated kinase. Another Wnt receptor, Ror2, has also been shown to activate cdc42 and JNK. The pathway is involved in

tissue polarity and cell migration; however, its exact role in mammals is not well understood (Kestler and Kuhl, 2008; Kikuchi *et al*., 2009).

The Wnt/Ca^{2+} pathway increases the intracellular Ca^{2+} concentration and increases the activity of the calcium sensitive enzymes calcium/calmodulin-dependent protein kinase (CaMK) II and protein kinase C (PKC), as well as the phosphatase calneurin which can activate NFAT further downstream (Kestler and Kuhl, 2008).

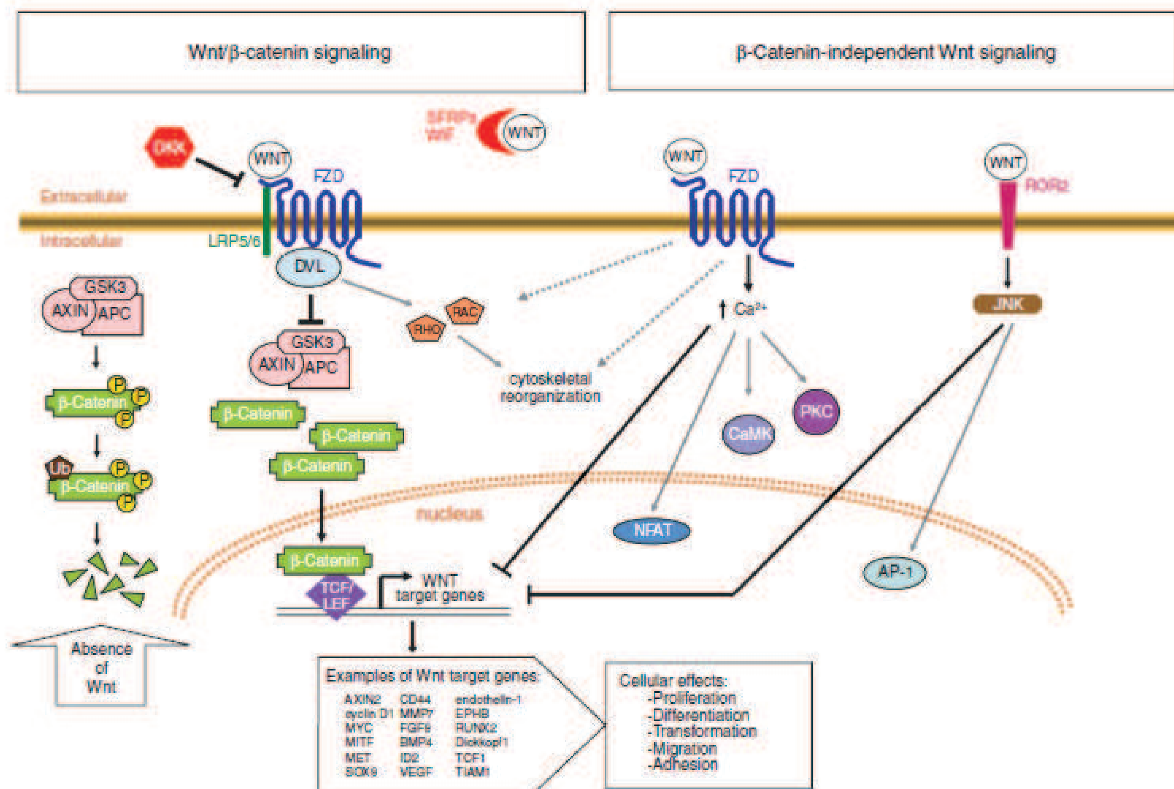


Figure 8. Wnt/β-catenin signalling and β-catenin-independent Wnt signalling (Chien *et al.*, 2009). (left panel) In the absence of Wnt ligands the destruction complex, composed of Axin, GSK3 and APC mediates the phosphorylation of β-catenin leading to its ubiquitination and degradation. (middle panel) Wnt ligands bind LRP5/6 co-receptor FZD mediating recruitment of DVL and the destruction complex, therefore preventing β-catenin degradation. Stabilized β-catenin accumulates in the nucleus where it binds transcription factors of the TCF/LEF family and activates transcription of target genes. (right panel) Non-canonical/β-catenin-independent Wnt signalling is independent of the LRP5/6 receptor and can also be activated by binding of Wnt to ROR2. The Ca^{2+}/Wnt pathway activates Ca^{2+}-sensitive downstream effectors, including CaMK and PKC and might activate transcriptional activity of NFAT. Activation of the Wnt/PCP pathway includes activation of JNK mediating transcriptional activity of AP-1. Of note, β-catenin-independent Wnt signalling has been demonstrated to inhibit β-catenin signalling.

In addition, it has been demonstrated that heparin sulfate proteoglycans (HSPG) can regulate Wnt signalling. For example, Syndecan-4, a transmembrane HSPG, has been shown to promote the non-canonical Wnt/PCP pathway by binding Fz7 and Dvl (Kikuchi *et al*., 2009).

Whether canonical or non-canonical Wnt pathways will be activated not only depends on the presented Wnt ligand(s), but also on the present receptors and is therefore very context and cell type-specific (Kestler and Kuhl, 2008; Kikuchi *et al*., 2009).

1.4.2 Inhibitors of Wnt signalling

The Wnt signalling pathway can be modulated by secreted, soluble inhibitors, including Wnt inhibitory factor (WIF), secreted frizzled related proteins (sFRP) and Dickkopf proteins (DKK) which antagonize the function of Wnt ligands. Within the sFRP family there are five members described, of which sFRP1, sFRP2 and sFRP3 are reported to bind Wnt and act as antagonist of Wnt signalling (Kawano and Kypta, 2003). sFRPs can either sequester Wnts and therefore prevent them from binding the LRP5/6-Frizzled receptor complex or sFRPs form non-functional receptor complexes by binding to Fz (**Figure 9**) (Cruciat and Niehrs, 2012). sFRPs can inhibit both canonical and non-canonical Wnt signalling. In addition, sFRP1 has been shown to bind Fz and act also as a Wnt agonist at low concentrations. Whether sFRP4 and sFRP5 act as Wnt inhibitors is not described so far (Kawano and Kypta, 2003).

Four members of the DKK family exist; however, DKK3 shares less sequence similarities as the other three group members with each other (Glinka *et al*., 1998). DKK proteins bind LRP5/6 and its co-receptor Kremen1/2, leading to the internalization of the receptor complex and its removal from the plasma membrane. However, new studies rather suggest that the disruption of the Wnt-induced Fz-LRP6 complex induced by DKK is sufficient for effective Wnt antagonism not requiring Kremen (**Figure 9**) (McDonald *et al*., 2009; Cruciat and Niehrs, 2012). DKK proteins inhibit only the canonical Wnt pathway.

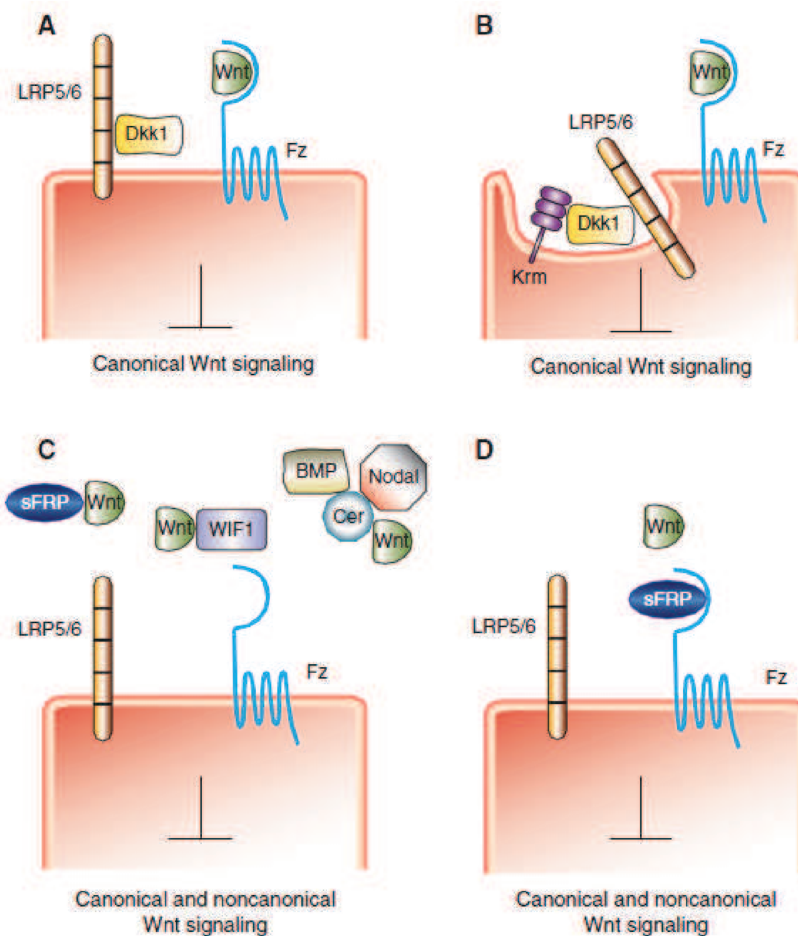


Figure 9. Mechanism of Wnt signalling inhibition. (A) DKK1 binds to LRP6 and thereby disrupts the Wnt-induced Fz-LRP6 complex formation) (B) and/or induces LRP6 endocytosis in the presence of its coreceptor Kremen (B). (C) sFRPs sequester Wnt, thereby inhibiting both canonical and non-canonical Wnt signalling. (D) sFRPs may also bind to Fz and thereby inhibiting canonical and noncanoncial Wnt signalling (Cruciat and Niehrs, 2012).

DKK1 has been described to exert β-catenin independent effects by activating the non-canonical Wnt/JNK pathway. In the β-catenin deficient H28 mesothelioma cell line and human placental choriocarcinoma cells DKK1-induced apoptosis was dependent on the JNK pathway (Lee *et al.*, 2004; Peng *et al.*, 2006). In other studies it has been shown that activation of DKK1 mediated activation of the Wnt/JNK pathway is involved in cardiogenesis, neurite outgrowth and β-amyloid toxicity (Pandur *et al.*, 2002; Endo *et al.*, 2008; Killick *et al.*, 2012). In xenopus and zebrafish it was demonstrated that the HSPG Knypek binds DKK1 and potentiates the ability of DKK1 in activating the Wnt/JNK pathway (Caneparo *et al.*, 2007).

DKK1 inhibits the migration of intestinal epithelial Caco-2 cells. DKK1 increases cdc42 activity (Koch *et al.*, 2009), which is a known downstream effector of the Wnt/PCP pathway. Cdc42 is also well-known to promote migration (Evers *et al.*, 2000), however, DKK1 leads to random

redistribution of cdc42 along the plasma membrane, away from the leading edge of the cell, which explains the defect in directional orientation of migrating cells in the presence of DKK1 (Koch *et al*., 2009).

Depending on the cellular context DKK2 can either function as an inhibitor or as an activator of the Wnt/β-catenin pathway. The expression levels of LRP5/6 seem to determine whether DKK2 acts an inhibitor or activator (Niehrs, 2006). Furthermore, it has been suggested that expression of the DKK co-receptor Kremen determines the activity of DKK2 to be an inhibitor of Wnt signalling (Niehrs, 2006). Induction of Wnt signalling by DKK2 can be inhibited by DKK1 (Wu *et al*., 2000).

The role of DKK3 in regulating Wnt signalling is not clear so far. In contrast to the other DKK family members it does not bind to LRP6 and Kremen (Niehrs, 2006). While there are studies showing that DKK3 does not exert any effect on Wnt signalling (Krupnik *et al*., 1999; Mao and Niehrs, 2003), other reports suggest that DKK3 acts as an inhibitor of Wnt signalling (Yue *et al*., 2007; Lee *et al*., 2009) or potentiates Wnt3A signalling by interacting with Kremen (Nakamura *et al.*, 2010). DKK3 has been also shown to regulate transforming growth factor-β (TGF-β) signalling (Pinho and Niehrs, 2007).

DKK4 binds LRP6 and hereby inhibits Wnt signalling (Krupnik *et al.,* 1999; Mao and Niehrs, 2003). It might also stimulate the non-canonical Wnt/JNK pathway (Hirata *et al*., 2011).

1.4.3 Regulation of DKK1 expression

DNA damage (Wang *et al*., 2000) and oxidative stress through induction of JNK (Colla *et al*., 2007) have been reported to induce DKK1 expression. Matrix rigidity, but not mechanical strain, represses DKK1 expression and induces Wnt signalling, probably through SRC activity (Barbolina *et al*., 2013).

Several transcription factor binding sites have been identified in the DKK1 promoter, including p53 (Wang *et al*., 2000; Killick *et al*., 2012), Nanog (Zhu *et al*., 2009), c-jun (Grotewold and Rüther, 2002), GATA6 (Zhong *et al*., 2011), SOX2 (Park *et al*., 2012) and glucocorticoids (Ohnaka *et al*., 2004). Interestingly, DKK1 is a Wnt target itself harbouring functional β-catenin/TCF-binding sites in its promoter (Gonzalez-Sancho *et al*., 2005) and DKK1 is transcriptional repressed by the Wnt target gene c-myc binding the DKK1 promoter

(Cowling *et al*., 2007). In addition, msx1 has been reported to inhibit DKK1 transcription (Menezes *et al*., 2012).

DKK1 expression can be regulated by several microRNAs, including miR-31 in lung cancer cells (Xi *et al*., 2010), miR-372 and miR-373 in breast cancer and CRC cells (Zhou *et al*., 2012), the miR-290 cluster in mouse embryonic stem cells (Zovoilis *et al*., 2009) as wells as miR-29 and miR-335-5b in osteoblasts (Kapinas *et al*., 2010; Zhang *et al*., 2011).

In many cancers the expression of DKK1 is reduced by various epigenetic mechanisms including i) CpG island promoter hypermethylation in CRC (Aguilera *et al*., 2006; Sato *et al*., 2007; Rawson *et al*., 2011), glioma (Foltz *et al*., 2010; Götze *et al*., 2010) breast cancer (Suzuki *et al*., 2008), lung cancer (Licchesi *et al*., 2008), multiple myeloma (Kocemba *et al*., 2012) and leukaemia (Suzuki *et al*., 2007; Valencia *et al*., 2009; Griffiths *et al*., 2010; Hou *et al*., 2011), ii) tobacco-smoke induced polycomb-mediated repression in lung cancer cells (Hussain *et al*., 2009) and iii) ASH1-mediated deacetylation and repressive trimethylation of lysine 27 of histone H3 in lung cancer (Osada *et al*., 2008).

1.4.4 Role of Wnt/β-catenin signalling in tumorigenesis, angiogenesis and metastasis

As especially documented for CRC, Wnt/β-catenin signalling activity can be highly elevated in human tumors due to mutations in the genes of Wnt/β-catenin signalling pathway members (e.g. APC, Axin2 or β-catenin) and contributes to tumor progression (Polakis, 2000). In other cancers epigenetic silencing of Wnt inhibitor genes contribute to the increased signalling activity of the Wnt/β-catenin pathway (Barker and Clevers, 2006). Wnt signalling has been documented to be important for survival and proliferation of various cancer cells (reviewed in Anastas and Moon, 2013).

As Wnt ligands, receptors and inhibitors are also expressed by endothelial cells (Goodwin *et al*., 2006) and Wnt/β-catenin signalling has been shown to be important for the control of vessel density in the retina of mice (Phng *et al*., 2009), it is likely that the pathway also plays a role in tumor angiogenesis. The Wnt inhibitors sFRP1 and WIF1 negatively regulate tumor angiogenesis (Hu *et al*., 2009). Interestingly, gene expression of *VEGFA*, playing an important role in formation of blood vessels in tumors (Yancopoulos *et al*., 2000), is regulated by β-catenin (Easwaran *et al*., 2003). However, only one study so far analysed the direct role of

Wnt/β-catenin in tumor angiogenesis. In a glioblastoma tumor model overexpression of Wnt1 in tumor cells leads to vessel normalization characterized by a better pericyte coverage resulting in reduced tumor growth (Reis *et al*., 2012). In addition, intracranial injection of GBM cells into mice expressing a constitutive active form of β-catenin in the endothelium resulted in reduced tumor vessel density. Vessels were more quiescent, shown by a smoother appearance of the basal lamina and closer distribution of collagen IV to the vessels. In addition, vessels were better covered with pericytes. Overall this study showed that Wnt/β-catenin signalling hampers GBM angiogenesis leading to quiescent and normalized vessels (Reis *et al*., 2012).

Several studies implicate also a role of Wnt/β-catenin signalling in metastatic progression of cancer. Wnt/β-catenin signalling enhances the migration and the invasive capability of cancer cells. In addition, Wnt/β-catenin signalling has been implicated in supporting the process of EMT, as well as upregulating the expression of MMPs and other factors necessary for cells to migrate through the ECM (reviewed in Anastas and Moon, 2013). Upon shRNA mediated β-catenin downregulation the incidence of metastatic lesions in a HCC xenograft model was reduced (Liu *et al*., 2010). In a melanoma mouse model β-catenin promoted the formation of metastasis (Damsky *et al*., 2011). Wnt target genes, such as MYC, CD44 and S100A4 have been also implicated in metastasis formation (Fodde *et al*., 2001; Stein *et al*., 2006). Other studies have shown that the expression of the ECM components POSTN and TNC enhances Wnt signalling in metastatic breast cancer cells which is important for the survival and growth of cancer cells at the metastatic site (Oskarsson *et al*., 2011; Malanchi *et al*., 2011; Oskarsson and Massagué, 2012).

1.4.5 Role of DKK1 in angiogenesis and metastasis

Several evidences exist that DKK1 is a potent regulator of the behaviour of endothelial progenitors and endothelial cells in healthy tissue as well as endothelial cells in pathological situations as cancer.

DKK1 has been demonstrated to stimulate the proliferation and tube formation of endothelial progenitor cells due to upregulation of VEGFR2 and CXCR4, receptors involved in promoting angiogenesis (Smadja *et al*., 2010). Furthermore, by inhibition of Wnt signalling in the bone marrow niche intra-peritoneally injected recombinant DKK1 mobilizes vasculogenic

progenitor cells and promotes their recruitment to newly formed blood vessels (Aicher *et al*., 2008).

In contrast several studies describe DKK1 as an inhibitor of endothelial cell proliferation, migration or tube formation *in vitro* (Ohlmann *et al*., 2010; Min *et al*., 2011; Kim *et al*., 2012), while, DKK2 promotes the ability of endothelial cells to proliferate, migrate, sprout and form tubes on matrigel. DKK2 exerts these functions by activation of cdc42 signalling via LRP6 but independently of Wnt/Fz signalling. Therefore, DKK1 might both inhibit angiogenesis by antagonizing canonical Wnt/β-catenin and/or antagonizing DKK2 functions (Min *et al*., 2011).

Recent studies suggest that DKK1 rather promotes than inhibits tumor angiogenesis. In breast tumors DKK1 transcript levels correlate with stromal derived factor-1 (SDF-1) and VEGF receptor 2 expression as well as the von Willebrand factor (vWF) content. In addition, DKK1 expression is colocalized with CD31 in human breast cancer tissue. In a breast cancer xenograft model injection of recombinant DKK1 in the tumor increases tumor size and CD31 staining (Smadja *et al*., 2010). Subcutaneous grafting of GBM cells overexpressing DKK1 results in an increase in tumor size. In addition, tumors are less hypoxic and have an increased vessel density than control tumors. Less nuclear β-catenin in the tumor endothelium is observed, suggesting that DKK1 functions in this context as an inhibitor of canonical Wnt/β-catenin signalling in vessels (Reis *et al*., 2012).

The effect of DKK1 on angiogenesis might be dependent on the growth factors present in the microenvironment. While DKK1 promotes bFGF-induced angiogenesis (Aicher *et al*., 2008; Smadja *et al*., 2010; Reis *et al*., 2012) it strongly blocks VEGFA-induced angiogenesis (Min *et al*., 2011) in *in vivo* matrigel plug assays. In addition, in other models and pathological situations DKK1 rather exerts anti-angiogenic functions. Treatment with recombinant DKK1 negatively affects lung vascularization in E11.5 organ cultures (De Langhe *et al*., 2005). DKK1 transgenic mice exhibited reduced vascular density in retinas and in an aortic ring assay recombinant DKK1 reduced sprouting (Min *et al*., 2011). In a mouse corneal pocket assay recombinant DKK1 inhibits VEGF-induced angiogenesis and in Tie2-DKK1 transgenic mice reduced angiogenesis in the developing bone occured compared to control mice (Oh *et al*., 2012).

Depending on the tumor type DKK1 exerts different effects on the metastatic progression of the cancer. In head and neck squamous cell carcinomas, oral squamous cell carcinoma and

nasopharyngeal carcinoma there is evidence that DKK1 inhibits metastasis (Katase *et al*., 2010; Li *et al*., 2010; Ogoshi *et al*., 2011). In gastric cancer, lung cancer and intrahepatic cholangiocarcinoma studies suggest that DKK1 promotes metastasis (Sheng *et al*., 2009; Gao *et al*., 2012; Shi *et al*., 2013). In hepatocelluar carcinoma and colon cancer conflicting studies exist, if DKK1 promotes or inhibits metastasis (Qin *et al*., 2007; Hao *et al*., 2010; Qi *et al*., 2012; Tao *et al.,* 2013).

In prostate and breast cancer DKK1 promotes osteolytic lesions while it inhibits Wnt-mediated osteoblastic bone metastasis. DKK1 inhibits Wnt signalling in the skeleton and therefore also the osteogenic activity of Wnts resulting in osteolysis at the metastatic site. If DKK1 levels are low enhanced Wnt signalling leads to osteoblastic activity resulting in osteosclerosis at the metastatic site in prostate cancer (Hall *et al*., 2006). Also in breast cancer DKK1 expression mediates osteolytic bone metastases (Voorzanger-Rousselot *et al*., 2007) as well as in multiple myeloma, where DKK1 reduces osteoblast proliferation and new bone formation (Clines *et al*., 2007).

2 Aims

My thesis was based on a microarray screening approach which had been aimed at identifying novel signalling pathways downstream of TNC *in vitro* and that are potentially implicated in tumor progression (Ruiz *et al*., 2004). By this approach it was found that TNC inhibits gene expression of Dickkopf 1 (*DKK1)*, encoding an inhibitor of canonical Wnt signalling, in T98G glioblastoma cells. While I worked on my thesis an *in vivo* insulinoma tumor model (Rip1Tag2 (RT2)) had been established in the laboratory where tumors with defined TNC expression levels had been analysed (Saupe, Schwenzer *et al.,* accepted). It was found that DKK1 expression levels inversely correlated with the TNC copy number. In the RT2 model it was also observed that TNC levels and tumor angiogenesis and lung micrometastasis correlated, providing the first proof of a promoting role of TNC in a stochastic and immune competent established tumor model to promote important early and late events in tumorigenesis (Saupe, Schwenzer *et al.,* accepted). Together with the *in vitro* results (Ruiz *et al*., 2004) these *in vivo* results suggested that TNC regulates DKK1 expression and that this regulation might have some implication in tumorigenesis. Therefore, I investigated the regulation of DKK1 by TNC in more detail.

The specific aims of my thesis were to:

1. Analyse the effect of DKK1 downregulation in tumor and stromal cells on Wnt signalling *in vitro*.
2. Develop a xenograft model to elucidate whether and how DKK1 and TNC impact on tumor angiogenesis.
3. Determine the mechanism of DKK1 downregulation by TNC.

3 Material and Methods

3.1 Molecular biology methods

3.1.1 Transformation of cells

Approximately 1 ng of plasmid DNA was added to 50 µl XL1b competent bacteria thawed on ice. After 30 minutes on ice, bacteria heat shocked placing the tube for 90 seconds at 42°C. Bacteria were stored again for 90 seconds on ice. 450 µl of LB-medium were added and bacteria were incubated for 60 minutes at 37°C. Bacteria were centrifuged (5 min, 3000 x rpm), the bacterial pellet was resuspended in 200 µl LB and plated on LB agar plates containing 100 µg/ml Ampicillin.

3.1.2 Plasmid DNA preparation

To obtain plasmids of a quantity and purity sufficient for analysis of successful cloning by restriction digest (plasmid Mini-preparation) bacteria were grown in 2 ml LB-medium containing 100 µg/ml Ampicillin overnight at 37°C, 220 x rpm. Bacteria were centrifuged (2.5 minutes at 5000 x rpm) and the pellet was resuspended in 200 µl STET buffer (8% sucrose/5% Triton X-100/ 50mM EDTA/50 mM Tris pH 8.0), 20 µl of fresh 10 mg/ml lysozyme was added. The suspension was heated for 40 seconds at 100°C and centrifuged 10 minutes at $1.6x10^3$ x g. DNA was precipitated by addition of 200 µl Isopropanol. The solution was incubated 10 minutes at room temperature followed by centrifugation at $1.2x10^3$ x g for 5 minutes. The pellet was washed with 70% Ethanol, dried and resuspended in H_2O.

To obtain plasmids of higher quantity and purity (plasmid Midi-preparation) 100 ml LB-medium containing 100 µg/ml Ampicillin were inoculated with transformed XL1b cells and grown overnight at 37°C. Plasmids were purified with the NucleoBond Xtra Midi Kit according to the manufacturer protocol (Macherey-Nagel).

3.1.3 PCR amplification of the promoter fragment

The proximal human DKK1 promoter was amplified from HCT116 genomic DNA using the following primers which possess restriction sites for the subsequent cloning in the pGL3-basic Luciferase reporter vector (Promega).

Fw-3K-BamHI-DKK1-Pm	5´- CGGGATCCGCTGTAAGAGAGACTCAGACAA - 3’
Rev-DKK1-Pm-NcoI	5’- GATCCATGGCTCAGAAGGACTCAAGAGGGAGA -3’

The PCR reaction mix (final volume 50 µl) was composed of:

150 ng genomic DNA
0.6 µl dNTP (25 nMol)
1 µl Pfx50 DNA Polymerase (Invitrogen)
5 µl 10x Pfx50 PCR Mix
0.2 µl Fw Primer
0.2 µl Rev Primer

The following PCR program was used:

94 °C – 2 minutes
94 °C – 15 seconds
59.6 °C – 30 seconds } 35 cycles
68 °C – 3.5 minutes
68 °C – 5 minutes

The PCR product was purified from the reaction mix with the NucleoSpin Extract Kit (Macherey-Nagel).

3.1.4 Restriction digest of DNA and cloning

The PCR product and the pGL3b vector were digested with BamH1/NcoI and BglII/NcoI, respectively. The reaction mix was composed of:

5 µl of NEBuffer (New England Biolabs)
0.5 µl BSA (if needed for enzyme activity; New England Biolabs)
1 µl Enzyme (New England Biolabs)
2 µg Insert or plasmid
Ad 50 µl H_20

The reaction was carried out for 2 hours at 37°C. Depending on the restriction enzymes used the restriction digest with two enzymes was carried out in parallel or sequential with an intermediate purification step with a spin column (NucleoSpin Extract II, Macherey Nagel). The digested plasmid and insert were purified by agarose gel electrophoresis. The DNA was extracted from the gel using the NuceoSpin Extract Kit.

Plasmid and Insert were ligated using the following conditions.

3 µl T4 DNA Ligase (0.1 Unit/µl) (Invitrogen)
4 µl 5x Ligase buffer (Invitrogen)
16 ng vector
3.33 ng Insert
Ad 19 µl H_2O

The reaction was incubated overnight at 18°C.

XL1-blue competent bacteria were transformed with 10 µl of the ligation reaction. Isolated bacterial colonies were grown overnight and plasmids were isolated by plasmid Mini-preparation. Insertion of the DKK1 promoter fragment was determined by Xho1 and BglII restriction enzyme analyses. Bacterial clones with the insert were amplified and plasmids were purified with a plasmid Midi-preparation. Plasmids were sequenced by GATC Biotech with the following primer:

pGL2 (forward) 5'-TCTTTATGTTTTTGGCGTC-3'
pGL3-RV (reverse) 5'-CTAGCAAAATAGGCTGTCC-3'

3.2 Cell biology methods

3.2.1 Culture of cell lines

Cell lines used in this study are described in **Table 1**.

Table 1. Cell lines used in this study.

CELL LINE	ORIGIN	REFERENCE
Tumor cell lines		
T98G	Human, GBM	ATCC: CRL-1690
KRIB	Human, osteosarcoma	Berlin *et al*., 1993
MDAMB 435	Human, melanoma (previously described as breast cancer)	ATCC: HTB
Caco2	Human,colorectal adenocarcinoma	ATCC: HTB37
βT2	Mouse, derived from RT2 mice	Gerhard Christofori, University of Basel
T98G:TPM1 C6	T98G overexpressing mouse Tropomyosin 1	Ruiz *et al*., 2004
T98G:shTPM1 4F3	T98G knockdown for Tropomyosin 1	Lange *et al*., 2008
T98G:Syndesmos	T98G overexpressing chicken (ch) Syndesmos	Lange *et al*., 2008
HEK293 cell line derivates		
HEK293 c18	Human, kidney, transformed with adenovirus 5 DNA, express EBNA1	ATCC: CRL-10852
HEK293 c18 : TNC	Overexpress human TNC	Lange *et al*., 2007
BOSC 23	Human, kidney; derived from ANJOU 65/HEK293 T; ecotropic envelope-expression packaging cell line	ATCC: CRL-11270
Endothelial cell lines		
HMVEC	Human dermal microvascular endothelial cells, immortalized with hTERT	Ellen van Obberghen-Schilling, Institute of Biology Valrose, Nice
cdc HMEC-1	Human dermal micovascular endothelial cells, immortalized	Ellen van Obberghen-Schilling, Institute of Biology Valrose, Nice

	with SV40	
BAEC (primary)	Bovine aortic endothelial cells	Ellen van Obberghen-Schilling, Institute of Biology Valrose, Nice
HUVEC (primary)	Human umbilical vein endothelial cells	Promo cell, C-12203
Pericytes		
Pericytes/HBVP (primary)	Human brain vascular pericytes	ScienCell, 1200
Fibroblasts		
L control (CTR)	Mouse	ATCC: CRL-2648
L Wnt3A	Mouse	ATCC: CRL-2647
MEF	Mouse embryonic fibroblasts	Lange *et al*., 2008
Cancer-Associated Fibroblasts		
ABD CT 5.1	Stromal cells from colon tumor obtained by tissue culture plastic adhesion	Olivier de Wever, Ghent University Hospital
ABD CT 14	Stromal cells from colon tumor obtained by tissue culture plastic adhesion	Olivier de Wever, Ghent University Hospital
hCT5.3-hTERT	Myofibroblasts obtained from colon tumor, hTERT immortalization	Olivier de Wever, Ghent University Hospital
Monocyte/Macrophages		
RAW 264.7	Derived from BALB/c mice, Abelson murine leukaemia virus-induced tumor	ATCC: TIB-71
J774A.1	Derived from BALB/c mice, reticulum cell sarcoma	ATCC: TIB-67

T98G, KRIB, MDAMB-435, HEK293 c18, L CTR, L Wnt3A, pericytes, hCT5.3-hTERT, MEF and βT2 cells were cultured in DMEM 4.5 g/l glucose with 10% FCS, 1% Pen/Strep, 40 µg/ml gentamicin. Macrophage cell lines were cultured in the same medium without addition of Pen/Strep. BOSC23 cells were grown in DMEM 4.5 g/l glucose with 10% FCS, 1% Pen/Strep, 1 mM NaPyruvate and 10 mM HEPES. HUVEC were cultured in Endothelial Cell Growth Medium (PromoCell, C-22010). ABD CT5.1/14 were cultured in DMEM 1 g/l Glucose with 10% FCS, 1% Pen/Strep. BAEC were cultured in DMEM 1 g/l glucose with 5% FCS, 1% Pen/Strep, 40 µg/ml gentamicin. Cdc.HMEC and HMVEC were cultured in MCDB 131 with 12.5% FCS, 1% Pen/Strep, 1µg/ml hydrocortisone, 10 ng/ml heparin, 10 ng/ml EGF, 10 ng/ml bFGF. T98G:Syndesmos, T98G:TPM1, T98G:sh TPM1 and control cell lines were cultured in the same medium as above upon adding 400 µg/ml G418. HEK293 c18:TNC were cultured with 2.5 µg/ml Puromycin. For indicated experiments cells were starved overnight with 1% serum or no serum with the same medium as described above. HUVEC were starved overnight in M199 with 1% FCS, 1µg/ml hydrocortisone, 10 ng/ml heparin, 10 ng/ml EGF, 10 ng/ml bFGF.

All cells were passaged by trypsinization (0.25% trypsin in versene solution), except monocyte/macrophage cells that were passaged by scrapping with a cell scrapper.

3.2.2 Collection of conditioned medium

L CTR and L Wnt3A cells, as well as KRIB:CTR and KRIB:mDKK1 cells were grown in DMEM 4.5 g/l glucose, 10% FCS, but without selection antibiotic. Conditioned medium (CM) was collected from confluent grown cells after 3 days of culture and which was stored at 4°C. After another 3 days of growth CM was collected and mixed with the first batch, filter-sterilized, aliquoted and stored at -20°C until use.

3.2.3 Growth factor and drug treatment

For growth factor or drug treatment cells were starved overnight. The respective solvent was used as control treatment, see **Table 2**. If growth factor treatment was carried out on FN or FN/TNC, cells were first seeded on the matrix and growth factors were added after 1 hour. The following drugs and growth factors were used in this study:

Table 2. Conditions used for drug and growth factor treatment.

Drug/Growth Factor	Solvent	Concentration Used	Company
Lysophosphatidic Acid (LPA)	H_20	1 – 30 µM	Santa-Cruz, Biotechnology
Platelet derived growth factor BB (PDGF-BB)	H_20/BSA	80 ng/ml	Sigma-Aldrich
Actinomycin D	DMSO	5 µg/ml	Sigma-Aldrich
Latrunculin B	DMSO	0.5 – 2 µM	Calbiochem
Cytochalasin D	DMSO	0.2 – 2 µM	Calbiochem
Jasplakinolide	DMSO	0.1 – 2 µM	Santa-Cruz, Biotechnology

3.2.4 Retroviral particle production and cell transduction

Retroviral Particles were used in order to establish HEK293 cells and HEK293:TNC cells stably expressing mDKK1 or control plasmid. To generate retroviral particles BOSC cells were transfected with pQXIB empty or pQXIB:mDKK1 plasmid, together with pCl Ampho encoding for the viral envelop (JetPei transfection reagent). After 24 hours medium of BOSC was refreshed. After another 24 hours the CM of BOSC was collected and filter-sterilized (0.45 µM filter). HEK293 cells were transduced by incubation with BOSC CM containing retroviral particles. After 24 hours of incubation medium was removed and transduced cells were selected with 4 µg/ml Blasticidin.

3.2.5 Lentiviral transduction of cells

The silencing of DKK1 and MKL1/2 was done by use of short hairpin (sh) mediated gene expression knockdown (kd). For sequences and clone IDs used in this study, see **Table 3**. For generation of KRIB shDKK1 cells, KRIB cells were transduced with MISSION lentiviral transduction particles (Sigma-Aldrich) or MISSION non-target shRNA control transduction particles (SHC002V, Sigma-Aldrich) with a MOI=10, transduced cells were selected with 10 µg/ml puromycin.

For generation of T98G shMKL1/2 and KRIB shMKL1/2 cells were transduced with MISSION lentiviral transduction particles (Sigma-Aldrich) or MISSION non-target shRNA control transduction particles (SHC002V, Sigma-Aldrich) with a MOI=1, transduced cells were selected with 2.5 µg/ml puromycin.

Table 3. TRC numbers and shRNA sequence.

GENE	NAME	CLONE ID	SEQUENCE (5'-3')
DKK1	Sh1	TRCN0000033385	CCGGCGGTTCTCAATTCCAACGCTACTCGAGTAGCGTTGGAATTGAGAACCGTTTTTG
	Sh2	TRCN0000033386	CCGGCCTGTCCTGAAAGAAGGTCAACTCGAGTTGACCTTCTTTCAGGACAGGTTTTTG
	Sh3	TRCN0000033387	CCGGGCCAGTAATTCTTCTAGGCTTCTCGAGAAGCCTAGAAGAATTACTGGCTTTTTG
	Sh4	TRCN0000033388	CCGGCCAGAAGAACCACCTTGTCTTCTCGAGAAGACAAGGTGGTTCTTCTGGTTTTTG
	Sh5	TRCN0000290003	CCGGCCAGAAGAACCACCTTGTCTTCTCGAGAAGACAAGGTGGTTCTTCTGGTTTTTG
MKL1	Sh1	TRCN0000083563	CCGGGCGGAGAAATTTCAGCAGATTCTCGAGAATCTGCTGAAATTTCTCCGCTTTTTG
	Sh2	TRCN0000083564	CCGGCCTCACCAATGGAACCACTATCTCGAGATAGTGGTTCCATTGGTGAGGTTTTTG
	Sh3	TRCN0000083565	CCGGGACTATCTCAAACGGAAGATTCTCGAGAATCTTCCGTTTGAGATAGTCTTTTTG
	Sh4	TRCN0000083566	CCGGCGCCACCTCTATCCTGCACAACTCGAGTTGTGCAGGATAGAGGTGGCGTTTTTG
	Sh5	TRCN0000083567	CCGGGCTCAAGTACCACCAGTACATCTCGAGATGTACTGGTGGTACTTGAGCTTTTTG
MKL2	Sh1	TRCN0000015393	CCGGCCAGTGTTAAAGAAGCAATTACTCGAGTAATTGCTTCTTTAACACTGGTTTTT
	Sh2	TRCN0000015394	CCGGGCAGACACTTTCACCGAGATTCTCGAGAATCTCGGTGAAAGTGTCTGCTTTTT
	Sh3	TRCN0000015395	CCGGCCTCATTAAGAGTGGAGAGATCTCGAGATCTCTCCACTCTTAATGAGGTTTTT
	Sh4	TRCN0000015396	CCGGGCCGGTTACAACACTACACAACTCGAGTTGTGTAGTGTTGTAACCGGCTTTTT
	Sh5	TRCN0000015397	CCGGGCCAGCTTAGAGAACCAACTACTCGAGTAGTTGGTTCTCTAAGCTGGCTTTTT

3.2.6 Transient and stable transfection of cells

For transient transfection (JetPEI, Polyplus) with plasmids encoding RhoA wt (Addgene Plasmid 12962: pRK5-myc-RhoA-wt), RhoA-Q63L (Addgene Plasmid 12964: pRK5-myc-RhoA-Q63L), RhoA-T19N (Addgene Plasmid 12963: pRK5-myc-RhoA-T19N), MKL1 wt (pEF fl MAL HA, provided by Guido Posern) and ΔN MKL1 (pEF HA-ΔN MAL provided by Guido Posern) $3x10^5$ cells were seeded in 6 well dishes the day before transfection with 800 ng plasmid per well. Empty pCB6 was used for control transfection. Successful transfection was monitored by co-

transfection of 200 ng pQCXIP-GFP. Transfected cells were starved overnight in medium without serum. 24 hours after transfection cells were either analysed by Western Blot and qRT-PCR or were seeded on FN or FN/TNC coated dishes and lysed after 5 hours.

In order to establish HEK:CTR and HEK:mKK1 cells stable expressing the pCEB control plasmid, cells were transfected with the pCEB plasmid and transfected cells were selected with 2.5 µg/ml Puromycin.

In order to establish KRIB:CTR and KRIB:mDKK1 cells stable overexpressing TNC the pQCXIB control or pQCXIB:TNC plasmid were linearized by digestion with SspI restriction enzyme. Linearization was checked on an agarose gel and linearized plasmid was purified by the NucleoSpin Extract II Kit (Macherey Nagel). KRIB:CTR and KRIB:mDKK1 cells were transfected with the linearized plasmid and transfected cells were selected by 5 µg/ml Blasticidin.

3.2.7 Immunofluorescence staining

Cells were fixed in 1% PFA for 10 minutes and permeabilized in PBS-Triton 0.1% for 10 minutes. Cells were stained with the in **Table 4** indicated antibodies or phalloidin probe. Cells were incubated with primary antibodies for 2 hours at RT, secondary antibodies for 1 hour and with the Phalloidin probe for 20 minutes. Between incubation steps cells were washed with PBS. Nuclei were stained with DAPI (1:50,000, Sigma D9542).

Table 4. Fluorescence probes and primary and secondary antibodies used for immunofluorescence staining.

TARGET	REFERENCE	CONCENTRATION	HOST
TPM1, TPM2, TPM3	Clone TM311, Sigma	1:1000	mouse
Vinculin	Abcam	1:50	mouse
Phalloidin-Tetramethylrhodamine B isothiocyanate	Sigma P1951	1:200	-
Cyanine 3	Jackson ImmunoResearch	1:800	
Alexa-488	Jackson ImmunoResearch	1:800	

3.3 Biochemical assays

3.3.1 Luciferase reporter assays

Luciferase reporter assays were carried with 80.000 cells/well seeded in 24 well plates.

For the β-catenin luciferase reporter assay cells were transiently transfected with the Super

8x TOPFlash (8 TCF/LEF binding sites) or control Super 8x FOPFlash (mutant TCF/LEF binding sites) (Veeman *et al.*, 2003 (obtained from Addgene, plasmids 12456 and 12457) as well as pRL-TK (TK-Renilla). Upon seeding for 5 hours on matrix-coated dishes, CM containing Wnt3A, mDKK1 or CTR medium was added for a total of 48 hours. TOPFlash luciferase activity was normalized to FOPFlash activity.

For DKK1 promoter luciferase assay T98G cells were transiently transfected with the pGL3-DKK1 promoter construct or empty pGL3-basic vector as well as pRL-TK. The luciferase assay was carried out 40 hours after transfection. Luciferase activity is presented as the ratio of pGL3-DKK1 to pGL3-basic.

For SRF luciferase reporter assays T98G were transfected with the 3DA.Luc plasmid (provided by Guido Posern) encoding for 2 c-fos derived SRF binding sites and pRL-TK plasmid for normalization of luciferase signal. Cells were starved overnight before drug treatment or seeding cells on FN or FN/TNC substrata.

Cells were lysed with Passive Lysis Buffer (Promega). Firefly and Renilla luminescence were detected by the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity.

3.3.2 Western Blotting

For obtaining protein lysates from cultured cells (6 well dishes), cells were washed with cold PBS and 100 µl of lysis buffer (150 mM NaCl/ 50 mM Tris pH 8/ 1% Igepal CA-630) was added. The lysate was vortexed and incubated 10 minutes on ice. After vortexing again the samples were centrifuged at 13000 rpm (centrifuge 5415D, Eppendorf), 4°C for 15 minutes. The supernatant containing the protein lysate was stored at -20°C until use. Protein concentration was determined by Bradford Assay using the Protein Assay reagent (500-006, Bio-Rad) with a BSA standard curve. 15 – 30 µg of protein were diluted 1:1 in 2x Laemmli buffer (125 mM Tris pH 6.8/4% SDS/10% Glycerol/5% β-Mercaptoethanol/Bromphenolblue) and loaded on SDS-Polyacrylamid gels (8% or 12% with a 4% stacking gel). Gels were run with the following running buffer: 1 Tris/Glycine with 0.2% SDS. Proteins were transferred onto PVDF membranes with a pore size of 0.45 µm (IPVH00010, Immobilon) for 90 minutes with 240 mA with cold 1x Tris/Glycine/20% Methanol blotting buffer. Successful blotting was verified by incubating the membrane with the Ponceau-S dye (81462, Sigma). The membrane was blocked with 5% Blocking-Grade Blocker (170-6404, Biorad) in PBS/0.1%

Tween for 30 minutes. The membrane was incubated with the primary antibody in 1.5% Blocking-Grade Blocker in PBS/0.1% Tween overnight at 4°C. After washing (3x 10 minutes) with PBS/0.1% Tween, the secondary antibody in 1.5% Blocking-Grade Blocker in PBS/0.1% Tween (Horseradish Peroxidase linked) was applied for 45 minutes at RT. Concentrations of the primary and secondary antibody can be found in **Table 5**.

Table 5. Primary and secondary antibodies for Western Blot.

TARGET	REFERENCE	CONCENTRATION	HOST
Primary antibodies			
DKK1	D3195, Sigma-Aldrich	1:400	rabbit
DKK1	AF1096, R&D	1:400	goat
MKL1	HPA030782, Sigma-Aldrich Prestige	1:500	rabbit
α-tubulin	CP06, oncogene research products	1:2000	mouse
6xHis tag	ab18184, Abcam	1:1000	mouse
TNC 1.2	Homemade	1:1000	rabbit
RhoA (26C4)	sc-418, Santa-Cruz	1:1000/1:5000	mouse
Secondary antibodies			
Anti-rabbit IgG	NA934V, GE Healthcare	1:10,000	donkey
Anti-mouse IgG	NXA931, GE Healthcare	1:10,000	sheep
Anti-goat IgG	sc-2020, Santa Cruz	1:5,000	donkey

Immunocomplexes were revealed by addition of detection reagents Amersham ECL Western Blotting detection reagent (RPN2106, GE Healthcare) or Amersham ECL Plus Western Blotting Detection System (RPN2132, GE Healthcare) and the emitted signal was captured in an Amersham hyperfilm ECL (28906837, GE Healthcare).

3.3.3 RNA isolation and quantitative RT-PCR

RNA was isolated (NucleoSpin RNA extraction kit, Macherey-Nagel or Trizol, Life Technologies) according to the manufacturer's instructions. RNA was reverse transcribed (MultiScript reverse transcriptase, Applied Biosystems). Quantitative polymerase chain reaction (qPCR) was done on cDNA diluted 1:2.5 in water with specific primers (for sequences see **Table 6** on a 7500 Real Time PCR system (Applied Biosystems) using SYBR green reaction mixture (Applied Biosystems). Relative gene expression levels of analysed target genes were determined by the $2^{-\Delta\Delta ct}$ method. The ct value of the gene of interest was normalized to the ct value of a housekeeping gene (ΔCt). This value was normalized to the

ΔCt of the control group. Determination of the fold change was done using the formula: Fold change = $2^{-\Delta\Delta Ct}$.

Table 6. Primers used for qRT-PCR and RT-PCR analysis. TPM1 and TPM2 primer sequences are from Lange *et al.*, 2007, RhoA primer sequences are from Sauzeau *et al.*, 2003.

GENE	FORWARD PRIMER (5'-3')	REVERSE PRIMER (5'-3')
Human Primers		
Axin2	CCACACCCTTCTCCAATCC	TGCCAGTTTCTTTGGCTCTT
β2-Microglobulin	GTGGGATCGAGACATGTAAGCA	AATGCGGCATCTTCAAACCT
Cdc42-effectorprotein 3	GCATCTCAGTCCAGCCAAG	GCCAGTCGGGGTACTGTTC
c-fos	GTCCTTACCTCTTCCGGAGATGT	ACTAACTACCAGCTCTCTGAAGTGTCA
DKK-1	GACCATTGACAACTACCAGCCG	TACTCATCAGTGCCGCACTCCT
DKK-2	GGCAGTAAGAAGGGCAAAAA	CCTCCCAACTTCACACTCCT
DKK-3	GAGGACACGCAGCACAAA	TGCCAGGTTCACTTCTGATG
DKK-4	AGGAGGTGCCAGCGAGAT	CATCTTCCATCGTAGTACAAACATC
Fos-like antigen 1	AGTTACCCCAGGCCTCTGAC	CTTCCTCCGGGCTGATCT
MKL1	TAGTGAGCGGAAGAATGTGC	ATCCCTTGGCTCACCAGTT
MKL2	CTTACCCCCTCTGAACGAAA	CTCTCGTCCTCCTTTGTTGC
SFRP1	GCTGGAGCACGAGACCAT	TGGCAGTTCTTGTTGAGCA
SFRP2	GCTTGAGTGCGACCGTTT	CAGGCTTCACATACCTTTGGA
SFRP3/FRZB	GGGCTATGAAGATGAGGAACG	CTGAGTCCAAGATGACGAAGC
SFRP4	CGATCGGTGCAAGTGTAAAA	ACCACCGTTGTGACCTCATT
SRF	AGACGGGCATCATGAAGAAG	TGATCATGGGCTGCAGTTT
TNC	GACTCAGAAGCCTTGGCCA	CCTGTGTAGGAGATGACATA
Tropomyosin 1	GCACCGAAGATGAACTGGACAA	CATCGGTGGCCTTTTTCTCTG
Tropomyosin 2	CCAACAACTTGAAATCCCTGG	CTTTGGTGGAATACTTGTCCGC
RhoA	GCAGGTAGAGTTGGCTTTATGG	CTTGTGTGCTCATCATTCCGA
Zyxin	CATGAAGTGTTACAAGTGTGAGGAC	AGTGTGGCACTTCCGACAG
Mouse Primers		
DKK1	CTGAAGATGAGGAGTGCGGCTC	GGCTGTGGTCAGAGGGCATG
MKL1	GCCAACCTGGATGACATGA	GAAGGGACCGCAACTTCA
Tropomyosin 1 (also human)	CCCGTAAGCTGGTCATCATC	TTCTTCAAGCTCGGCACATT

For mRNA expression analysis by RT-PCR RNA was reverse transcribed as described above and 3 µl of the cDNA were used for the following PCR reaction mix and PCR program, primer sequences are shown in **Table 7**.

12.5 µl 2x Hot Goldstar Red Master (Eurogentec)
0.25 µl forward Primer (100 µM)
0.25 µl reverse Primer (100 µM)
3 µl cDNA
Ad 25 µl H_2O

95 °C – 1 minute
95 °C – 30 seconds
60 °C – 30 seconds 35 cycles
72 °C – 1 minute
72 °C – 7 minutes

Table 7. Primers used for RT-PCR.

GENE	FORWARD PRIMER (5'-3')	REVERSE PRIMER (5'-3')
Syndesmos (chicken)	AACTTCCTGGCCAACTCCTT	GCGTATTCATTGCCTGCTCT
GAPDH (human)	ATCTTCTTTTGCGTCGCCAG	AATCCGTTGACTCCGACCTTC
DKK1 (bovine)	AGCCGGTGCGCAAATCTG	CGATTTCCCCTCGATGGAAATG
β2-Microglobulin (bovine)	ACATCTGGGTGTCTTCTGC	GCAAGATGCTTCATCTACAATCTC

3.4 Protein purification

Protein purification was carried out with the ÄKTA Prime Plus (GE Healthcare) with the Prime view 5.0 Software.

3.4.1 FN protein purification

FN was purified from filter-sterilized horse serum (Amimed, Bioconcept, Allschwil). A gelatine-agarose (Sigma) column was equilibrated with PBS. The serum was loaded on the column and the column was washed with PBS until the OD280 reached again the baseline. Triton-buffer (1 M NaCl/0.01 M Tris-HCl pH 8.3/0.05% Triton X-100) was applied on the column. The column was washed with PBS before applying Elution buffer (PBS/4M Urea). The protein concentration of the collected samples was determined by measurement of the OD280. Fractions with an OD280 higher than 0.2 were pooled. Pooled fractions were dialyzed (Cellu Sep T3 cellulose tubular membrane, nominal MWCO: 12,000 – 14,000) 2x for 2 hours and 1x overnight against PBS at 4°C. Aliquots were frozen in liquid nitrogen and stored at -80°C.

3.4.2 TNC protein purification

Conditioned medium containing human TNC with a C-terminal Histag (Lange *et al*., 2007) was collected from HEK293 c18:TNC grown for 2 days in DMEM without supplements. Conditioned medium was filtered over a bottle top filter (0.22 µM, Stericup, Millipore) and protein was precipitated by adding 29.1 g Ammonium sulphate per 100 ml CM and stirring for 2 hours at 4°C. Precipitated protein was enriched by centrifugation at 12000 x g for 20 minutes. The precipitate was resuspended in PBS/T (0.01% Tween-20) and dialyzed 2x for 2 hours and one time overnight at 4°C against PBS/T.

Dialysed protein was centrifuged at 12000 x g for 10 minutes. In order to remove FN from the sample, the supernatant was passed over a gelatine-agarose column equilibrated with PBS/T. The flow through was collected and adjusted to the same concentrations as the equilibration buffer (250 mM Sodium Phosphate/450 mM NaCl/20 mM Imidazol/500 mM Urea). Ni^{2+} resin (Nalgene, Jena Bioscience) equilibrated with Equilibration buffer was incubated with the protein sample on an overhead-rotator at 4°C overnight.

The Ni^{2+} beads were packed in a column. The flow through was again passed over the column. The column was washed with the equilibration buffer until optical density was back to baseline. The column was washed with 250 mM Sodium Phosphate/450 mM NaCl/20 mM Imidazol and protein was eluted with 250 mM Sodium Phosphate/450 mM NaCl/300 mM Imidazol. Fractions with an OD280 higher than 0.2 were pooled. Pooled fractions were dialyzed 2x for 2 hours and 1x overnight against PBS/T at 4°C. Aliquots were frozen in liquid nitrogen and stored at -80°C.

Protein fractions were analysed by Western Blotting or on an 8% SDS gel, which was stained with a 45% Methanol/15% Acetic Acid/0.025% (w/v) Coomassie Blue solution and destained with a 5% Methanol/7.5% Acetic Acid solution.

3.4.3 Coating with FN and TNC

Plastic culture dishes were coated with FN alone (1 µg/cm²) for 1 hour at 37°C, or first with FN (1 µg/cm²) for 1 hour at 37°C and after washing with PBS with TNC (1 µg/cm²) in PBS/0.01% Tween-20 for 1 hour at 37°C. Uncoated surfaces were saturated with 1% heat-inactivated BSA/PBS for 1 hour at 37°C. Before cell seeding dishes were dried under a cell culture hood.

3.5 Heterotopic xenograft model

5 x 10^6 HEK293 control, HEK293:TNC, HEK293:mDKK1 or HEK293:TNC:mDKK1 (in 100 µl PBS) were injected with a 25G needle in both flanks subcutaneously of 6 weeks old female nude mice (Charles River). Tumor size was measured twice weekly with a calliper. Mice were sacrificed 11 weeks after injection.

3.6 Statistical analysis

The GraphPad Prism software (version 5) was used for graphical representations of data and statistical analyses to assess significance of observed differences. Differences were considered significant when p-values were less then 0.05 as analysed by an unpaired student's p-test.

4 Results

4.1 TNC induces Wnt signalling in tumor cells by downregulation of DKK1

In order to identify novel downstream signalling mechanisms, implicated in tumor progression and regulated by TNC, potential downstream targets had been identified by a microarray profiling approach (Ruiz *et al*., 2004). Therefore, T98G cells had been seeded on dishes either coated with FN alone or FN and TNC together for 12 hours. By this approach DKK1 was identified as a gene whose expression was differentially regulated on the FN/TNC substratum as compared to FN. DKK1 is a soluble inhibitor of Wnt signalling (Glinka *et al*., 1998) and was 2.8-fold reduced in the presence of TNC (Ruiz *et al*., 2004).

4.1.1 TNC downregulates DKK1 in tumor cells

In order to analyse if DKK1 downregulation by TNC is a general effect on tumor cells several tumor cell lines of different origin, including T98G GBM, KRIB osteosarcoma, MDAMB 435 melanoma, Caco2 colorectal cancer and MCF-7 breast cancer cells, were seeded on FN or FN/TNC coated dishes (**Figure 10A**) and DKK1 mRNA expression was analysed by qRT-PCR and Western Blot. In addition, HEK293 cells and βT2 cells, derived from tumors of the RT2 mouse model, were included in the analysis. Whereas T98G, KRIB, MDAMB435, Caco2, MCF7, βT2 and HEK293 cells adhered on the FN substratum they remained rounded or were poorly spread (all but MCF7) or were smaller and exhibited a mesenchymal-like phenotype (MCF7), respectively when grown on FN/TNC (**Figure 10A**). In all analysed cell lines, except HEK293 and βT2, DKK1 expression levels were reduced in the presence of TNC (**Figure 10B**). In particular, in KRIB cells *DKK1* gene expression was 10-fold reduced on FN/TNC, while in Caco2 DKK1 levels were only 2-fold reduced (**Figure 10B**). In HEK293 cells DKK1 levels were not reduced, while βT2 cells did not express DKK1. In T98G grown for up to 12 days on FN/TNC DKK1 levels were still 5-fold reduced (**Figure 10C**). In contrast, already after 5 hours of seeding cells on FN/TNC, *DKK1* gene expression was 33-fold reduced, demonstrating a fast and long-lasting effect of TNC on *DKK1* gene expression levels in tumor cells (**Figure 10C**). As shown by Western Blot analysis of cell lysates DKK1 protein expression was also reduced in the presence of TNC in KRIB and T98G cells (**Figure 10D**).

In order to analyse if TNC might regulate the expression of other Wnt inhibitors in a similar manner, gene expression of secreted Wnt inhibitors of the DKK and sFRP families in T98G,

KRIB and MDAMB 435 grown on FN or FN/TNC were analysed by qRT-PCR (**Figure 10E**). SFRP1 was 1.6- and 3-fold downregulated in T98G and KRIB respectively, while it was not expressed in MDAMB 435. Also SFRP4 was 3-fold downregulated in T98G, while it was not expressed in KRIB and MDAMB 435. In contrast, SFRP3 was significantly upregulated, both in T98G and MDAMB 435, but no change in its expression was observed in KRIB. DKK2 was only expressed in KRIB, but no difference in expression was observed. DKK3 was 1.8-fold downregulated in T98G; conversely it was upregulated in KRIB and not expressed in MDAMB 435. I did not detect expression of DKK4 or SFRP2 in any of the cell lines.

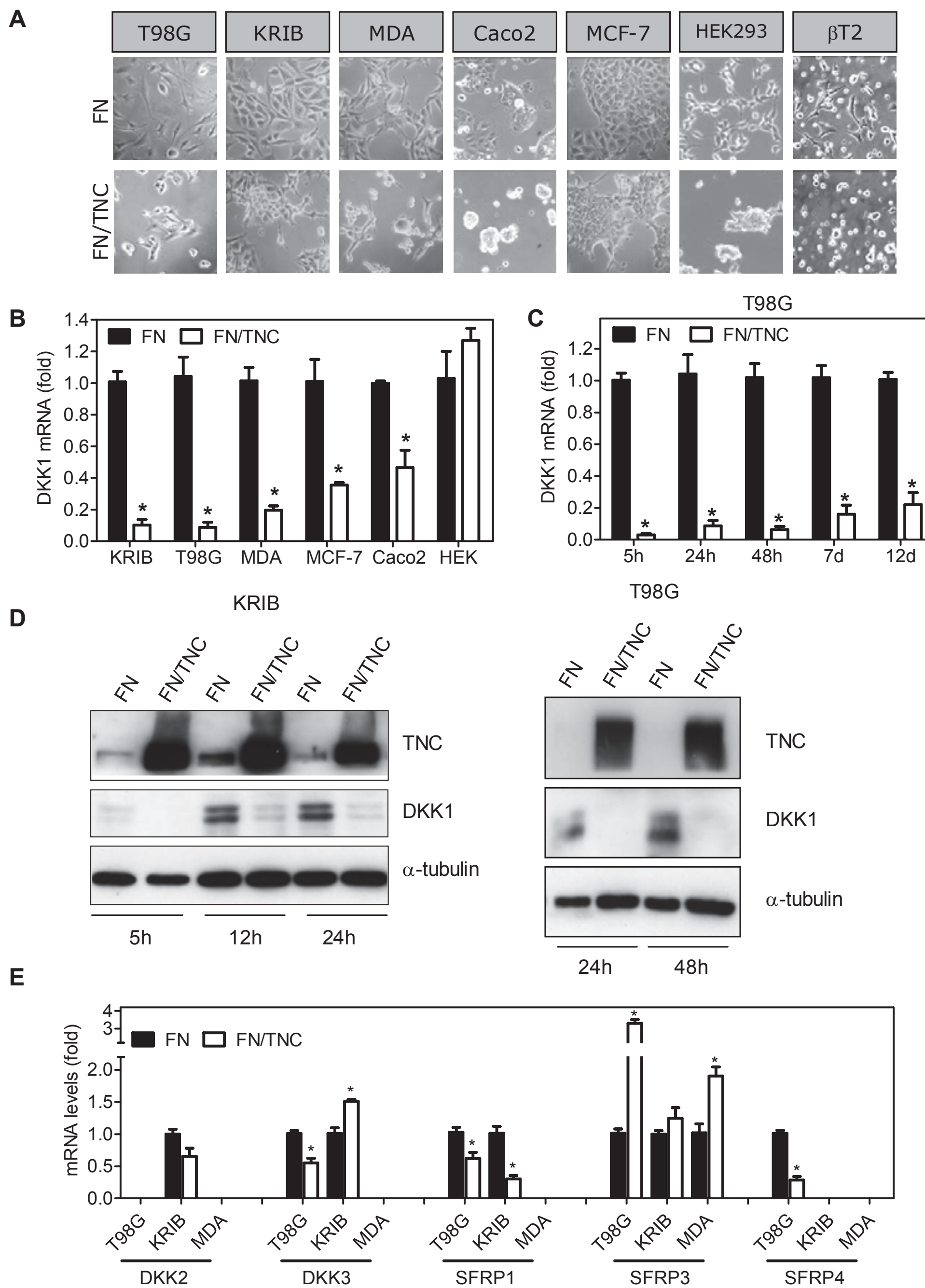


Figure 10. TNC reduces *DKK1* gene and protein expression in tumor cells *in vitro*.
(A) T98G, KRIB, MDAMB 435 (MDA), Caco2, MCF-7, HEK293 and βT2 cell lines were seeded on FN or FN/TNC coated dishes for 24h and 48h (HEK293). **(B)** DKK1 mRNA levels were quantified by qRT-PCR and are represented relative to expression on FN after 24h or 48h (HEK293) on FN or FN/TNC. **(C)** *DKK1* gene expression analysis by qRT-PCR in T98G cells on FN or FN/TNC for the indicated time points. **(D)** DKK1 protein expression analysis by Western Blotting in KRIB and T98G cells plated on FN or FN/TNC. **(E)** Gene expression analysis by qRT-PCR of Wnt inhibitors in T98G, KRIB and MDAMB-435 (MDA) cells upon plating on the indicated substrata for 24h (KRIB, MDAMB-435) or 48h (T98G). There was no expression of SFRP2 or DKK4 detectable in any of the cell lines. Error bars represent SEM and asterisks (*) indicate p-values < 0.05.

In summary gene expression analysis revealed that TNC reduces the expression of DKK1 RNA and protein in multiple tumor cell lines. Regulation of other Wnt inhibitors by TNC in a subset of analysed cell lines was also observed. But those inhibitors were either less downregulated than compared to DKK1, not expressed at all or were upregulated (sFRP3) but not in all cell lines. Therefore, I conclude that TNC is able to specifically target *DKK1* gene expression *in vitro*.

In order to analyse if DKK1 is targeted *in vivo*, *DKK1* gene expression has been analysed in tumors of RT2 mice overexpressing TNC (RT2/TNC) and that are KO for TNC (RT2/TNC KO). A lower percentage of RT2/TNC tumors than RT2 control tumors expressed DKK1 (**Figure 11A**). Furthermore, comparing *DKK1* gene expression levels in tumors expressing DKK1 revealed that RT2/TNC tumors expressed less DKK1 (**Figure 11B**). In contrast, tumors derived from RT2/TNC KO expressed higher levels than tumors from RT2 mice expressing only one TNC allele (**Figure 11C**). This indicates that also *in vivo* DKK1 expression inversely correlates with TNC expression levels.

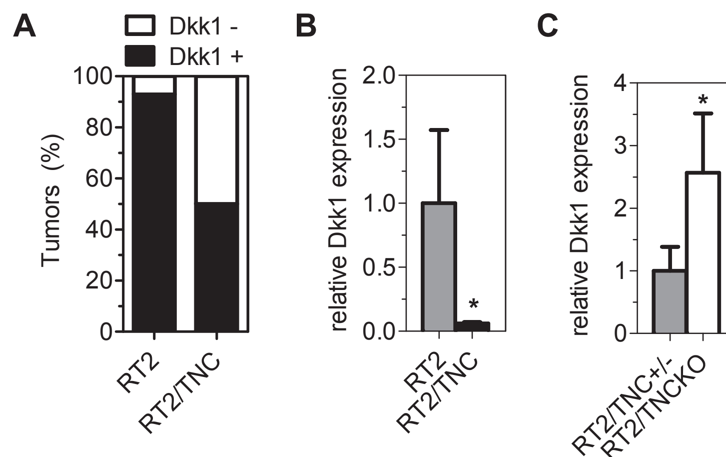


Figure 11. TNC copy number negatively correlates with *Dkk1* gene expression *in vivo* in the RipTag2 (RT2) tumor model.
(A – C) Dkk1 expression was analysed by qRT-PCR in tumors isolated from the pancreata of RT2 or RT2 TNC +/- (control), RT2/TNC and RT2/TNCKO mice. **(A)** Tumors were stratified according to Dkk1 levels, as expressing (Dkk1 +) or not expressing (Dkk1 -). Dkk1 was found to be expressed in 26 of 27 RT2 tumors and in 7 of 13 RT2/TNC tumors. Difference between genotypes, $p < 0.05$. **(B)** *Dkk1* gene expression in those RT2 and RT2/TNC tumors with detectable *Dkk1* gene expression. **(C)** In RT2/TNCKO tumors (15 of 24 tumors were Dkk1 positive) Dkk1 expression was higher compared to RT2/TNC+/- control tumors (16 of 23 tumors were Dkk1 positive). Error bars represent SEM and asterisks (*) indicate p-values < 0.05. (Experiments were performed and analysed by F. Saupe.)

4.1.2 TNC induces Wnt signalling

DKK1 is an inhibitor of canonical Wnt signalling (Glinka *et al*., 1998). Therefore, I aimed to analyse whether TNC is able to stimulate Wnt signalling activity. Wnt signalling activity can be analysed by expression analysis of canonical Wnt signalling target genes, e.g. the prototypical Wnt target gene *Axin2*, staining for nuclear β-catenin or by the TOPFlash Assay. The TOPFlash Assay relies on a synthetic reporter plasmid containing tandem repeats of the TCF/LEF transcriptional response element upstream of the luciferase coding sequence and therefore allows monitoring Wnt/β-catenin signalling in cultured cells.

KRIB and T98G cells did not show cell-autonomous Wnt signalling as analysed in a TOPFlash Assay (**Figure 12A**), however Wnt signalling activity could be stimulated by transfection with a plasmid encoding mutant β-catenin, lacking the phosphorylation site for GSK3β, or by treatment with Wnt3A containing conditioned medium (CM) (**Figure 12A**). Therefore, the following experiments were all performed in the presence of CM containing Wnt3A.

It was not possible to include T98G cells in the following analysis, as in the presence of Wnt3A CM I did not observe the TNC induced DKK1 downregulation in this cell line. This is not surprising, knowing that DKK1 has also been described to be induced by Wnt signalling (Gonzalez-Sancho *et al*., 2005).

TOPFlash activity was analysed in KRIB grown for 48 hours on FN or FN/TNC. In the presence of TNC TOPFlash activity was 3.5-fold enhanced (**Figure 12B**). In addition, Axin2 mRNA levels were 2-fold increased when KRIB cells were grown for 5 hours on FN/TNC (**Figure 12C**). Furthermore, if KRIB cells were treated with CM of KRIB cells previously grown for 48 hours on FN or FN/TNC, CM from cells grown on FN/TNC increased TOPFlash activity 1.5-fold (**Figure 12D**). To address whether Wnt signalling is potentially activated in RT2 tumors with different TNC levels, Axin2 expression was determined by qRT-PCR and it was observed that indeed Axin2 mRNA levels were 1.4-fold increased in tumors of transgenic RT2/TNC mice compared to control RT2 mice (**Figure 12E**).

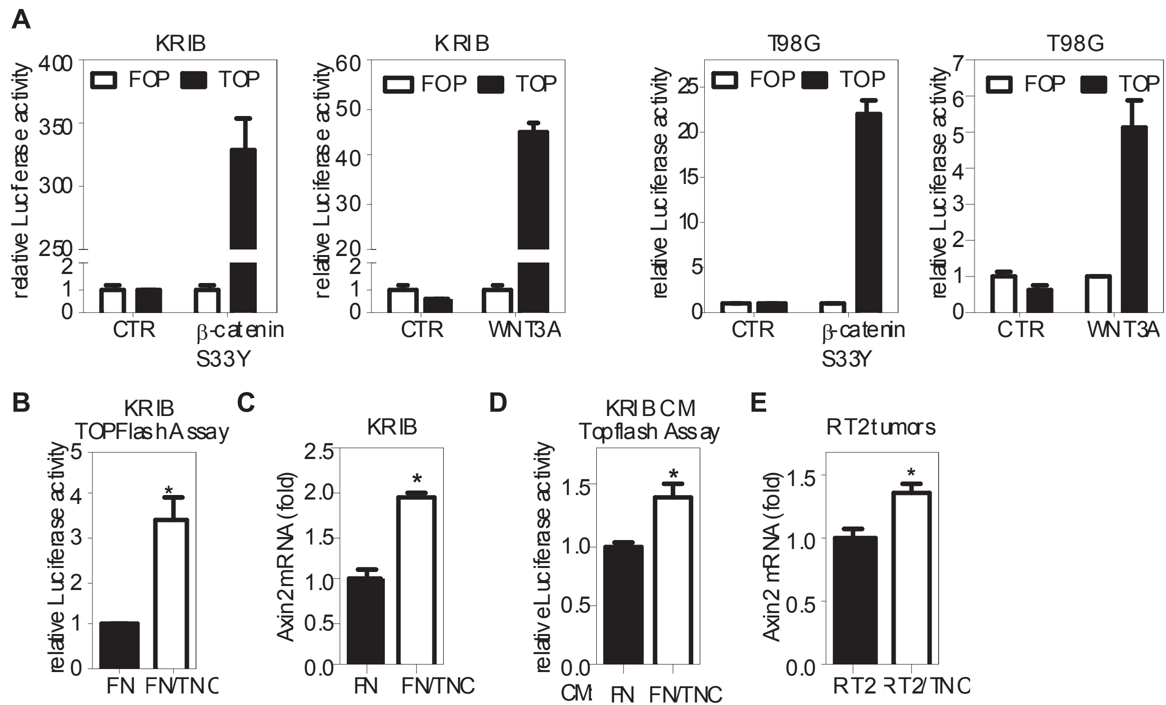


Figure 12. TNC enhances TOPFlash activity and Axin2 expression in Wnt3A treated KRIB cells and enhances Axin2 expression in tumors of RT2/TNC mice.

(A) TOP- and FOPFlash activity of KRIB and T98G cells co-transfected with constitutively active (S33Y) β-catenin (left panel) or upon treatment with CM containing Wnt3A (right panel). **(B)** TOPFlash activity of KRIB cells grown on FN or FN/TNC for 48h. WNT3A containing CM or CTR CM was added after 5h of cell seeding on the substrata. **(C)** Axin2 expression was determined by qRT-PCR in KRIB grown for 5h on FN or FN/TNC in presence of Wnt3A containing CM. **(D)** The experiment was performed as described in (B) except that KRIB cells were seeded on uncoated dishes and treated in parallel with CM from KRIB cells that were previously grown for 48h on FN or FN/TNC. **(E)** Axin2 expression was determined by qRT-PCR from RNA isolated from RT2 control and RT2/TNC mice. (The experiment was performed and analysed by F. Saupe.) Error bars represent SEM and asterisks (*) indicate p-values < 0.05.

In conclusion, these results demonstrate that TNC stimulates canonical Wnt signalling as shown by increased TOPFlash activity and enhanced Axin2 expression levels in the presence of TNC *in vitro* and *in vivo*. Furthermore, treatment with CM from KRIB grown on a FN/TNC was sufficient to stimulate TOPFlash activity, indicating that a soluble factor secreted by these cells is responsible for the observed pro-stimulatory effect of TNC on Wnt signalling.

4.1.3 Is enhanced Wnt signalling activity in the presence of TNC mediated by TNC induced DKK1 downregulation?

To address whether Wnt signalling activity in the presence of TNC is causally linked to DKK1 downregulation on a TNC substratum Wnt activity was determined by both overexpression and knockdown of DKK1 in KRIB.

Expression of mDKK1 harbouring a C-terminal Histidine-tag was confirmed by Western Blotting in both lysates and CM from KRIB cells (**Figure 13A**). In order to analyse if ectopically expressed mDKK1 inhibits Wnt signalling activity both TOPFlash assay activity (**Figure 13B**) and *Axin2* gene expression (**Figure 13C**) were analysed. KRIB cells expressing mDKK1 had 11-fold reduced TOPFlash activity and 2.9-fold reduced expression of Axin2. In addition, treatment of KRIB with CM of KRIB:mDKK1 also reduced TOPFlash activity 5-fold (**Figure 13D**).

DKK1 blocked Wnt activation in a concentration dependent manner, as the inhibitory effect was lost with enhanced dilution of mDKK1 CM (**Figure 13E**). In order to establish KRIB cells with reduced DKK1 expression, a shRNA-mediated strategy was used. Reduced expression of DKK1 was confirmed by quantifying DKK1 mRNA levels by qRT-PCR (**Figure 13F**) and protein levels on Western Blot (**Figure 13G**). A knockdown of DKK1 resulted in a higher TOPFlash activity (**Figure 13H**).

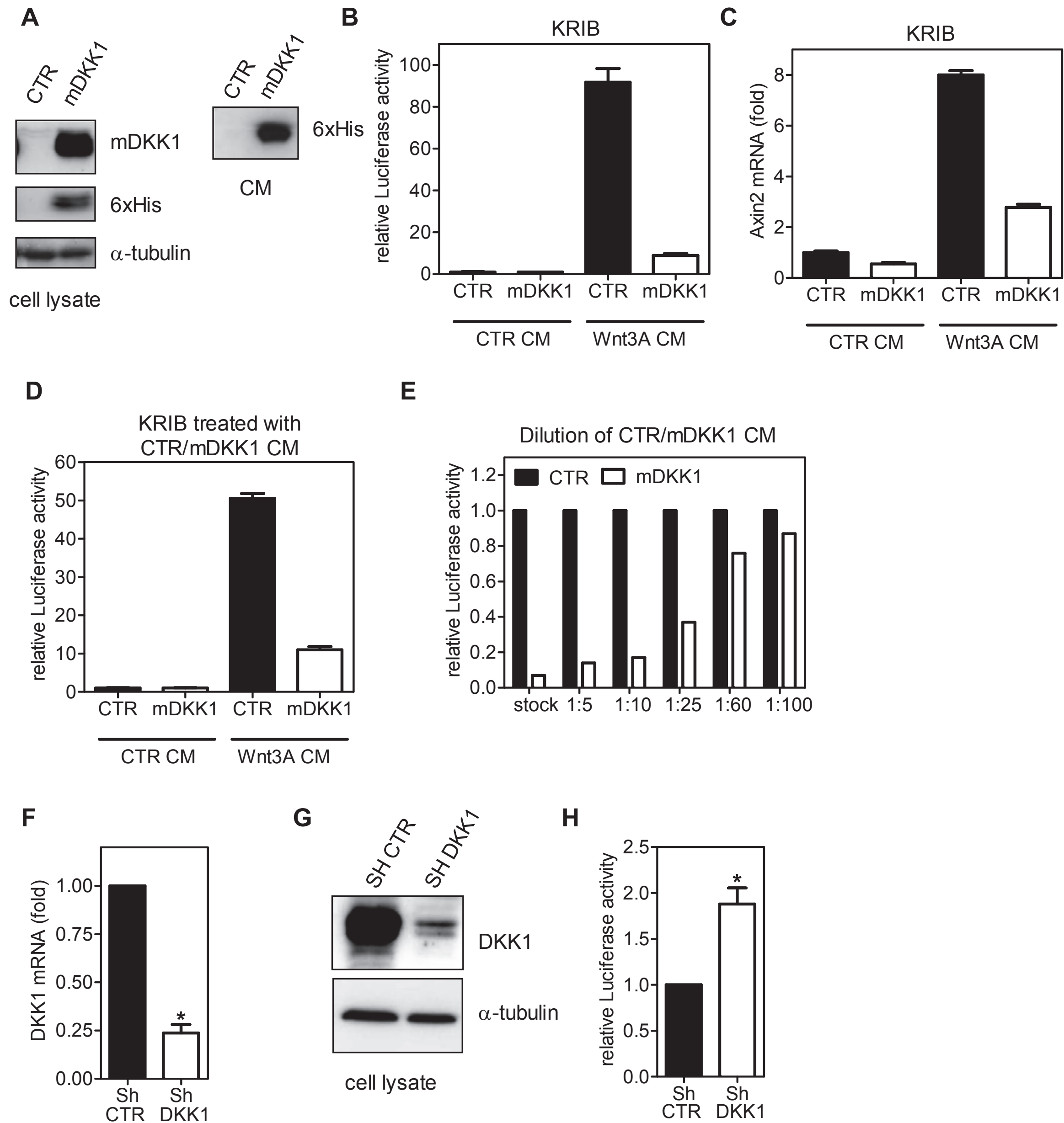


Figure 13. Ectopic expression of mDKK1 reduces Wnt signalling activity while knockdown of DKK1 enhances Wnt signalling in KRIB cells.
(A) Expression of murine DKK1 was analysed by Western Blotting with antibodies against DKK1 or the poly-histidine tag (6xHis) in lysates and CM from KRIB cells control (CTR) or overexpressing mDKK1. **(B)** TOPFlash activity in Wnt3A treated KRIB cells upon mDKK1 overexpression. **(C)** Axin2 expression was determined by qRT-PCR in Wnt3A treated KRIB cells upon mDKK1 overexpression. **(D)** TOPFlash activity in KRIB cells upon addition of Wnt3A and/or mDKK1 containing CM. **(E)** TOPFlash activity in KRIB cells upon addition of Wnt3A CM and different dilutions of KRIB CTR CM or mDKK1 CM. **(F-G)** Expression of DKK1 was analysed by qRT-PCR **(F)** and Western Blotting **(G)** upon shRNA mediated DKK1 knockdown (shDKK1) in KRIB cells. **(H)** TOPFlash activity in Wnt3A treated KRIB control (shCTR) and DKK1 knockdown (shDKK1) cells. Error bars represent SEM and asterisks (*) indicate p-values < 0.05.

In order to assess the contribution of TNC induced DKK1 downregulation to enhanced Wnt signalling by TNC, KRIB cells were grown on FN or FN/TNC and treated in parallel with control CM or CM containing mDKK1 (**Figure 14A**). TNC enhanced TOPFlash activity upon treatment

with control CM and treatment with mDKK1 containing CM was able to repress TOPFlash activity on both substrata. Importantly, in the presence of mDKK1 containing CM TNC was still able to induce TOPFlash activity but to a lesser extent than in control conditions, suggesting that DKK1 downregulation by TNC is indeed a mechanism by which TNC induces TOPFlash activity.

In addition, KRIB:sh control (CTR) and KRIB:sh DKK1 cells were seeded on FN or FN/TNC (**Figure 14B**). As expected, in control cells TNC enhanced TOPFlash activity and DKK1 knockdown enhanced TOPFlash activity on FN compared to control cells on FN. Of note, TNC was not able to further increase TOPFlash activity in KRIB knockdown for DKK1, indicating that downregulation of DKK1 by TNC is indeed the major mechanism by which TNC stimulates Wnt signalling activity.

Axin2 expression, as an additional readout for Wnt signalling activity from cells plated on FN and FN/TNC and upon treatment with CTR or mDKK1 containing CM, was also analysed (**Figure 14C**). In the control treatment TNC increased the expression of Axin2 mRNA levels by 2.3-fold in comparison to FN. Addition of mDKK1 containing CM repressed Axin2 expression both on FN and FN/TNC. In the presence of mDKK1 CM, TNC still enhanced Axin2 expression by approximately the same fold as in the control treatment, indicating that induction of Axin2 expression by TNC is independent of TNC induced DKK1 downregulation.

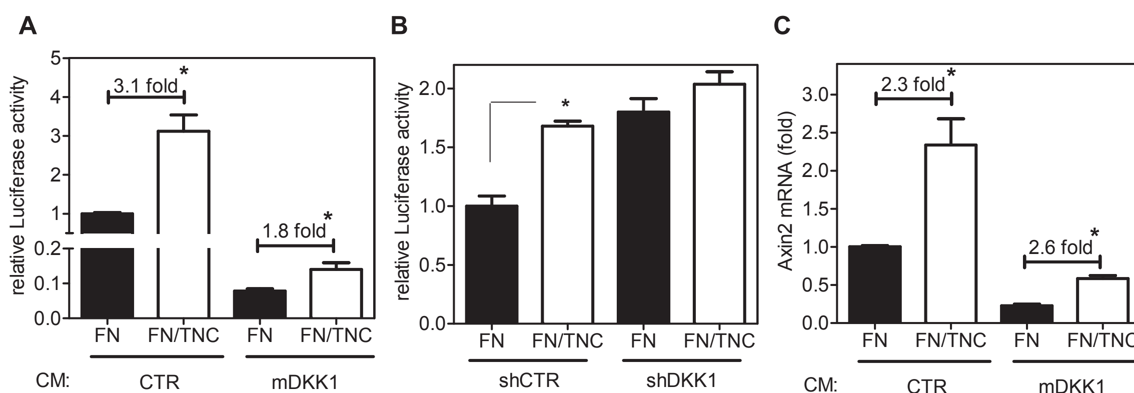


Figure 14. Regulation of TNC mediated Wnt signalling activity by DKK1.
(A) TOPFlash activity of Wnt3A treated KRIB cells seeded on FN or FN/TNC upon addition of CM from CTR or mDKK1 overexpressing KRIB cells. **(B)** TOPFlash activity of Wnt3A treated KRIB cells seeded on FN or FN/TNC upon knockdown of DKK1. **(C)** Axin2 expression determined by qRT-PCR in Wnt3A treated KRIB cells seeded on FN or FN/TNC upon addition of CTR CM or CM containing mDKK1. Error bars represent SEM and asterisks (*) indicate p-values < 0.05.

In summary here it was shown that TNC strongly downregulates the expression of the Wnt inhibitor DKK1 in several tumor cell lines and that this is causally linked to Wnt signalling

activation as shown by a TOPFlash activity and, by experimentally modulating DKK1 levels (overexpression and knockdown). In addition, *in vivo* in RT2 tumors we observed an inverse correlation between TNC copy number and *DKK1* gene expression levels, which was also linked to an increased expression of the Wnt target gene *Axin2*.

4.2 Regulation of DKK1 expression by TNC in stromal cells

In many tumors, including tumors of the RT2 model, TNC is highly expressed in the vicinity of stromal cells. This suggests that stromal cells might preferentially interact with TNC. Therefore, here it was addressed whether TNC has an impact on DKK1 expression and Wnt signalling in stromal cells.

4.2.1 TNC downregulates DKK1 in stromal cells

In order to analyse if TNC is able to regulate DKK1 expression also in stromal cells, different primary and immortalized stromal cells, as endothelial cells, pericytes and (cancer-associated) fibroblasts were plated on FN or FN/TNC. A 2-fold downregulation of DKK1 mRNA expression in HUVEC grown on FN/TNC was observed (**Figure 15A, B**). In addition, DKK1 protein levels were reduced in the presence of TNC as analysed by Western Blot (**Figure 15C**). In contrast, in immortalized endothelial cells TNC only slightly decreased DKK1 levels (cdc-HMEC) or did no affect DKK1 levels (HMVEC) (**Figure 15D, E**). BAEC did not express DKK1. In two different CRC-derived primary cancer-associated fibroblasts (CAFs) lines 3-fold and 1.6-fold reduced DKK1 levels were observed on FN/TNC (**Figure 15F, G**), while in a CRC-derived hTERT-immortalized CAF cell line DKK1 levels were upregulated on FN/TNC (**Figure 15 H, I**). Primary fibroblasts (MEF) did not express DKK1. In pericytes a FN/TNC substratum reduced DKK1 levels 5-fold (**Figure 15 J, K**). In addition, I analysed *DKK1* gene expression in murine monocyte/macrophage cellular models (RAW264.7 and J774A.1 cell lines) seeded on FN or FN/TNC, but observed no or very low expression of DKK1.

Whereas DKK1 is reduced by TNC in primary endothelial cells, pericytes and CAFs, DKK1 is not at all expressed in macrophages, MEF, BAEC, its expression was not changed in immortalized EC or it was increasingly expressed in immortalized CAFs. In a tumor, I consider endothelial cells, primary pericytes and CAFs as more relevant than experimentally

immortalized CAFs and EC or normal fibroblasts. Therefore, the results for those cells that I found to downregulate DKK1 expression on the TNC substratum is presumably more relevant in a tumor context. These results suggest that some tumor relevant stromal cells are a source of DKK1 and that TNC blocks DKK1 expression in these cells. Altogether contact of stromal cells with TNC may generate a microenvironment with low DKK1 levels.

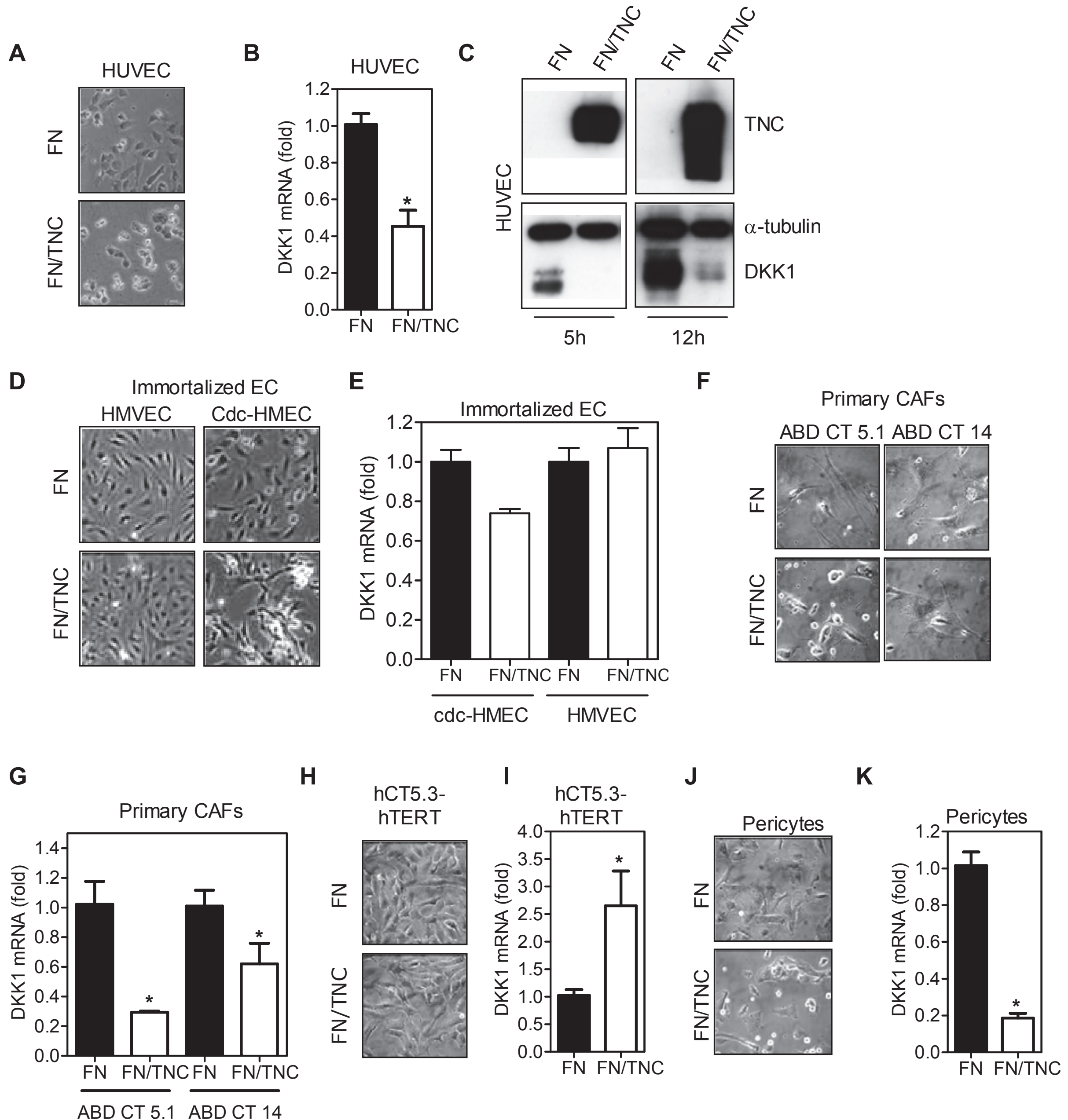


Figure 15. TNC downregulates DKK1 in primary endothelial cells (HUVEC), pericytes and CRC-derived CAF.
(A) HUVEC were plated on FN or FN/TNC (5h) and *DKK1* gene expression (5h) was analysed by qRT-PCR. **(B)** DKK1 protein expression was analysed by Western Blotting **(C)**. **(D)** Immortalized microvascular endothelial cells were plated on FN or FN/TNC (12h) and *DKK1* gene expression (12h) was analysed by qRT-PCR **(E)**. CRC-derived primary CAF were plated on FN or FN/TNC (5h) and *DKK1* gene expression (5h) was analysed by qRT-PCR **(G)**. CRC-derived immortalized CAF were plated on FN or FN/TNC (24h) and *DKK1* gene expression (24h) was analysed by qRT-PCR **(I)**. **(J)** primary pericytes were plated on FN or FN/TNC (5h) and *DKK1* gene expression (5h) was analysed **(K)**. Error bars represent SEM and asterisks (*) indicate p-values < 0.05.

4.2.2 Does TNC induce Wnt signalling in stromal cells?

Due to difficulties in transfection of primary cell lines with the TOPFlash reporter construct I had to limit the analyses of Wnt/β-catenin signalling activity in HUVEC and pericytes to the analysis of *Axin2* gene expression. In HUVEC Axin2 mRNA levels were 2-fold increased in the presence of TNC (**Figure 16A**), indicating stimulation of Wnt signalling activity by TNC. In pericytes TNC did not induce Axin2 expression (**Figure 16B**). In order to stimulate Wnt signalling in pericytes the experiment was repeated in the presence of Wnt3A containing CM. Wnt3A was able to stimulate Axin2 expression in pericytes, however I did not observe any difference in Axin2 expression between cells grown on FN or FN/TNC (**Figure 16C**).

In summary, I showed that TNC activates Wnt signalling in HUVEC but does not regulate Wnt signalling in pericytes upon stimulation with Wnt3A.

In order to analyse if enhanced *Axin2* gene expression levels were linked to DKK1 downregulation by TNC, HUVEC were treated with CM containing mDKK1. DKK1 was not able to repress TNC-induced Axin2 expression (**Figure 16D**). As previous experiments in HUVEC had been performed without adding Wnt3A (since Wnt activity was detectable without Wnt3A), it was analysed if Wnt3A was able to induce Axin2 expression in HUVEC. Upon treatment with Wnt3A CM Axin2 was only induced after 5 hours of treatment, while at later time points Wnt3A was not able anymore to induce Axin2 expression (**Figure 16E**). Therefore, the following experiments were performed with HUVEC treated for 5 hours with Wnt3A. HUVEC were treated in addition with CM from HUVEC (**Figure 16F**) or KRIB (**Figure 16G**) cells previously grown for 48 hours on FN or FN/TNC, in which DKK1 levels are reduced in the presence of TNC. It was analysed whether CM from cells grown on FN/TNC was able to further stimulate Axin2 expression. However, no difference in Axin2 expression was observed in Wnt3A stimulated HUVEC. These results indicate that while a direct contact with a TNC substratum can induce Axin2 expression in HUVEC there is no effect on Axin2 expression if HUVEC are treated with the CM from cells grown on FN/TNC.

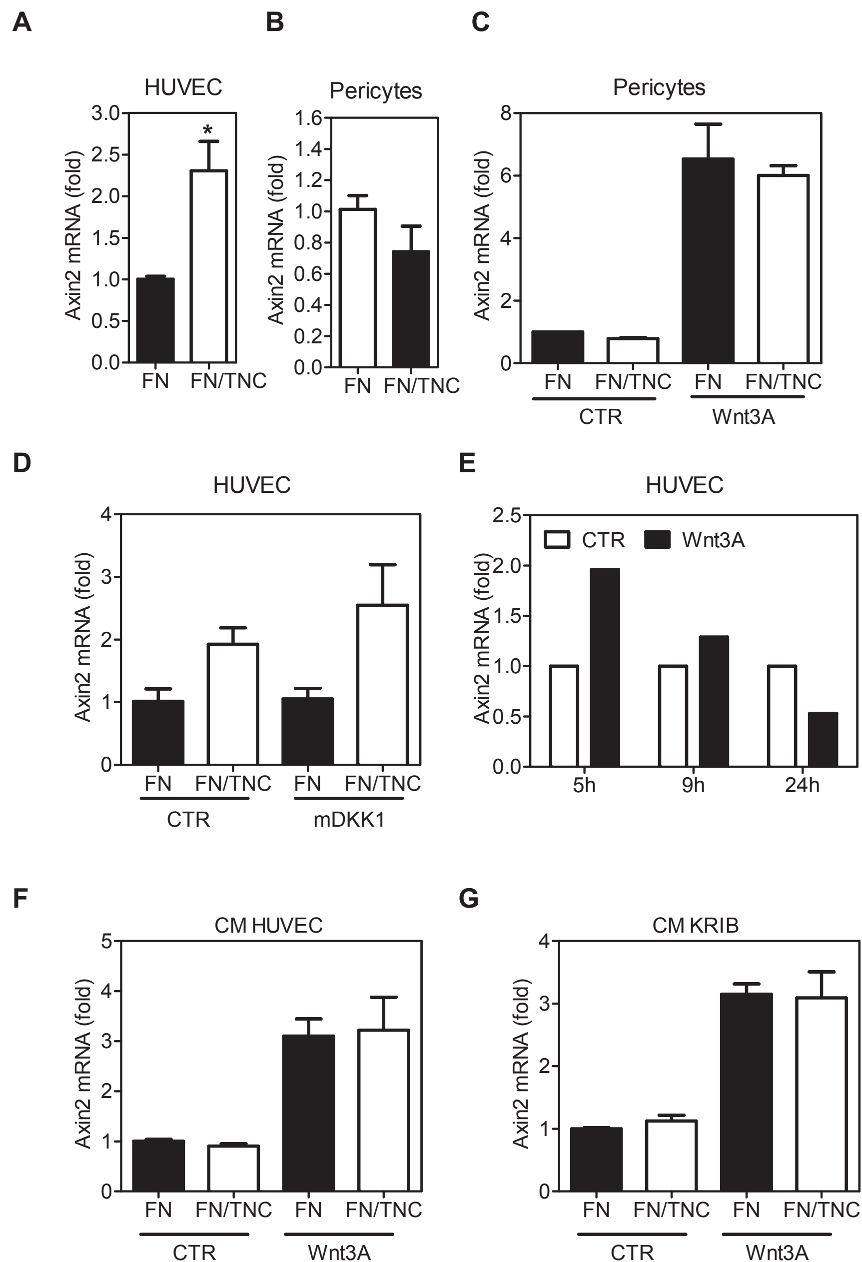


Figure 16. TNC induces *Axin2* expression in HUVEC, but not in pericytes.
Axin2 gene expression was analysed: **(A, B)** in HUVEC and pericytes without Wnt3A stimulation after 5h on FN or FN/TNC. **(C)** in pericytes after 24h on FN or FN/TNC including 12h Wnt3A stimulation. **(D)** in HUVEC after 5h on FN or FN/TNC and stimulation with conditioned medium (CM) from KRIB:CTR or KRIB:mDKK1 cells. **(E)** in HUVEC after Wnt3A stimulation for 5h, 9h and 24h. **(F)** in Wnt3A stimulated HUVEC treated for 5h with CM from HUVEC grown on FN or FN/TNC for 48h. **(G)** in Wnt3A stimulated HUVEC treated for 5h with CM from KRIB cells grown on FN or FN/TNC for 48h. Error bars represent SEM and asterisks (*) indicate p-values < 0.05.

In summary, these analysis revealed that not only in tumor cells (see chapter 4.1) but also in different stromal cell types TNC downregulates DKK1 expression. While in endothelial cells TNC induces Axin2 expression, this does not appear to apply to pericytes. What the functional relevance of reduced DKK1 expression in stromal cells for tumor progression needs to be analysed in more detail.

4.3 DKK1 as regulator of tumor angiogenesis

We had demonstrated that TNC promotes tumor angiogenesis in the RT2 model. In particular, we had shown that RT2/TNC mice harboured a higher numbers of angiogenic islets (**Figure 17A**), while RT2/TNC KO mice harboured a lower number of angiogenic islets compared to control mice (**Figure 17B**), indicating that TNC is promoting the angiogenic switch. Furthermore, quantification of CD31-positive EC revealed a higher expression of CD31 in tumors of RT2/TNC mice and a lower expression of CD31 in tumors of RT2/TNC KO mice suggesting that TNC promotes tumor angiogenesis (**Figure 17C, D**).

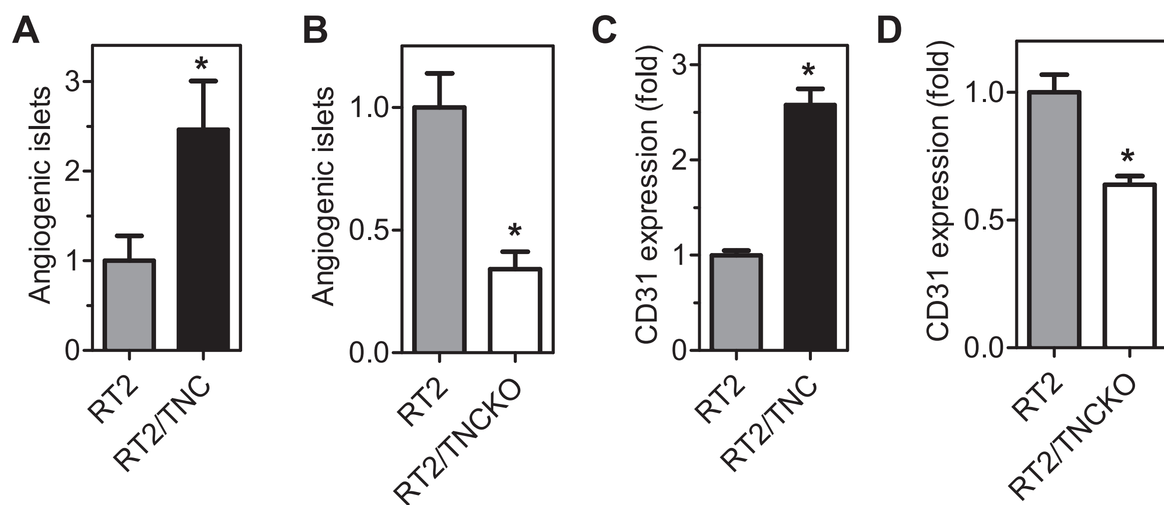


Figure 17. TNC enhances the number of angiogenic islets in the RT2 model of tumor progression and increases the amount of CD31 positive endothelial cells.
(A, B) Number of angiogenic islets per mouse normalized to RT2 controls in RT2/TNC (**A**; N=9 control and N=7 RT2/TNC mice) and RT2/TNCKO (**B**; N=7 control and N=7 RT2/TNCKO mice) upon isolation of islets from the pancreata of 8-weeks old mice. **(C, D)** Tumor blood vessel quantification upon CD31 staining of tumor sections from 12 week old mice as CD31 positive area fraction per tumor normalized to RT2 controls. **(C)** RT2 (N = 6 mice, n = 34 tumors, 203 images), RT2/TNC (N = 4, n = 17, 106 images), **(D)** RT2 (N = 3, n = 71), RT2/TNCKO (N = 3, n = 111). Error bars represent SEM and asterisks (*) indicate p-values < 0.05. (Experiments were performed and analysed by F. Saupe.)

We have observed a negative correlation between DKK1 expression and TNC expression in tumors of the RT2 model which coincided with increased tumor angiogenesis. Therefore, we asked whether DKK1 might also impact on tumor angiogenesis.

KRIB:CTR and KRIB ectopically expressing mDKK1 (**Figure 13A**) were subcutaneously injected in nude mice. Tumors expressing mDKK1 (**Figure 18A**) were significantly smaller than control tumors (**Figure 18B**) and tumors were white reminiscent of lacking blood vessels compared to vascularised control tumors (**Figure 18C**). Indeed, by quantifying CD31 expression upon

immunofluorescence staining, it was observed that mDKK1 expressing tumors express less CD31 (**Figure 18D**). Further analysis revealed that DKK1 did not have an impact on tumor cell proliferation *in vitro* and *in vivo* (**Figure 18E, F**), suggesting that DKK1 was inhibiting tumor growth mainly by inhibiting angiogenesis and not by hampering tumor cell proliferation directly.

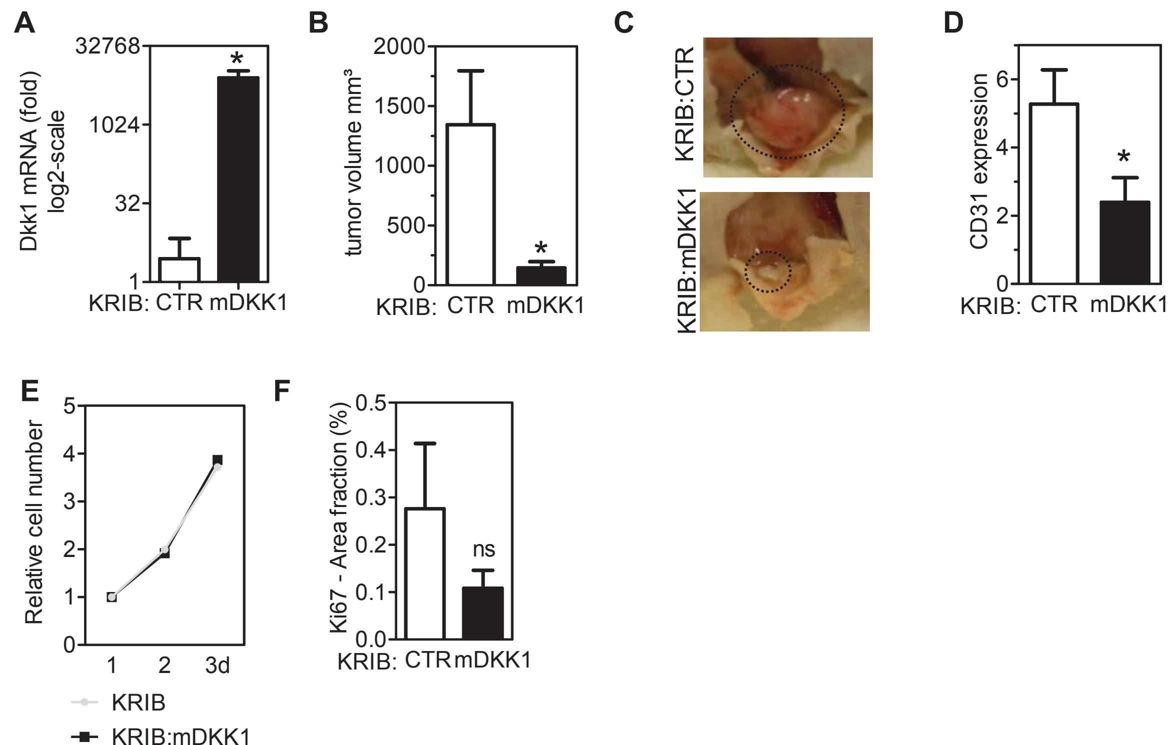


Figure 18. Ectopic expression of mDKK1 in KRIB inhibits tumor angiogenesis in a xenograft model.
(A) Quantification of murine *DKK1* gene expression by qRT-PCR in control or mDKK1 overexpressing KRIB derived tumors. **(B)** Mean tumor volume of control (CTR, n = 10) and mDKK1 overexpressing (n = 9) KRIB tumors upon subcutaneous injection of the corresponding cells into nude mice. **(C)** Examples of macroscopic images from the tumors. **(D)** Tumor microvessel density was determined by CD31 staining and quantification in KRIB:mDKK1 tumors (n = 8) control KRIB tumors (n = 10). **(E)** DKK1 does not change tumor cell proliferation *in vitro*. Proliferation of KRIB (parental) and KRIB:mDKK1 cells was analysed with a MTS Assay. Data are normalized in each group to values of day 1. **(F)** DKK1 does not change tumor cell proliferation *in vivo*. Proliferating cells were quantified in tumors derived from KRIB control or KRIB:mDKK1 cells. Ki67 positive areas were determined using ImageJ software upon staining for Ki67 and reported to the DAPI positive areas per tumor. No significant (ns) difference was observed (n = 5 per group). Error bars represent SEM and asterisks (*) indicate p-values < 0.05. (Experiments were performed and analysed by I. Gasser, except **(E)** was performed by Thomas Hussenet.)

This experiment suggested that DKK1 acts as a suppressor of tumor angiogenesis. However, whether TNC indeed is regulating tumor angiogenesis by repressing DKK1 was not shown. Therefore, I aimed to (i) analyse tumor growth and angiogenesis upon overexpression of TNC and (ii) wanted to analyse whether overexpression of mDKK1 is able to block TNC induced tumor growth and angiogenesis in a xenograft model.

I established both HEK293 and KRIB cells overexpressing TNC or mDKK1 alone or both expressing TNC and mDKK1. Expression of TNC and mDKK1 were analysed both by qRT-PCR and Western Blot (**Figure 19A-F**). However, overexpression of TNC in KRIB only resulted in approximately 2-fold increase of secreted TNC protein level as analysed by Western Blot of CM (**Figure 19E**). Therefore, I assessed the role of TNC, DKK1 and combined expression of TNC and DKK1 on tumor growth and angiogenesis only with HEK293 cells.

HEK293 cells were subcutaneously grafted into both flanks of nude mice and tumor size was analysed over a time period of 11 weeks. However, only 4 of the 10 injected sites gave rise to tumors. Due to the low numbers of tumors, it is not possible to draw final conclusions from this experiment, although a slight trend towards bigger tumors upon TNC expression and smaller tumors upon additional expression of mDKK1 was observed (**Figure 19G, H**).

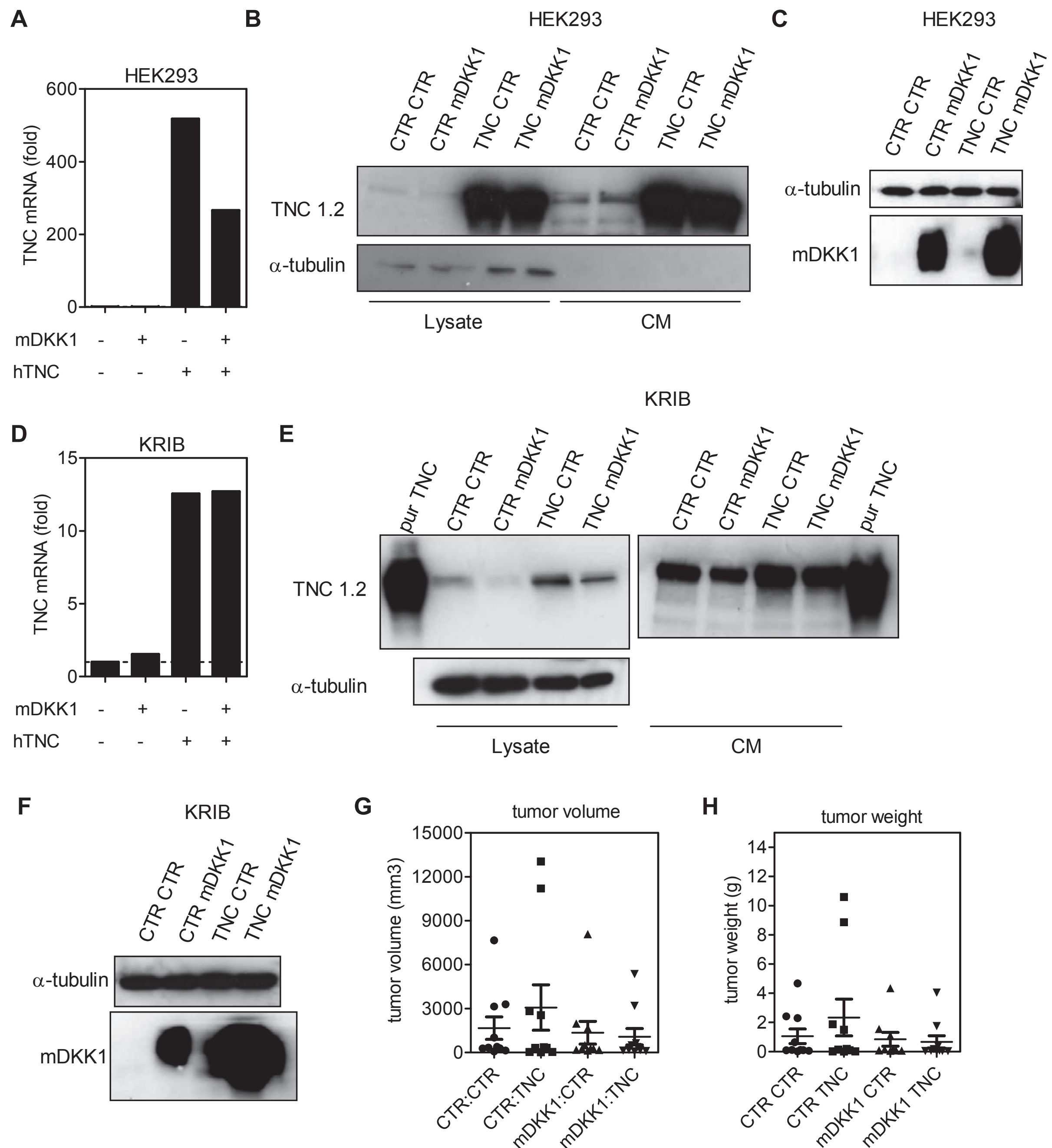


Figure 19. Establishment of HEK293 and KRIB cells overexpressing TNC and mDKK1.
(A-C) HEK293:CTR were generated by transfection with pCEB plasmid, HEK293 overexpressing mDKK1 or control plasmid (pQXCIP) were generated by transducing cells with pQXCIP empty plasmid or pQXCIP:mDKK1 containing retroviral particles. Overexpression of TNC in HEK293 (Lange *et al.,* 2007) was verified by qRT-PCR **(A)** or by Western Blotting from cell lysate and CM **(B)**, expression of mDKK1 was verified by Western Blotting **(C). (D-E)** KRIB:CTR and KRIB:mDKK1 cells overexpressing TNC were generated by transfection with linearized pQXCIB control or pQXCIB:TNC plasmid. Overexpression of TNC was verified by qRT-PCR **(D)** or Western Blotting from cell lysate and CM **(E)**, expression of mDKK1 was verified by Western Blotting **(F)**. **(G, H)** HEK293 cells overexpressing TNC, mDKK1 or both TNC and mDKK1 were implanted subcutaneously in nude mice and tumor volume and weight was determined after 11 weeks of growth. Error bars represent SEM.

Therefore, I aim to repeat the experiment with KRIB cells. We are in progress to knockdown TNC in control cells, in order to reach a significant difference in secreted TNC levels compared to TNC overexpressing cells.

4.4 Mechanism of DKK1 expression regulation by TNC

In the second aim I wanted to identify the mechanisms by which a FN/TNC substratum causes reduced expression of DKK1.

4.4.1 TNC regulates DKK1 promoter activity

As described above (**Figure 10B, C**), downregulation of DKK1 by TNC occurred at mRNA level. Therefore, the expression of DKK1 mRNA might be regulated either by modulation of its mRNA stability (e.g. through miRNAs or other mechanisms) or at the promoter level due to changed activity/expression of a transcription factor activating or repressing the expression of the gene. Regulation of *DKK1* gene expression by different miRNA and transcriptional activators or repressors has been demonstrated (see chapter 1.4.3).

Analysis of the expression of several miRNAs predicted or known to regulate the expression of DKK1 (e.g. miR31, miR 590-3p, miR-186, miR-103) were not regulated by TNC (M. Marko, personal communication). In order to assess whether *DKK1* gene expression levels are regulated through a change in mRNA stability T98G cells were treated with Actinomycin D at different time points after cells were seeded on FN or FN/TNC (**Figure 20A**). Actinomycin D inhibits transcription by binding DNA at the transcription initiation complex and preventing the elongation of the mRNA chain by the RNA polymerase II. In control treated cells DKK1 expression was induced over time after cells were seeded on FN. On a FN/TNC substratum a reduced and delayed induction of DKK1 mRNA expression was observed. In contrast, upon treatment with Actinomycin D no induction of *DKK1* gene expression both on a FN and FN/TNC substratum was observed. Importantly, no difference in *DKK1* gene expression levels between cells grown on FN or FN/TNC occurred, suggesting that DKK1 regulation by TNC is transcription-dependent.

Next it was addressed whether TNC affects DKK1 transcription at promoter level. Therefore, cells were transfected with a plasmid containing the DKK1 promoter upstream of a luciferase

reporter gene. Indeed, a 2.5-fold reduced luciferase activity was measured when cells were seeded on FN/TNC compared to cells on FN alone (**Figure 20B**), demonstrating that TNC downregulates *DKK1* gene expression by reducing DKK1 promoter activity.

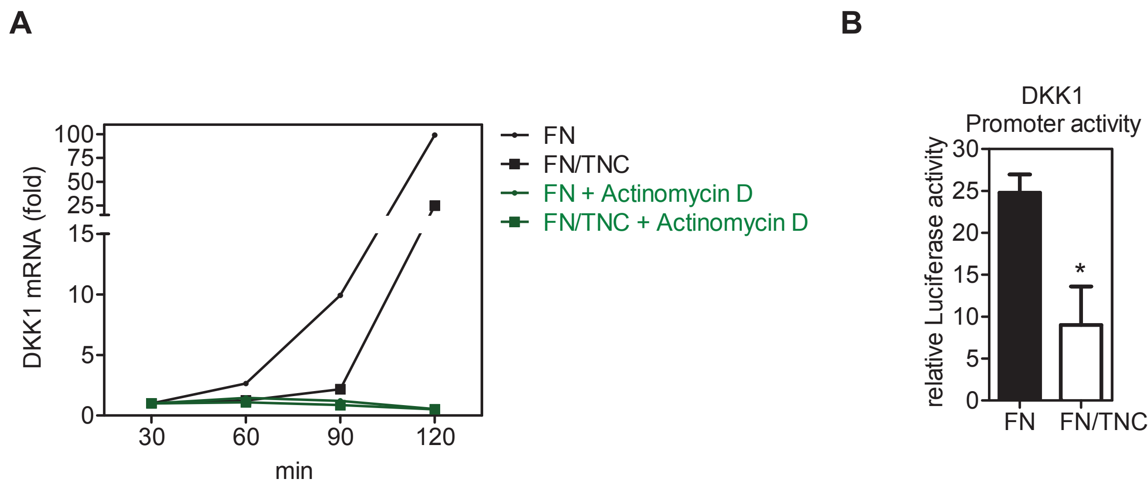


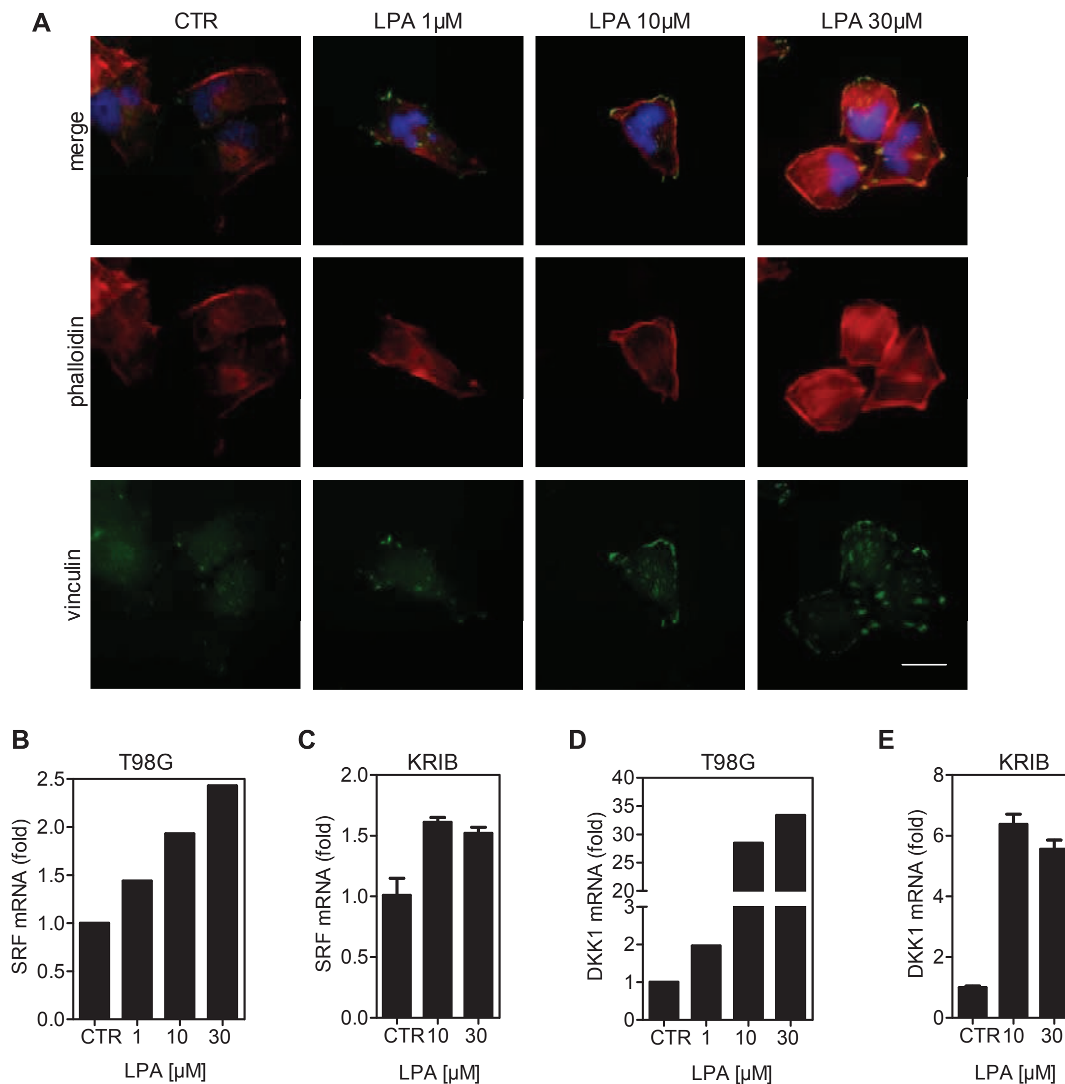
Figure 20. TNC reduces DKK1 promoter activity in T98G cells.
(A) T98G cells were starved overnight in 1% FCS and seeded on FN or FN/TNC. After 30 min cells were treated with 5 µg/ml Actinomycin D and were harvested after 30 min, 60 min and 90 min of treatment for RNA isolation and *DKK1* gene expression quantification by qRT-PCR. One representative experiment of two experiments is shown. **(B)** T98G were starved overnight in 1% FCS, trypsinized cells were transfected with the DKK1 promoter luciferase construct. Cells were seeded on FN or FN/TNC for 40 hours. Error bars represent SEM and asterisks (*) indicate p-values < 0.05.

4.4.2 *DKK1* gene expression can be induced by stabilization of stress fibres

As reduced DKK1 expression in the presence of TNC occurred already after 1 hour (see **Figure 20A**), and as TNC rapidly blocks adhesion to FN by preventing the formation of stress fibres I hypothesised that TNC could regulate DKK1 expression directly by a transcription factor, which activity or nuclear localization is controlled by the actin cytoskeleton. Therefore, to further support this hypothesis, I first investigated whether DKK1 expression is correlated with the integrity of the actin cytoskeleton.

T98G and KRIB cells were treated with LPA and *DKK1* gene expression levels were analysed. LPA is a growth factor inducing actin polymerisation and stress fibre formation (Ridley and Hall, 1992; **Figure 21A**). The expression of SRF, a known target of LPA and regulated by actin polymerisation (Spencer and Misra, 1999; Gineitis and Treisman, 2001), was induced by LPA treatment in T98G cells in a dose-dependent manner (**Figure 21B**). Also in KRIB cells LPA induced *SRF* gene expression (**Figure 21C**). Of note, in T98G cells the treatment with LPA induced *DKK1* gene expression in a dose-dependent manner. After 3 hours of treatment a

strong induction of *DKK1* gene expression by more than 30-fold was observed (**Figure 21D**) and likewise LPA treatment enhanced DKK1 expression in KRIB cells (**Figure 21E**). To prove that induction of *DKK1* gene expression by LPA was indeed due to enhanced stress fibre formation, LPA-treated T98G cells were pre-incubated with Latrunculin B, a drug inhibiting actin polymerisation (Spector *et al*., 1983). Indeed actin stress fibres were disrupted (**Figure 21F**). Both *SRF* and *DKK1* gene expression were not anymore induced by LPA when actin was depolymerised upon Latrunculin B treatment (**Figure 21G, H**).



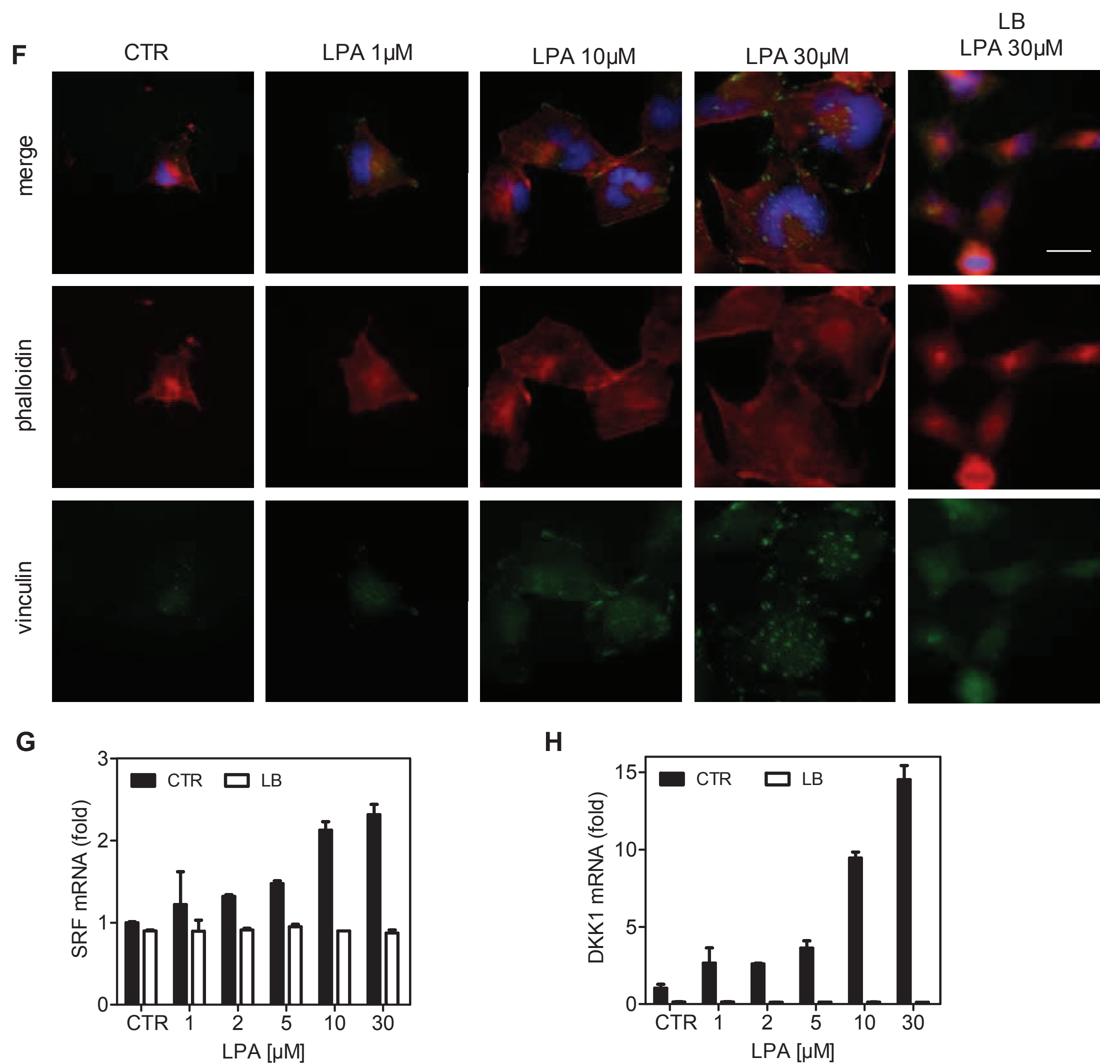


Figure 21. LPA induces stress fibre formation and DKK1 gene expression.
Serum starved T98G and KRIB cells were treated with the indicated concentrations of LPA for 3 hours. **(A)** IF staining of T98G for F-actin (phalloidin) and vinculin. Nuclei are stained in blue (DAPI). Scale bar 20 µm. **(B-E)** qRT-PCR analysis of *SRF* **(B, C)** and *DKK1* **(D, E)** gene expression in T98G and KRIB cells. **(F, G)** Serum starved T98G were pre-treated for 30 minutes with 5 µM Latrunculin B (LB) if indicated and treated for 3 hours with the indicated concentrations of LPA. **(F)** IF staining of T98G for F-actin (Phalloidin) and vincullin. Nuclei are stained in blue (DAPI). Scale bar 20 µm. **(G, H)** qRT-PCR analysis of *SRF* **(G)** and *DKK1* **(H)** gene expression. Error bars represent SEM.

Previous studies from our laboratory have shown that ectopic expression of chicken (ch) Syndesmos and TPM1 restore cell spreading on FN/TNC, while knockdown of TPM1 in T98G hampered cell spreading on FN (Ruiz *et al.*, 2004; Lange *et al.*, 2008). Syndesmos is a Syndecan-4 binding protein and is localized in focal adhesions, and provides a molecular bridge to the integrin adhesome through binding to paxillin; and TPM1 is an actin-binding and stabilizing protein (see also chapter 1.3.1 and 1.3.2).

Therefore, it was analysed if ectopic expression of chSyndesmos has an impact on DKK1 expression. First expression of chSyndesmos in T98G cells was confirmed by RT-PCR (**Figure**

22A). T98G cells expressing chSyndesmos had more and larger focal adhesions than control cells as shown by IF staining for vinculin (**Figure 22B**). Interestingly, *DKK1* gene expression was 4.7-fold upregulated upon ectopic expression of chSyndesmos (**Figure 22C**).

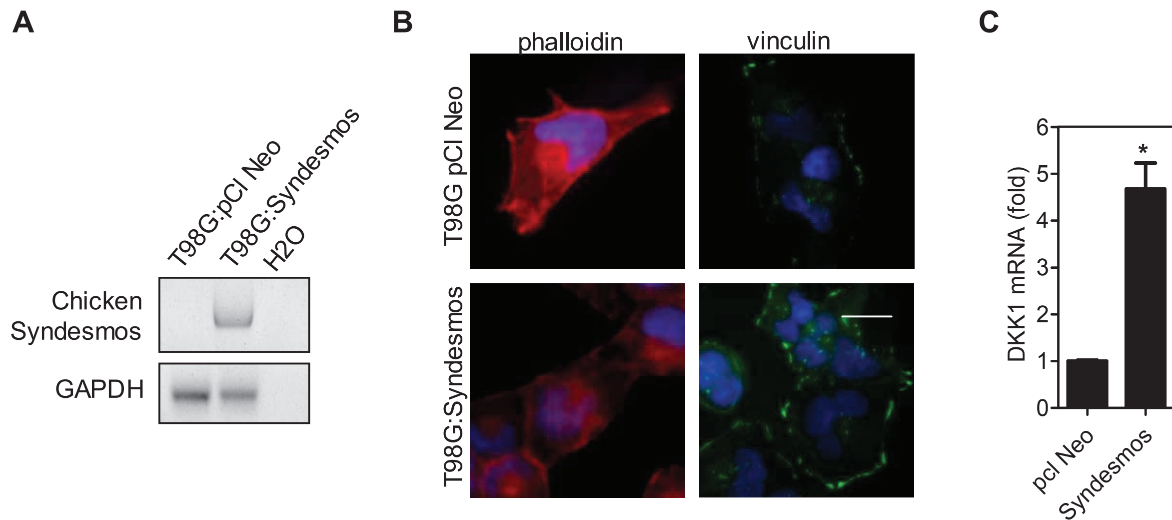


Figure 22. Ectopic expression of chicken (ch) Syndesmos in T98G enhances DKK1 gene expression.
(A) Analysis of chSyndesmos expression in T98G cells by RT-PCR. **(B)** IF staining of T98G:pCL Neo and chSyndesmos expressing T98G for F-actin (phalloidin) and vinculin. Nuclei are stained in blue (DAPI). Scale bar 20 µm. **(C)** *DKK1* gene expression was analysed by qRT-PCR in T98G:pCl Neo and T98G:chSyndesmos cells. Error bars represent SEM and asterisks (*) indicate p-values < 0.05.

Overexpression and knockdown of TPM1 were confirmed by qRT-PCR (**Figure 23A**) and IF (**Figure 23B**). TPM1 overexpression induced the formation of focal adhesions and stress fibres (**Figure 23C**). Upon TPM1 overexpression *DKK1* gene expression was induced 3.7-fold. Conversely, the knockdown of TPM1 in T98G cells strongly reduced *DKK1* gene expression (**Figure 23D**).

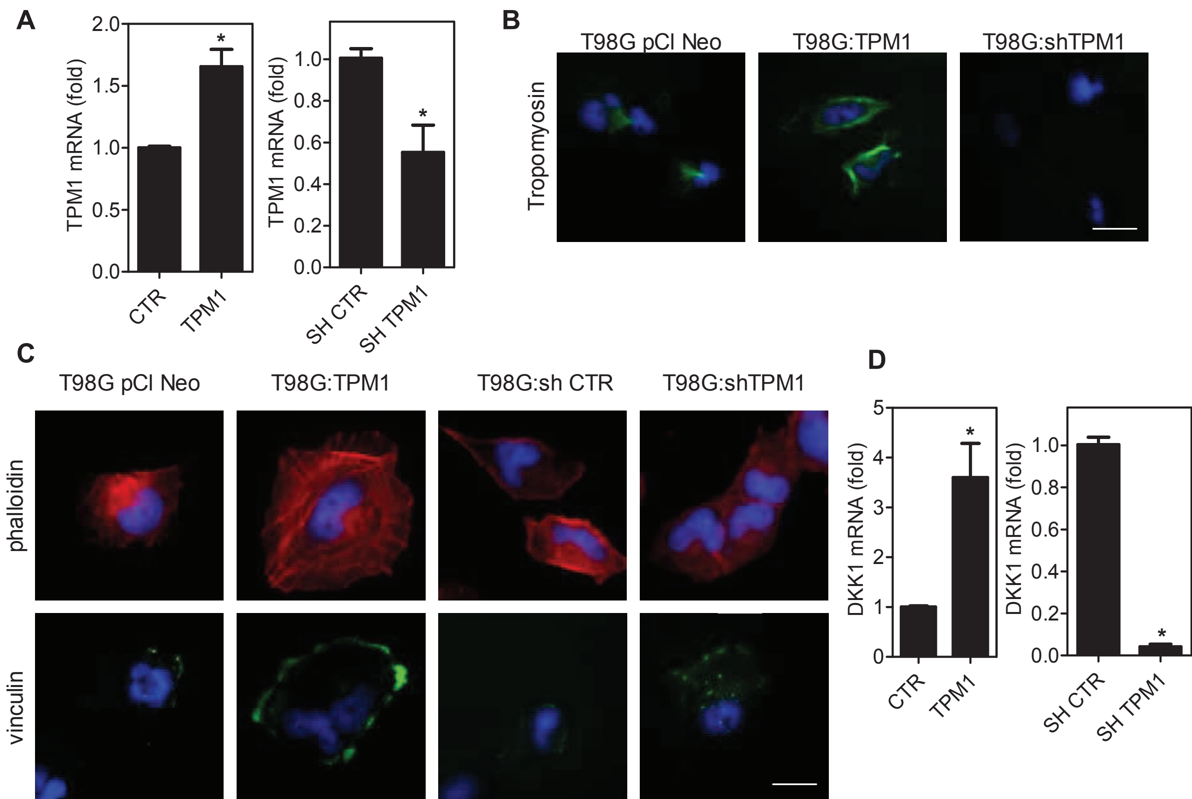


Figure 23. Tropomyosin1 (TPM1) enhances DKK1 expression in T98G cells.
(A, B) Analysis of TPM1 expression in T98G knockdown for TPM1 or overexpressing TPM1 by IF **(A)** or qRT-PCR **(B)**. **(C)** IF staining for F-actin (Phalloidin) and Vinculin in T98G cells knockdown for TPM1 or overexpressing TPM1. Nuclei are stained in blue (DAPI). Scale bar 20 µm. **(D)** *DKK1* gene expression levels analysed by qRT-PCR in T98G cells overexpressing TPM1 (left panel) or T98G cells knockdown for TPM1 (right panel). Error bars represent SEM and asterisks (*) indicate p-values < 0.05.

In order to elucidate whether reduced stress fibre formation in the presence of TNC is responsible for reduced DKK1 levels, T98G grown on FN or FN/TNC were treated with LPA. LPA treatment restored cell spreading on FN/TNC (**Figure 24A**). In addition, by analysing *DKK1* gene expression, it was observed that LPA restored DKK1 expression on FN/TNC (**Figure 24B**).

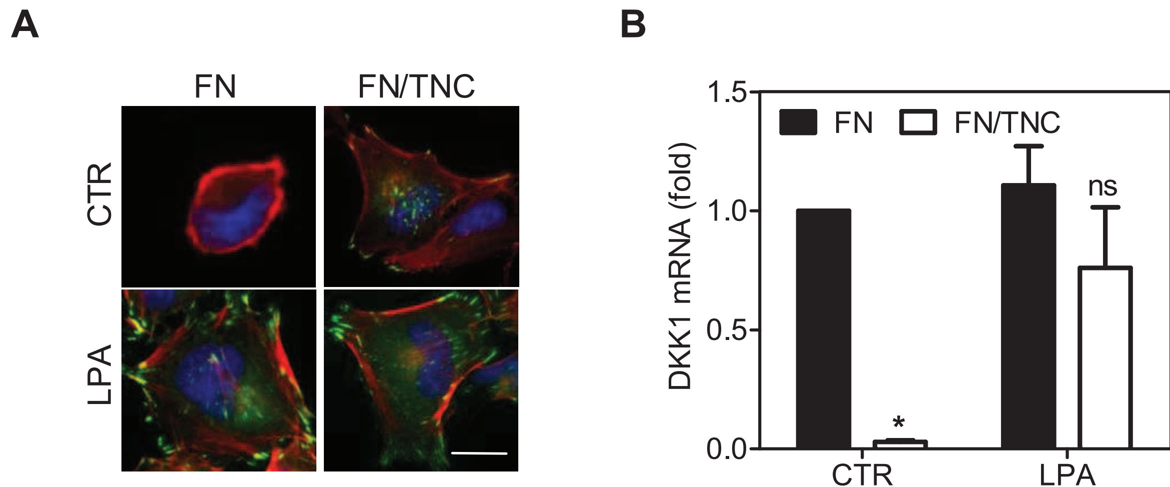


Figure 24. LPA restores cell spreading and DKK1 expression on FN/TNC in T98G cells.
(A-B) Serum starved T98G cells were seeded on FN or FN/TNC and after 1 hour LPA (30 µM) was added for 4 hours. **(A)** IF staining for F-actin (phalloidin – red) and vinculin (green). Nuclei are stained in blue (DAPI). Scale bar 20 µm. **(B)** *DKK1* gene expression was analysed by qRT-PCR. Error bars represent SEM and asterisks (*) indicate p-values < 0.05.

These results suggest that DKK1 downregulation by TNC is mediated by TNC-induced impairment of stress fibre formation, as LPA treatment both restored cell spreading and stress fibre formation and DKK1 levels on a FN/TNC substratum to levels as on FN. Altogether, these experiments strongly support the notion that DKK1 expression is regulated by an intact actin cytoskeleton.

4.4.3 RhoA alone is not implicated in DKK1 downregulation on a FN/TNC substratum

RhoA is a central regulator of actin polymerisation (see section 1.3.2). Its activity can be induced by several external stimuli, including integrin signalling and growth factor treatment e.g. with LPA (Ridley and Hall, 1992). Previous studies have demonstrated that RhoA activity or protein levels are reduced in the presence of TNC, indicating that reduced RhoA activity might be central in mediating the TNC specific lack of actin stress fibre formation (Wenk *et al.*, 2000; Lange *et al.*, 2007). In order to analyse whether RhoA is also involved in regulation of DKK1 expression, I transiently overexpressed wt RhoA, constitutively active (CA) RhoA (Q63L) or dominant negative (DN) RhoA (T19N) in T98G cells. CA-RhoA cannot hydrolyse GTP and therefore signals constitutively to its effector proteins (Heasman and Ridley, 2008). DN-RhoA allows binding to GEFs but inhibits downstream interactions with effector proteins (Heasman and Ridley, 2008). I confirmed overexpression of RhoA by qRT-PCR analysis (**Figure**

25A) and Western Blot (**Figure 25B**). *SRF* gene expression and SRF luciferase reporter activity were used as readouts as expected RhoA-induced downstream signalling (**Figure 25C, D**). However, neither the expression of wt RhoA nor CA-RhoA, induced DKK1 expression, and DKK1 expression was not downregulated by expression of DN-RhoA (**Figure 25E**). Furthermore, overexpression of CA-RhoA did not rescue *DKK1* gene expression in T98G cells grown on FN/TNC, nor did overexpression of DN-RhoA reduce *DKK1* gene expression on FN. In addition, overexpression of CA-RhoA did not restore cell spreading on a FN/TNC substratum (**Figure 25F**). These results demonstrate that activation of RhoA alone is not sufficient to restore T98G cell spreading on FN/TNC. Apparently, other molecules or additional Rho GTPases are blocked on a FN/TNC substratum and mediate TNC-induced cell rounding and DKK1 downregulation.

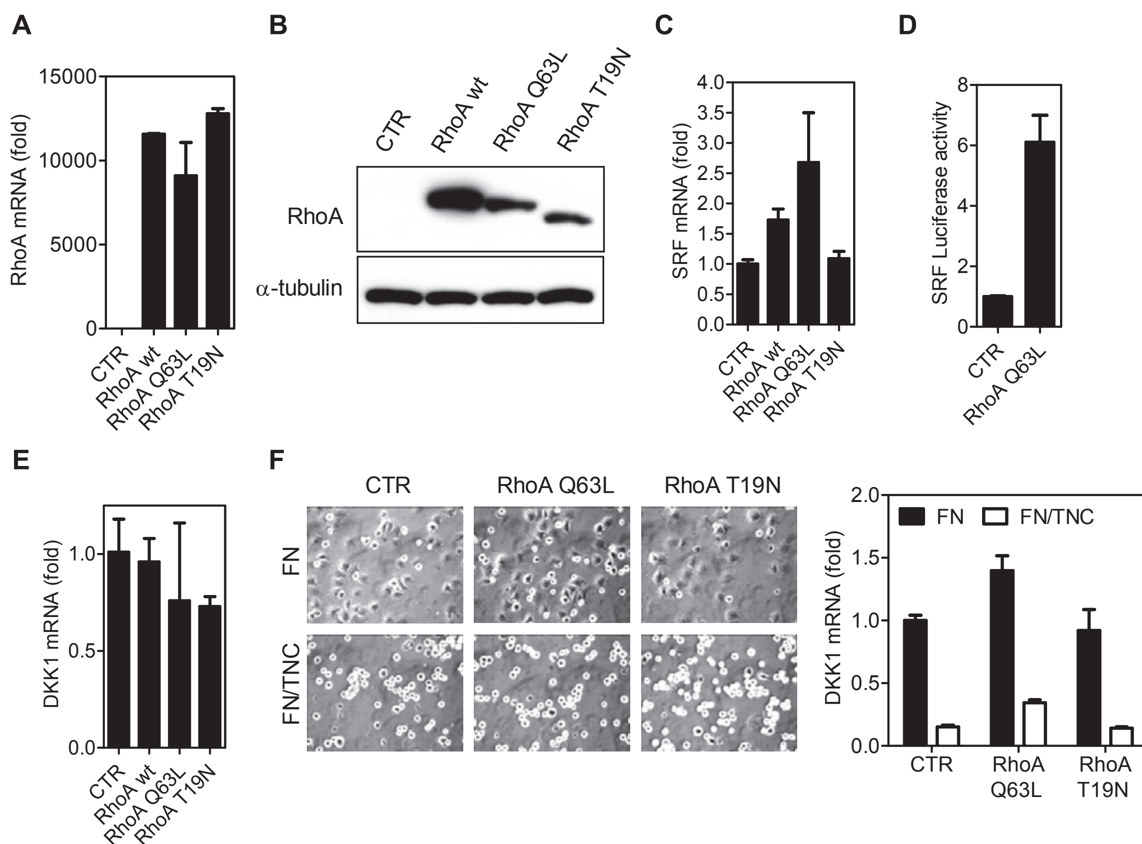


Figure 25. RhoA signalling does not induce DKK1 gene expression and does not restore cell spreading and *DKK1* gene expression on FN/TNC in T98G cells.
T98G cells were transiently transfected with RhoA wt, CA RhoA or DN RhoA. **(A-B)** Overexpression of wt, CA or DN RhoA was verified by qRT-PCR **(A)** and by Western Blotting **(B)**. **(C-D)** *SRF* gene expression **(C)** and SRF luciferase activity **(D)** was analysed upon overexpression of RhoA. **(E)** *DKK1* gene expression was analysed upon overexpression of RhoA. **(F)** Serum starved T98G cells transiently transfected with RhoA CA or RhoA DN were seeded for 5 hours on FN or FN/TNC and analysed for *DKK1* gene expression. Error bars represent SEM.

4.4.4 TNC reduces SRF/MKL1 activity

My previous observations support a direct molecular link between actin stress fibres and the regulation of *DKK1* gene expression. To try to answer to the question of which transcription factor(s) is/are involved in the regulation of *DKK1* gene expression on a FN/TNC substratum, I first analysed in further detail the results from a previous microarray screening from the laboratory (Ruiz *et al*., 2004). Interestingly, I noticed that several SRF/MKL1 target genes were downregulated on a FN/TNC substratum. As the nuclear localisation of the SRF co-transcription factor MKL1 can be regulated by actin polymerisation, e.g. induced by serum or LPA (Miralles *et al*., 2003), my hypothesis was that TNC may induce DKK1 downregulation through impairment of SRF/MKL1 activity.

A SRF/MKL1 specific luciferase reporter assay (3D.A-Luc) was used to examine SRF/MKL1 transcriptional activity in the presence of TNC. The 3D.A-Luc reporter system contains SRF binding sites (derived from the *c-fos* gene) upstream of a Xenopus leavis actin TATA box regulating the expression of the firefly luciferase encoding cDNA. The promoter lacks sequences needed for TCF (Ternary Complex Factors; note that these are different to the T-Cell Factors, also abbreviated TCF, implicated in β-catenin signalling) binding and therefore is only responsive to the SRF/MKL1 pathway. The activity of the SRF reporter was 25% reduced in T98G cells seeded on FN/TNC compared to T98G grown to FN alone, indicating reduced SRF/MKL1 transcriptional activity on FN/TNC (**Figure 26A**). Furthermore, downregulation of different SRF/MKL1 target genes by TNC was confirmed by qRT-PCR in three different tumor cell lines grown on FN or FN/TNC (**Figure 26B-D**). In contrast, *c-fos*, a SRF/TCF target gene, was upregulated in the presence of TNC in T98G and KRIB cells (**Figure 26B, D**). In summary, these findings indicate that TNC inhibits transcriptional SRF/MKL1 activity thus leading to reduced expression of SRF/MKL1 target genes.

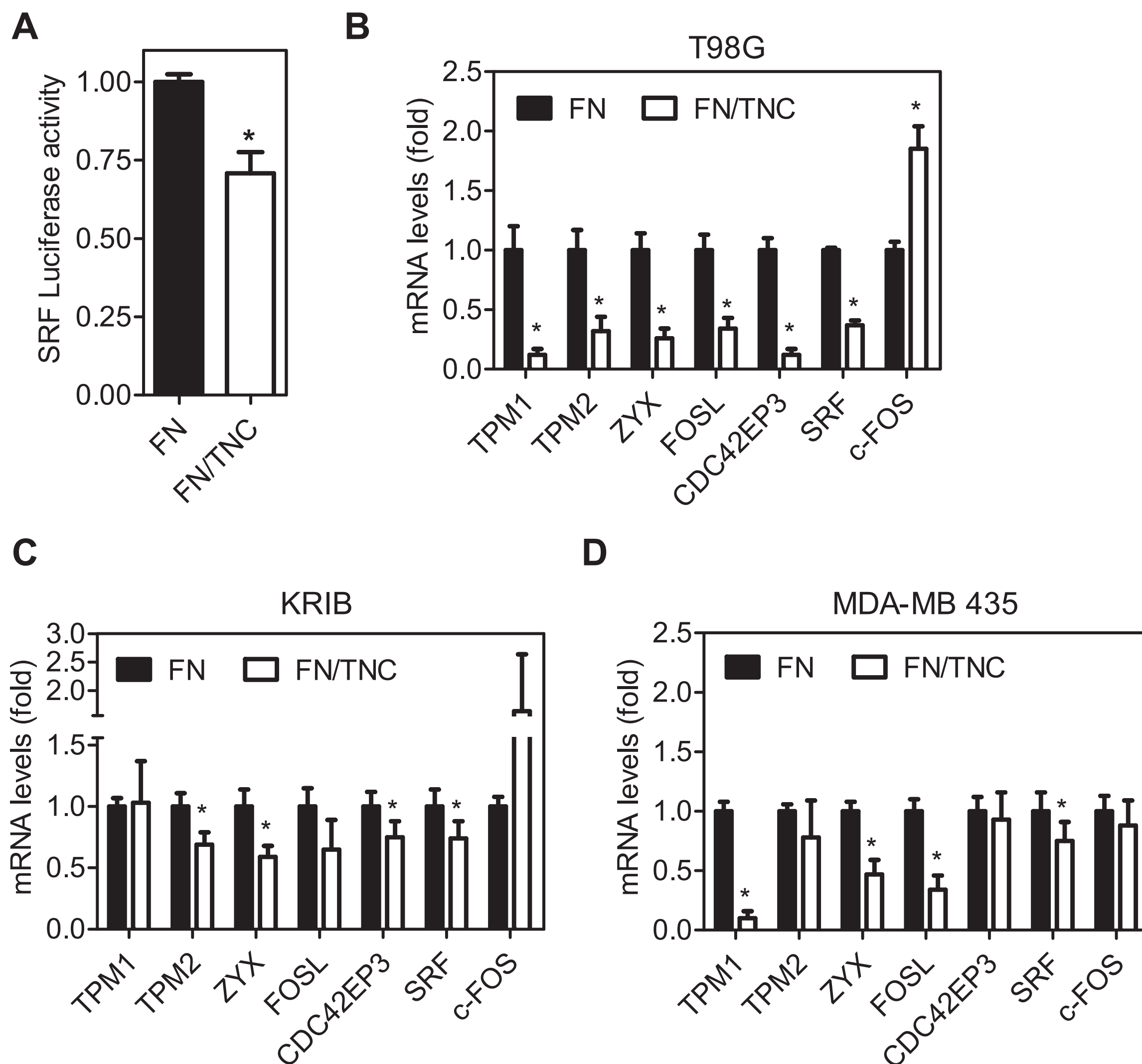


Figure 26. TNC regulates transcriptional activity of SRF/MKL1.
(A) Serum starved T98G cells were grown on FN or FN/TNC (24h) and SRF luciferase activity was analysed. Error bars represent SEM. **(B-C)** qRT-PCR analysis of known MKL1/SRF target genes and a SRF/TCF target gene (*c-fos*) in T98G **(B)**, KRIB **(C)** and MDA-MB 435 **(D)** grown for 24 hours or 48 hours (T98G) on FN or FN/TNC. Error bars represent SD. Asterisks (*) indicate p-values < 0.05.

Next, it was addressed whether low MKL1 levels (mimicking the TNC substratum) had an impact on the expression of MKL1 target genes. Therefore, T98G cells were engineered with a stable shRNA mediated knockdown of MKL1 (**Figure 27A**). Since MKL1 and MKL2 are similarly regulated and might be functionally redundant (Posern and Treisman, 2006) MKL2 was also silenced (**Figure 27A**). For two independent shRNAs targeting MKL1 (SH3 and SH5) a specific knockdown of MKL1 was obtained without affecting MKL2 expression levels. Similarly for MKL2, two different shRNAs (SH2 and SH4) resulted in a decreased MKL2 mRNA expression, but these also impacted on MKL1 expression. Therefore, for further analysis of

the effects of MKL1 knockdown, the MKL1 shRNAs SH3 and SH5 were selected. Whereas MKL2 protein levels could not be assessed due to the lack of an appropriate antibody, the knockdown of MKL1 was confirmed by Western Blot (**Figure 27B**). By qRT-PCR I observed that the above-analysed SRF/MKL1 target genes were also downregulated upon MKL1 knockdown, suggesting that these genes are indeed SRF/MKL1 target genes, while expression of the SRF/TCF target gene *c-fos* was not changed (**Figure 27C**).

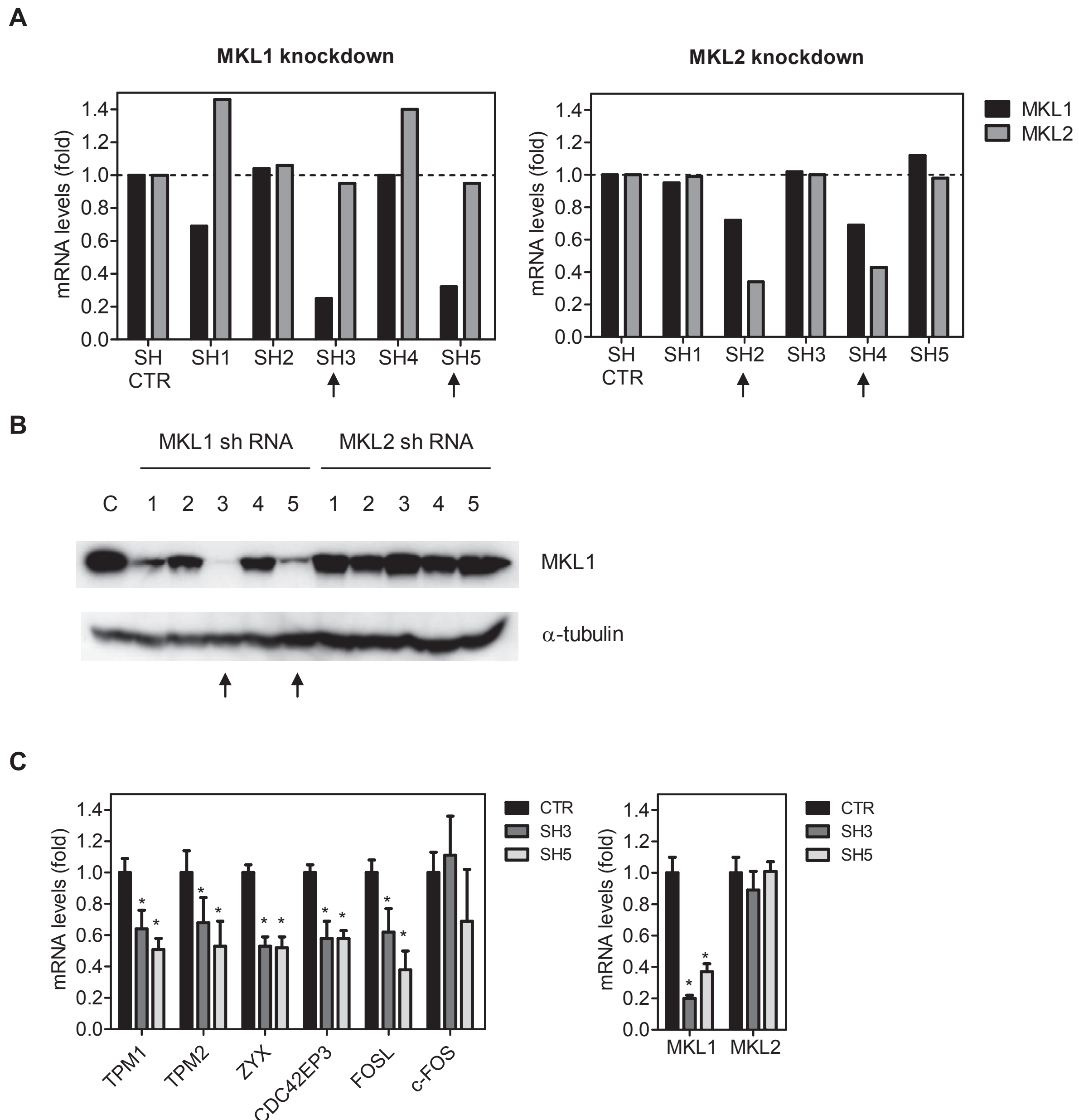


Figure 27. Sh-mediated MKL1 and MKL2 knockdown in T98G cells.
(A) Validation of MKL1 (left panel) and MKL2 knockdown (right panel) in T98G cells by qRT-PCR. **(B)** Validation of MKL1 knockdown in T98G cells by Western Blotting. **(C)** Gene expression analysis of SRF/MKL1 target genes and a SRF/TCF target gene (*c-fos)* in T98G upon MKL1 knockdown with two different shRNA (left panel). qRT-PCR analysis confirmed that *MKL1*, but not *MKL2* gene expression was reduced in analysed T98G cells. Error bars represent SD and asterisks (*) indicate p-values < 0.05.

4.4.5 Regulation of DKK1 expression by MKL1

The effect of MKL1 knockdown on gene expression of *DKK1* was analysed. Using two independent shRNAs for MKL1 knockdown resulted in a 5-fold reduced *DKK1* gene expression (**Figure 28A**). In addition, DKK1 protein levels were also reduced as shown by Western Blot (**Figure 28B**) and a luciferase based DKK1 promoter assay revealed a reduced DKK1 promoter activity upon knockdown of MKL1 (**Figure 28C**).

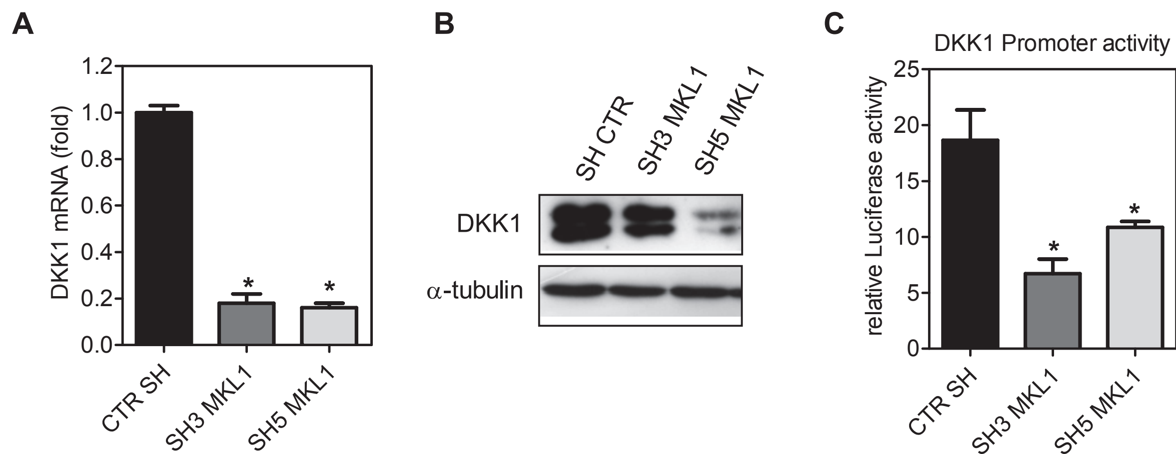


Figure 28. Stable knockdown of MKL1 in T98G cells reduces DKK1 gene expression, protein levels and promoter activity.
(A) Analysis of *DKK1* gene expression by qRT-PCR, **(B)** DKK1 protein expression by Western Blotting and **(C)** DKK1 promoter luciferase activity in T98G cells knockdown for MKL1. Error bars represent SD (A) or SEM (B) and asterisks (*) indicate p-values < 0.05.

In parallel the effect of transiently overexpressing full-length (fl) MKL1 or expressing a constitutively active form of MKL1 (MKL1 ΔN), lacking the G-actin binding site (Miralles *et al.*, 2003), on *DKK1* gene expression was analysed. Overexpression of wt and constitutively active MKL1 was confirmed by qRT-PCR (**Figure 29A**) and Western Blot (**Figure 29B**). To confirm MKL1 activity, SRF expression and SRF luciferase reporter activity were determined (**Figure 29C, D**). Surprisingly, the transient overexpression of MKL1 fl and MKL1 ΔN did not induce DKK1 expression (**Figure 29E**) and when cells were seeded on a FN or FN/TNC substratum DKK1 levels on FN/TNC were not restored (**Figure 29F**). This is in stark contrast to the data described before-hand demonstrating that MKL1 knockdown reduced *DKK1* gene expression and promoter activity. Possible explanations for these discrepancies will be presented in the discussion section.

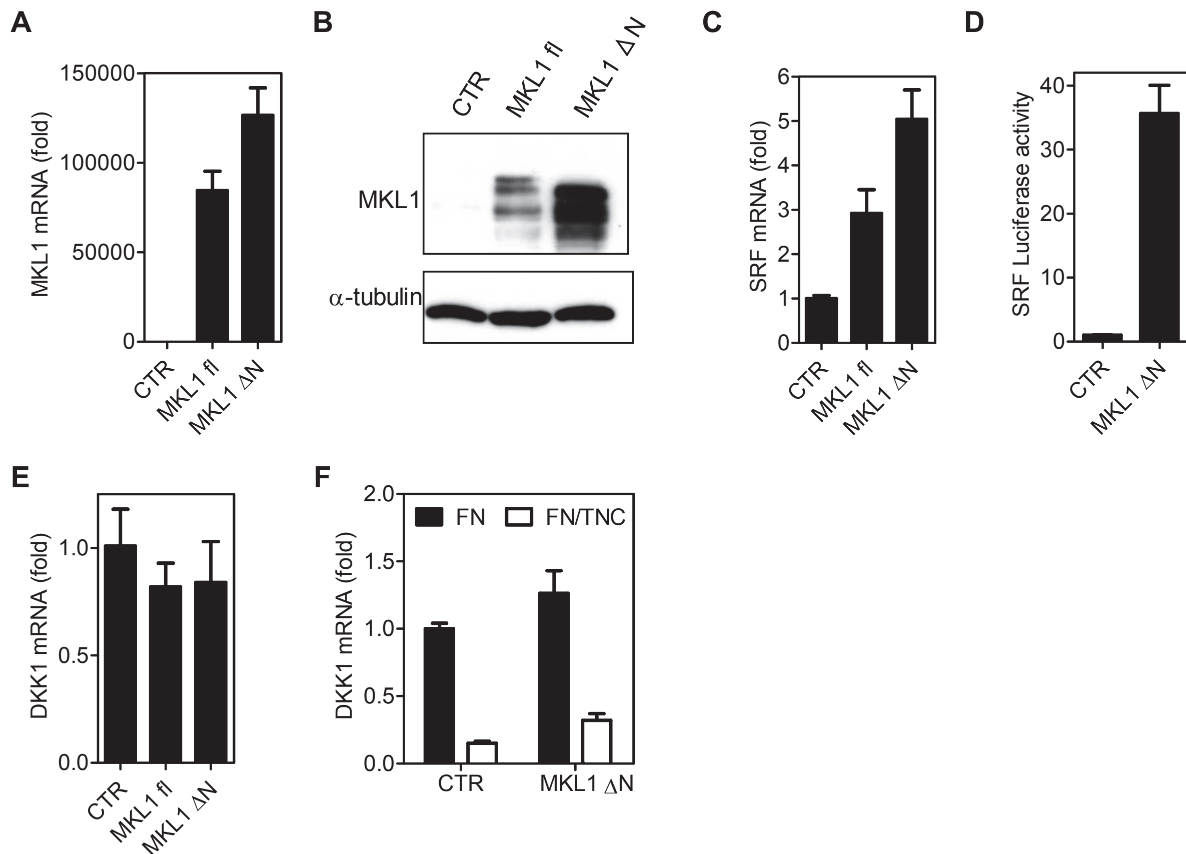


Figure 29. MKL1 overexpression does not restore DKK1 expression on a FN/TNC substratum.
T98G cells were transiently transfected with full-length (fl) MKL1 or constitutively active MKL1 lacking its G-actin binding site (MKL1 ΔN). **(A-B)** Overexpression of MKL1 fl and MKL1 ΔN was verified by qRT-PCR **(A)** and Western Blotting **(B)**. **(C-D)** *SRF* gene expression **(C)** and SRF luciferase activity **(D)** upon overexpression of MKL1 was analysed. **(E)** *DKK1* gene expression upon overexpression of MKL1 was analysed by qRT-PCR. **(F)** Serum starved T98G transiently transfected with MKL1 ΔN were seeded for 5 hours on FN or FN/TNC and analysed for *DKK1* gene expression. Error bars represent SEM.

4.4.6 Regulation of DKK1 expression by actin binding drugs

In order to analyse in more detail the contribution of F-actin stability to DKK1 regulation, T98G cells were treated with different drugs stabilizing or disrupting F-actin. Cytochalasin D and Latrunculin B both inhibit the formation of polymerised F-actin. Furthermore, co-treatment with Cytochalasin D and Latrunculin B is helpful to discriminate between genes either depending on an intact cytoskeleton or SRF/MKL1 regulated genes (**Figure 30**). MKL1 is sequestered by monomeric G-actin in the cytosol; when actin is polymerised into F-actin, MKL1 is released from G-actin and can enter the nucleus (see also chapter 1.3.3). Cytochalasin D depolymerises F-actin but also releases MKL1 from G-actin therefore activating MKL1-dependent transcription. Latrunculin B inhibits actin polymerisation and blocks the dissociation of MKL1 from G-actin. Therefore, in serum starved cells a pre-

treatment with Latrunculin B prevents Cytochalasin D-mediated induction of SRF/MKL1 target genes (**Figure 30**) (Miralles *et al.*, 2003; Descot *et al.*, 2009).

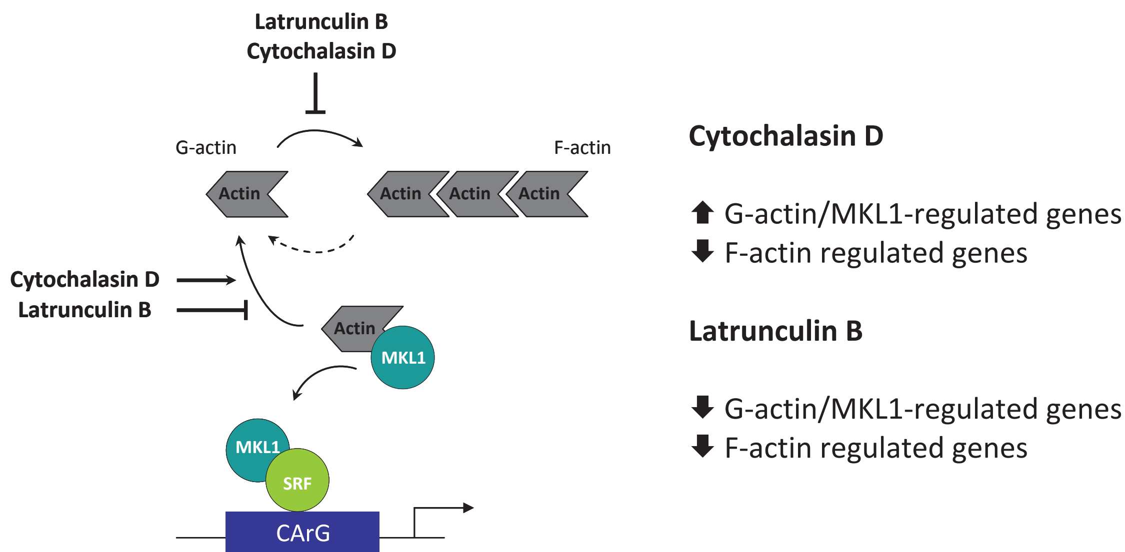


Figure 30. Effect of Latrunculin B and Cytochalasin D on actin polymerisation and SRF/MKL1 target gene expression.

Upon treatment with Latrunculin B and Cytochalasin D both stress fibres and vinculin-containing focal adhesions were removed in T98G (**Figure 31A**). Treatment with Cytochalasin D induced SRF expression as well as SRF-luciferase activity (**Figure 31B, C**). Interestingly, DKK1 expression was induced upon treatment with 0.2 µM and 0.5 µM Cytochalasin D (**Figure 31D**) despite a lack of stress fibres and focal adhesions (**Figure 31A**). However, if a higher concentration of Cytochalasin D was used, *DKK1* gene expression was not changed (1 µM) or even reduced (2 µM) (**Figure 31E**). As expected, treatment with Latrunculin B did neither induce *SRF* gene expression nor luciferase activity in serum starved T98G cells (**Figure 31E, F**). However, DKK1 expression was 2.5-fold reduced (**Figure 31G**). I analysed whether Latrunculin B represses the Cytochalasin D-induced target gene expression, which is characteristic for genes regulated by MKL1. Therefore, serum starved T98G cells were pre-treated for 15 minutes with Latrunculin B before treatment with Cytochalasin D. As expected, Latrunculin B reduced Cytochalasin D-induced *SRF* gene expression and luciferase activity (**Figure 31H, I**). *DKK1* gene expression was also significantly reduced by Latrunculin B when cells were treated with 0.5 µM Cytochalasin D (**Figure 31J**).

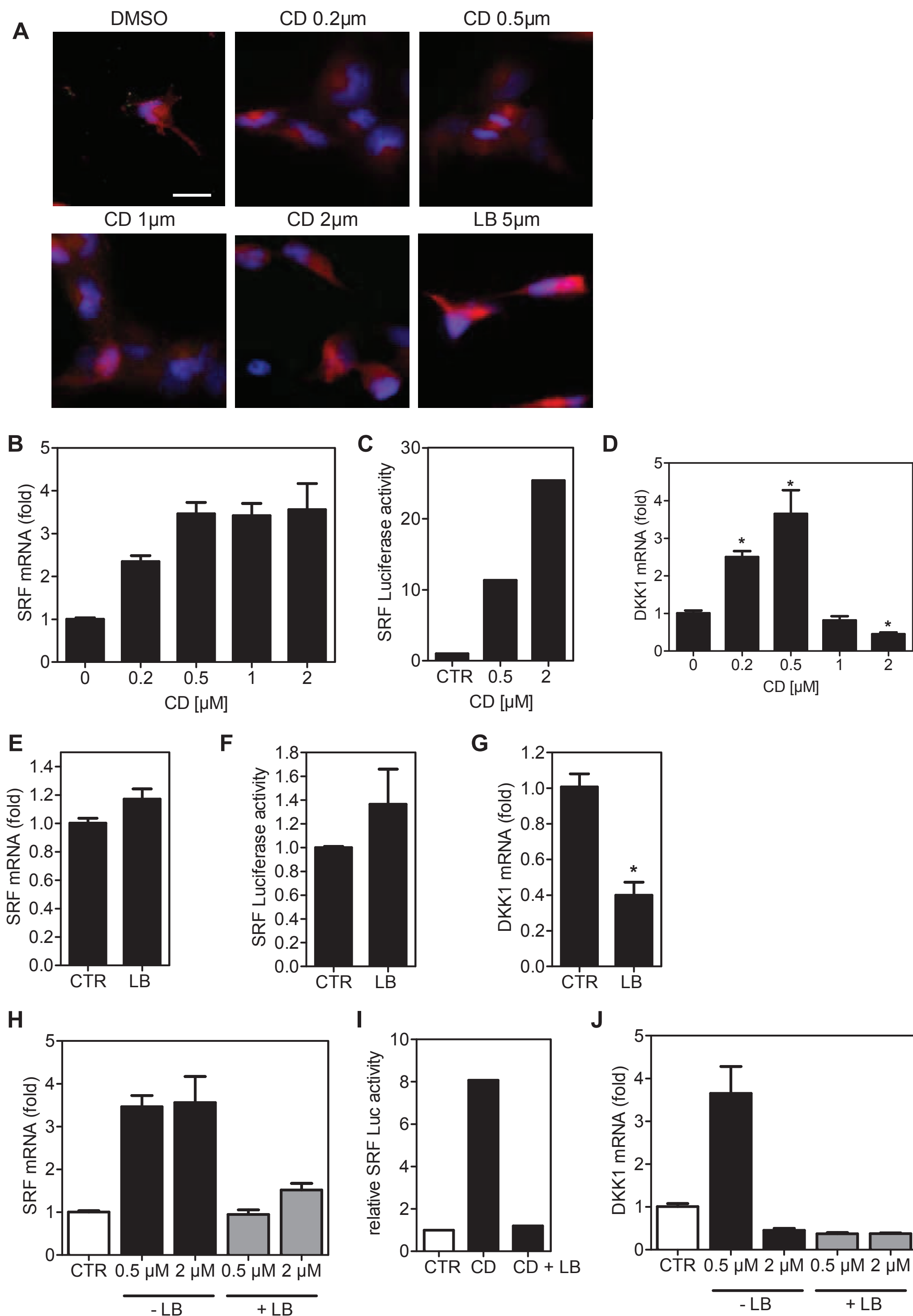


Figure 31. Regulation of DKK1 expression by F-actin depolymerising drugs: Cytochalasin D induces DKK1 expression at low and inhibits DKK1 expression at high concentration, while Latrunculin B reduces DKK1 expression.
Serum starved T98G cells were treated for 3 hours with the indicated concentrations of Latrunculin B (LB) and Cytochalasin D (CD). **(A)** IF staining of CD- and LB-treated T98G cells for F-actin (phalloidin) and vinculin. Nuclei are stained in blue (DAPI). Scale bar 20 µm. **(B, C)** *SRF* gene expression **(B)** and SRF luciferase activity **(C)** upon treatment with CD. **(D)** Gene expression analysis of *DKK1* after CD treatment. **(E, G)** *SRF* gene expression **(E)** and SRF Luciferase activity **(F)** upon treatment with LB were analysed. **(G)** Gene expression of *DKK1* was analysed after LB treatment. **(H-J)** Serum starved T98G cells were treated with 0.5 µM and 2 µM CD alone for 3 hours or pre-treated for 15 min with 5 µM LB. **(H, I)** *SRF* gene expression **(H)**, SRF luciferase activity **(I)** or *DKK1* gene expression **(J)** upon treatment with CD or CD and LB. Error bars represent SEM and asterisks (*) indicate p-values < 0.05.

In summary, those experiments revealed that DKK1 shows both characteristics of a SRF/MKL1 target gene (inducible by low concentrations of Cytochalasin D and inhibited by additional treatment with Latrunculin B) and an F-actin regulated gene (inhibited by 2 µM Cytochalasin D, inhibited by Latrunculin B).

In contrast to Cytochalasin D and Latrunculin B, Jasplakinolide stabilizes F-actin and thereby depletes the cellular G-actin pool. Yet Jasplakinolide also disrupts stress fibres (Bubb *et al.*, 2000). As expected, treatment with Jasplakinolide induces *SRF* gene expression and luciferase activity at different concentrations (**Figure 32A, B**). DKK1 expression did not change after 1 hour of treatment, while its expression was decreased after 3 hours of treatment with Jasplakinolide (**Figure 32C**). These data demonstrate that while SRF activity was induced by Jasplakinolide, induction of filamentous actin per se did not induce DKK1 expression, but decreased DKK1 expression.

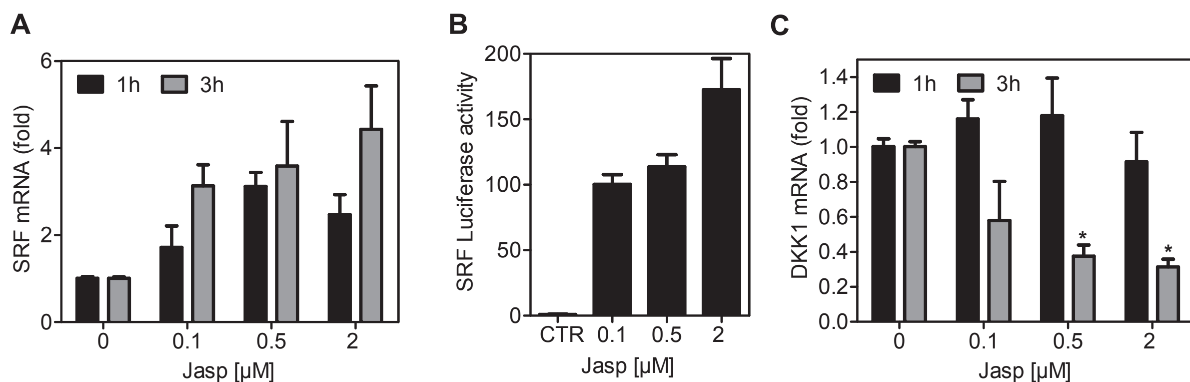


Figure 32. Treatment of T98G cells with Jasplakinolide reduces DKK1 gene expression while it enhances SRF activity.
(A-C) Serum starved T98G cells were treated with the indicated concentration and at the indicated time points with Jasplakinolide (Jasp). **(A,B)** *SRF* gene expression after 1 hour or 3 hours of treatment **(A)** and SRF Luciferase activity after 3 hours of treatment with Jasp **(B)**. **(C)** *DKK1* gene expression was analysed by qRT-PCR after 1 hour or 3 hours of Jasp treatment. Error bars represent SEM and asterisks (*) indicate p-values < 0.05.

In summary, SRF luciferase activity and *SRF* gene expression were induced by Cytochalasin D in a concentration-dependent manner and Cytochalasin D-induced SRF expression was repressed by Latrunculin B. Also Jasplakinolide induced SRF activity, indicating that polymerisation of actin and therefore depleting the G-actin pool is sufficient to induce SRF expression. Although *DKK1* gene expression was induced by low concentration of Cytochalasin D and Cytochalasin D-induced *DKK1* gene expression was repressed by

Latrunculin B treatment, high concentrations of Cytochalasin D reduced DKK1 expression. Of note, also upon induction of actin polymerisation by Jasplakinolide DKK1 expression was reduced, indicating that actin polymerisation alone is not sufficient for induction of *DKK1* gene expression.

Therefore, these experiments demonstrated that DKK1 is not regulated in the same manner as a canonical SRF/MKL1 target gene and other mechanisms may account for DKK1 regulation by the actin cytoskeleton.

In conclusion, I have several indications that DKK1 regulation depends on an intact actin cytoskeleton, however, I could not unravel the exact molecular mechanism(s) yet. Induction of stress fibre formation by LPA or overexpression of the stress fibre stabilizing protein TPM1 induced *DKK1* gene expression. RhoA which is a known central actor for actin dynamics downstream of LPA is not responsible for this effect. Furthermore, using knockdown approaches for the actin-regulated co-transcription factor MKL1 indicated that MKL1 participates to the regulation of *DKK1*; however and unexpectedly, I could not confirm that MKL1 directly and alone regulates DKK1 expression upon MKL1 overexpression and drug treatments modulating MKL1 activity.

5 Summary

Following the first aim of my thesis I observed that TNC downregulates the Wnt signalling inhibitor DKK1 in a wide range of tumor cells *in vitro*. In KRIB tumor cells DKK1 downregulation was linked to enhanced Wnt signalling as analysed by a TCF/LEF-based reporter assay and as shown by DKK1 knockdown and overexpression approaches. Furthermore, in a number of stromal cell types (endothelial cells, pericytes, CAF) I also observed downregulation of the Wnt signalling inhibitor DKK1. TNC induced Axin2 expression, indicative of elevated Wnt signalling, in HUVEC cells. In contrast to endothelial cells, in pericytes no induction of Axin2 was observed on a FN/TNC substratum, supporting published results that suggest that DKK1 might mainly exert β-catenin independent signalling effects in pericytes (**Figure 33A**).

In the second aim a potential correlation between reduced DKK1 levels and enhanced angiogenesis, observed in the RT2 and RT2/TNC tumor model, was addressed by a xenograft model with tumorigenic cells overexpressing TNC, mDKK1 or both molecules. But due to technical issues this aim could not be accomplished.

Third aim of my thesis was to analyse the mechanism(s) implicated in DKK1 downregulation downstream of TNC. I showed that TNC regulates DKK1 expression by reducing its promoter activity. Since cells on a FN/TNC substratum fail to establish actin stress fibres I mimicked disruption of the actin cytoskeleton by Latrunculin B or by knockdown of TPM1 and found that indeed the lack of an actin stress fibre network resulted in low *DKK1* gene expression. Treatment with the actin de-polymerising drug Cytochalasin D inhibited *DKK1* gene expression, however stimulated *DKK1* gene expression at low concentrations, which is characteristic for genes regulated by the SRF co-transcription factor MKL1. Indeed, knockdown of MKL1 resulted in decreased *DKK1* gene expression and reduced DKK1 promoter activity. However, MKL1 overexpression did not restore DKK1 levels on a FN/TNC substratum. Thus, despite regulation of DKK1 by MKL1, TNC might downregulate DKK1 transcription in a MKL1 independent manner. I also showed that DKK1 downregulation on a FN/TNC substratum can be rescued upon restoration of actin stress fibre formation by LPA. In addition, enforcement of actin stress fibres by overexpression of TPM1 largely increased DKK1 expression, while induction of actin polymerisation alone by Jasplakinolide treatment was not sufficient to induce *DKK1* gene expression. Altogether, my results showed that DKK1

expression is regulated by the formation of stress fibres and that TNC impairs DKK1 expression due to inhibition of actin stress fibre formation (**Figure 33B**).

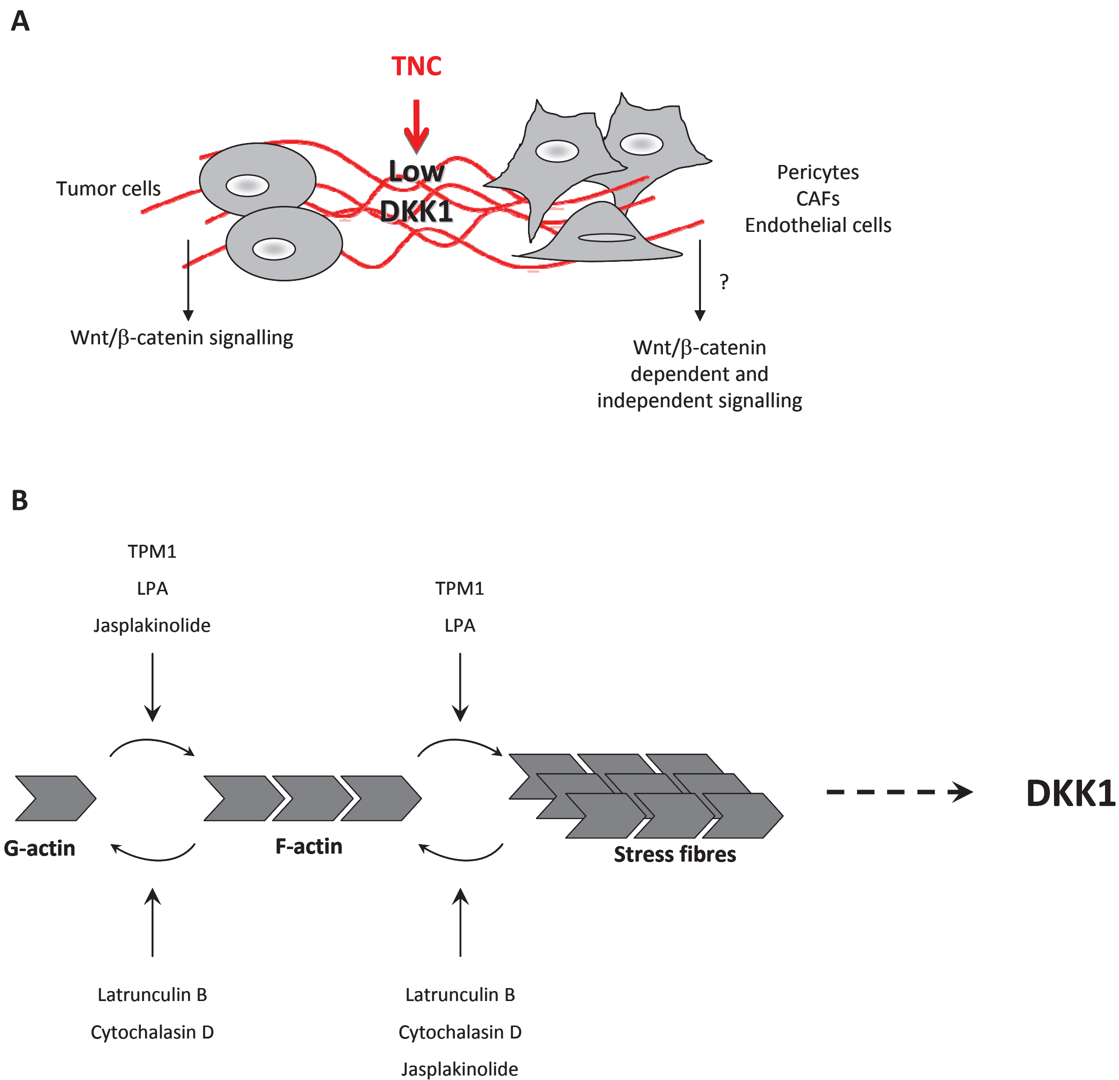


Figure 33. Summary of the major results on the consequences and mechanism of DKK1 downregulation by TNC. (A) TNC reduces *DKK1* gene expression in tumor and stromal cells. In tumor cells DKK1 downregulation mediates stimulation of Wnt/β-catenin signalling. In stromal cells the effect of DKK1 downregulation on Wnt//β-catenin signalling needs to be analysed in further detail. **(B)** Model of DKK1 regulation by the actin polymerisation status of the cell. Disruption of stress fibres and actin depolymerisation (Latrunculin B, Cytochalasin D) decreases *DKK1* gene expression, while induction of actin polymerisation and stress fibre formation (LPA, TPM1) increases *DKK1* gene expression. Jasplakinolide-mediated actin polymerisation, but stress fibre disruption decreases *DKK1* gene expression.

6 Discussion and Perspectives

6.1 Consequences of downregulation of DKK1 by TNC in tumor and stromal cells

The tumor microenvironment is a crucial driver of tumor progression. The expression of the microenvironmental ECM molecule TNC has been correlated with low patient survival, lung metastagenicity of breast cancer cells, tumor cell proliferation, migration and tumor angiogenesis (reviewed in Orend *et al.*, 2013).

In order to identify possible downstream signalling pathways regulated by TNC, by an *in vitro* microarray approach the Wnt inhibitor DKK1 was identified as a target gene downregulated by TNC (Ruiz *et al*., 2004), implicating a role of TNC in modulating Wnt signalling. Here I have analysed the role of TNC mediated DKK1 downregulation in tumor and stromal cells on Wnt signalling.

I will discuss in more detail:

1. The TNC mediated DKK1 downregulation on Wnt signalling in tumor cells and its possible consequences on tumor progression.

2. The role of TNC on DKK1 expression and Wnt signalling in stromal cells.

3. The potential impact of TNC on tumor angiogenesis through DKK1 and the possible mechanisms by which DKK1 regulates tumor angiogenesis.

6.1.1 TNC enhances Wnt signalling in tumor cells

TNC induced DKK1 downregulation augments Wnt signalling in tumor cells

DKK1 downregulation by TNC has been demonstrated here in several tumor cell lines indicating that DKK1 downregulation by TNC is a general effect on tumor cells. In HEK293 cells no impact of TNC on *DKK1* gene expression levels was observed. One could speculate that the integrin receptor-status needed for proper attachment and spreading on FN might play a role for TNC-induced DKK1 downregulation, as HEK293 cells do not express the FN-binding integrin $\alpha v\beta 3$ (Simon *et al*., 1997; Li *et al*., 2001; Taherian *et al*., 2011). Thus it would be interesting to analyse if overexpression or knockdown/blocking of the FN-binding

integrins $\alpha v\beta 3$-integrin and $\alpha 5\beta 1$-integrin might have an impact on *DKK1* gene expression levels.

Furthermore, TNC enhanced Wnt signalling activity in KRIB cells as shown by enhanced TOPFlash activity and increased expression of the bona fide Wnt target gene *Axin2*. By using *in vitro* assays with DKK1 overexpression and sh-mediated DKK1 knockdown approaches it was demonstrated that TNC enhances Wnt signalling in KRIB cells by downregulation of the Wnt inhibitor DKK1. Upon treatment with mDKK1 containing CM I showed that TNC induces TOPFlash activity to a lesser extent than in the control treatment, indicating that indeed DKK1 downregulation by TNC is the major mechanism by which TNC induces Wnt signalling in these cells. The experiment might have been biased by the fact that treatment with mDKK1 containing CM and not purified DKK1 was used. One cannot exclude that ectopic expression of mDKK1 in KRIB cells potentially has changed the expression of other secreted factors that might affect the readout. Another way to test the involvement of DKK1 in TNC-regulated TOPFlash activity, would be to use a DKK1 neutralizing antibody that inhibits DKK1 activity in the CM of cells seeded on FN or FN/TNC. Of note, knockdown of DKK1 did not allow TNC anymore to stimulate TOPFlash activity, demonstrating that indeed DKK1 downregulation by TNC is the main mechanism by which TNC induces TOPFlash activity. While I have demonstrated that enhanced TOPFlash activity in the presence of TNC is indeed regulated through TNC mediated repression of DKK1, I was not able to establish a direct link between DKK1 downregulation by TNC and enhanced Axin2 expression, because TNC induced Axin2 expression upon treatment with mDKK1 CM to the same levels as with control treatment. It is conceivable that Axin2 induction by TNC depends on TNC mediated DKK1 downregulation plus an additional mechanism. Possibly, the TNC mediated downregulation of other secreted Wnt inhibitors than DKK1 in KRIB cells, such as sFRP1, could enhance Axin2 expression in parallel. Another possibility could be that TNC regulates Axin2 expression by affecting directly intracellular signalling independent of secreted factors.

Does the TNC-mediated stimulation of Wnt signalling promote tumor progression?

Wnt signalling is a major pathway promoting tumor progression in several tumor types (Anastas and Moon, 2013). Therefore, one major mechanism by which TNC potentially promotes tumor progression is through enhancing Wnt signalling in tumor cells. Ectopic

expression of TNC in the RT2 tumor model is correlated with an increased Axin2 expression, suggesting that TNC may have enhanced Wnt signalling *in vivo*. However, a previous study showed that tumor progression due to the loss of E-cadherin in the RT2 model is independent from Wnt signalling in tumor cells, as both overexpression of a stabilized β-catenin and KO of TCF-1 in tumor cells did not impact on tumor progression (Herzig *et al*., 2007). Therefore, it should be questioned whether an increase of Axin2 expression/Wnt signalling in tumor cells upon ectopic TNC expression is the major mechanism by which TNC induces tumor progression in the RT2 model. Yet, we do not know which cells exactly contribute to the observed Axin2 induction. As the observed induction of Axin2 expression by TNC was only very moderate (1.35-fold), it is possible that Wnt signalling in stromal cells, in particular in endothelial cells, may have promoted tumor progression through an impact on tumor angiogenesis (discussed in chapter 6.1.3).

Despite a direct proof for canonical Wnt/β-catenin signalling in tumor cells of RT2/TNC tumors and an impact on tumor progression, it is conceivable that in other tumor types TNC might enhance Wnt signalling and tumor progression. A study by Oskarsson *et al*. (2011) showed that TNC increased the expression of the Wnt target gene *Lgr5* in metastatic breast cancer cells contributing to their survival (Oskarsson *et al*., 2011). That ECM may have an impact on Wnt signalling was shown in another study. POSTN, another ECM glycoprotein and component of the tumor microenvironment, presents Wnt ligands to metastatic breast stem cells and thereby enhances Wnt signalling and lung colonization (Malanchi *et al*., 2012). Furthermore, POSTN has been shown to enable the incorporation of TNC into the ECM (Kii *et al*., 2010) indicating that both molecules might cooperate in enhancing Wnt signalling activity (Oskarsson and Massagué, 2012).

Therefore, for further analysis it will be interesting to analyse the effect of a combined TNC and POSTN substratum on Wnt signalling activity in tumor cells. In addition, our group started to analyse the impact of pseudo-3D ECM substrata on tumor and stromal cell behaviour by using cell derived matrixes (CDM). In this assay, (cancer-associated) fibroblasts, which can be engineered for overexpression and knockdown of ECM molecules (such as FN, TNC and POSTN), deposit a layer of ECM on which then subsequently tumor cells or stromal cells can be cultured. In this assay the function and structure of ECM proteins might be better reflected than by coating a plastic dish with purified ECM proteins. Therefore, the

CDM represents an interesting tool to analyse *in vitro* the impact of ECM components, in particular TNC and POSTN, in tumor and stromal cell behaviour in the future.

Interestingly, TNC is a Wnt/β-catenin target gene itself. Expression of Wnt7b in the pulmonary vascular smooth muscle cell line Pac1 increased TNC expression while siRNA mediated β-catenin knockdown decreased TNC expression. Similarly, loss of β-catenin expression in smooth muscle cell precursors resulted in decreased TNC expression and TNC expression was enhanced in the airways of LiCl-treated embryos. Furthermore, TNC expression was reduced in Wnt7b-deficient embryos. The TNC promoter was responsive to β-catenin and β-catenin was bound to the TNC promoter (Cohen *et al*., 2009). In human CRC TNC was expressed in the ECM surrounding cells with nuclear β-catenin at the tumor invasion front. *In vitro* experiments demonstrated that TNC expression is reduced upon knockdown of β-catenin in CRC cell lines and that β-catenin associates with the TNC promoter (Beiter *et al*., 2005) altogether supporting that TNC itself is a Wnt target. Therefore, these data indicate that a positive feedback loop between Wnt/β-catenin signalling and TNC might exist in tumor cells, fuelling tumor progression.

In conclusion, I have demonstated a direct link between TNC induced DKK1 downregulation and activation of Wnt signalling in tumor cells, which may be essentiell for tumor progression.

6.1.2 Role of TNC on DKK1 expression and Wnt signalling in pericytes and CAFs

In a tumor also stromal cells are in contact with TNC. Therefore, I was interested to analyse whether TNC also regulates DKK1 and Wnt signalling in stromal cells. Indeed TNC induced DKK1 downregulation in endothelial cells, pericytes as well as in CAFs. Due to difficulties in transfection of primary cell lines with the TOPFlash reporter construct I so far had to limit the analysis of Wnt/β-catenin signalling activity in these cells to the analysis of *Axin2* gene expression. However, we are in progress to analyse TOPFlash activity with cells transduced with a lentiviral TOPFlash reporter construct, which will further help to elucidate if TNC is able to induce Wnt signalling in stromal cells.

Our results indicate no effect of TNC on Wnt signalling in pericytes, as no change in Axin2 expression upon Wnt3A stimulation was observed in the presence of TNC.

Very recently, a study using a murine model of kidney injury, demonstrated that DKK1 inhibits pericyte expansion, proliferation and transition to myofibroblasts. In particular, DKK1 inhibited PDGF-BB and TGF-β signalling, which respectively promote proliferation and migration of pericytes. Of note, DKK1 effects were independent of β-catenin signalling as the same results were obtained with β-catenin-deficient pericytes. But DKK1 effects were dependent on the secretion of Wnt ligands binding to LRP6 and co-receptors PDGFR and TGF-βR (Ren *et al*., 2013). As DKK1 seems to mostly target pathways independent of β-catenin in this cell type, these results might explain why in my experiments no enhanced Axin2 expression in pericytes occured in the presence of TNC and reduced DKK1 levels. Therefore, there is a need for further studies to analyse if DKK1 downregulation in the presence of TNC potentially promotes TGF-β and/or PDGF-BB signalling and thus eventually enhances migration and proliferation of pericytes and CAFs.

Interestingly, the authors demonstrated that DKK1 also blocks the activation and proliferation of myofibroblasts *in vitro*, which might be relevant in the tumor microenvironement, where activation of myofibrobasts plays an important role in driving tumor progression (Kalluri and Zeisberg, 2006; Hanahan and Weinberg, 2011). TNC might contribute to the activation and expansion of CAFs by reducing DKK1 levels and promoting β-catenin independent signalling.

In addition, Ren and coworkers (2013) demonstrated that DKK1 inhibited pericyte detachment from capillaries. This result might be relevant in a TNC context, as we observed less pericyte blood vessel coverage in tumors of RT2/TNC mice (Saupe, Schwenzer *et al.,* accepted) which correlated with reduced DKK1 levels. Interestingly, reduced pericyte coverage is assumed to promote extravasation of tumor cells and therefore metastasis (Hanahan and Weinberg, 2011; Mazzone *et al*., 2009). We observed indeed enhanced lung micrometastasis formation in RT2/TNC mice (Saupe, Schwenzer *et al.,* accepted). Altogether this might suggest that one way by which TNC promotes metastasis formation is by decreasing the coverage of tumor blood vessels with pericytes facilitating dissemination.

In conclusion, I have demonstrated that TNC downregulates DKK1 in pericytes and CAFs, which was not accompanied by enhanced Wnt/β-catenin signalling in pericytes. Decreased DKK1 expression in pericytes and CAF might contribute to tumor progression through β-catenin independent signalling mechanisms.

6.1.3 Potential impact of TNC on tumor angiogenesis through DKK1

Our results suggest that in the RT2 tumor model TNC promotes the angiogenic switch and tumor angiogenesis (Saupe, Schwenzer *et al.,* accepted). However, the underlying mechanisms are unknown. Several studies demonstrated a correlation between TNC and VEGFA expression (Tanaka *et al*., 2004; Behrem *et al*., 2005; Ishiwata *et al*., 2005). Using Plasmon surface resonance (Biacore) we and others (De Laporte *et al*., 2013) have demonstrated VEGFA binding to TNC which raises the possibility that a TNC rich ECM may impact on VEGFA signalling. Moreover, not only VEGFA but also other pro-angiogenic factors such as CTGF, PDGF and FGF were shown to bind TNC (De Laporte *et al*., 2013). Whether the binding of growth factors to TNC enhances their activity or blocks it, needs to be addressed in the future. It is possible that a TNC rich microenvironment contributes to the establishment of a gradient of pro-angiogenic growth factors that could attract endothelial cells or acts as a reservoir for pro-angiogenic growth factors, which may be released upon activation of MMPs degrading the ECM (Hynes *et al*., 2009). In order to analyse if binding of VEGFA by TNC is relevant in the RT2 tumor model, one could analyse by immunofluorescence staining whether TNC and VEGFA are co-localised in RT2 tumors.

DKK1 regulates tumor angiogenesis

As DKK1 has been demonstrated to inhibit angiogenesis *in vitro* and *in vivo* (De Langhe *et al.*, 2005; Ohlmann *et al.*, 2010; Min *et al.*, 2011) and we observed a negative correlation between the TNC copy number and DKK1 expression in RT2 tumors, I focused in this study on a possible contribution of reduced DKK1 levels to enhanced tumor angiogenesis in the presence of TNC. Indeed, here it was shown that DKK1 is repressing tumor angiogenesis in a tumor xenograft model, which might explain how TNC regulates tumor angiogenesis. However, we did not directly prove so far that the underlying mechanism by which TNC enhances tumor angiogenesis is due to downregulation of DKK1. Hence, I used tumor cells

overexpressing TNC and mDKK1 for xenograft experiments to analyse the role of TNC and TNC mediated DKK1 downregulation in tumor angiogenesis. Yet, I was not able so far to successfully establish a model that mimics TNC induced tumor angiogenesis in a xenograft model, in which we then could further elucidate the contribution of reduced DKK1 levels. I had faced several problems during the setup of the experiment:

(i) Injection of HEK cells overexpressing mDKK1 and/or TNC did not result in a sufficient number of tumors to draw final conclusions, although there was a slight trend towards higher tumor weight and volume upon overexpression of TNC and, reduced tumor weight upon ectopic expression of mDKK1.

(ii) By retroviral transduction I failed to established KRIB cells overexpressing TNC, probably due to the large size of the TNC cDNA encoding plasmid (more then 13 kbp). Therefore, I had transfected cells with the linearized plasmid, which usually results in much lower levels of ectopically expressed protein than upon retroviral transduction. Indeed, overexpression of TNC in KRIB cells did not result in a sufficient high amount of TNC in the CM of engineered cells to use those cells for *in vivo* grafting experiments. Although the episomal vector used for overexpression of TNC in HEK293 cells resulted in a high ectopic expression of TNC this approach has limitations, as the plasmid is not stably integrated in the genome and might get lost due to lack of selection pressure in the cells growing in the tumor, especially if tumors are grown for several weeks.

Therefore, for subsequent experiments I propose:

(i) To knockdown TNC in KRIB control cells and compare their tumorigenic and tumor-angiogenic potential to KRIB cells overexpressing TNC.

(ii) To circumvent the difficulties I faced with TNC overexpression in cell lines it would be also possible to inject syngenic breast cancer cell lines (recently established in our lab) overexpressing mDKK1 and knockdown for DKK1 in control and TNC KO mice. Altogether, this has two advantages: (a) TNC expression is modified in stromal cells, such as endothelial cells and fibroblasts, and (b) immune competent mice are used.

We demonstrated an anti-angiogenic role of DKK1 in tumor angiogenesis using a xenograft model. Yet, there are two studies describing a pro-angiogenic effect of DKK1 in breast cancer

and glioma (Smadja *et al*., 2010; Reis *et al*., 2012). In human breast tumors DKK1 expression correlated with the vWF content and DKK1 was co-localized around CD31 positive blood vessels (Smadja *et al*., 2010). In a murine glioma model, where cells were implanted subcutaneously in nude mice, overexpression of DKK1 led to a reduced nuclear β-catenin staining in the tumor endothelium, indicative of reduced canonical Wnt/β-catenin signalling in endothelial cells. Moreover, DKK1 induction led to increased vessel density and tumor volume when the cells were implanted subcutaneously or intra-cranially (Reis *et al*., 2012).

A possible explanation for these conflicting observations might be that DKK1 is exerting either pro- or anti-angiogenic effects depending on the local microenvironment and its ability to regulate additional pathways. Indeed, besides blocking canonical Wnt/β-catenin signalling, DKK1 can activate the non-canonical Wnt/JNK pathway (see section 1.4.2). In addition, in the study of Min *et al*. (2011) overexpression of DKK2 induced filopodia formation of HUVEC by increasing cdc42 activity, which could be inhibited by overexpression of DKK1. Interestingly, regulation of cdc42 activation by DKK1 in Caco2 cells has been also shown by Koch *et al*. (2009), where enhanced mislocalised activity of cdc42 inhibited migration of Caco2 cells. Furthermore, Min *et al*. (2011) observed decreased vascular density in the retina in DKK1/DKK2 double transgenic mice compared to DKK2 transgenic mice and recombinant DKK1 counteracted endothelial cell sprouting in aortic rings derived from DKK2 transgenic mice. The study by Ren *et al.* (2013) revealed that DKK1 inhibits the PDGF- and TGF-β-activation of MAPK and JNK signalling, resulting in reduced proliferation and migration independent of β-catenin signalling. Although this study has used pericytes and myofibroblasts to describe the effect of DKK1, it is possible that DKK1 inhibits PDGF and TFG-β signalling pathways in endothelial cells, thus changing their ability to proliferate, migrate and sprout.

Whether DKK1 acts as an inhibitor of canonical Wnt signalling or regulates other pathways might depend on the presence of certain cell surface receptors and co-receptors and probably their relative amounts. The presence of defined pro-angiogenic co-factors in the microenvironment might also play a role for the effect of DKK1 on tumor angiogenesis. While DKK1 has been shown to promote bFGF-induced angiogenesis (Aicher *et al*., 2008; Smadja *et al*., 2010; Reis *et al*., 2012) it reduces VEGFA-induced angiogenesis (Min *et al*., 2011) in a cell-free matrigel plug assay *in vivo*. As TNC has been demonstrated to bind several growth factors, including VEGFA, bFGF, PDGF-BB and TGF-β (De Laporte *et al*., 2013)

TNC could have a specific effect on presenting (VEGFA) or masking growth factors (bFGF) and therefore favour a special function of DKK1.

Does TNC stimulate tumor angiogenesis through enhanced Wnt signalling in endothelial cells?

It needs to be addressed which specific pathways are targeted by the TNC-induced downregulation of DKK1 in endothelial cells, including Wnt/β-catenin-dependent and Wnt/β-catenin independent pathways. We did not analyse so far, if the anti-angiogenic effect of DKK1 in our xenograft model and possibly in the RT2 model is due to regulation of canonical Wnt/β-catenin signalling pathway or, by affecting Wnt/β-catenin independent pathways. This question could be addressed upon injection of DKK1 overexpressing tumor cells into mice with a dominant negative form of β-catenin expressed in the endothelium (Cattelino *et al*., 2003). If overexpressed DKK1 still affects tumor angiogenesis, this will be independent of canonical Wnt/β-catenin signalling in endothelial cells.

In vitro, TNC reduced DKK1 expression and enhanced Axin2 expression in HUVEC cells, indicative of enhanced Wnt signalling activity. It needs to be further characterized in depth if TNC is indeed able to stimulate Wnt signalling in endothelial cells, e.g. by using the TOPFlash assay. Furthermore, it would be interesting to analyse if in addition to the upregulation of Axin2 TNC also regulates the expression of other Wnt targets with a functional role in angiogenesis, as VEGFA, Dll4 or Ang-2 (Easwaran *et al*., 2003; Corada *et al*., 2010; Ohlmann *et al*., 2010).

Enhanced Wnt signalling in endothelial cells in the presence of TNC might be instrumental for promoting tumor angiogenesis and might affect the functionality of blood vessels. Active Wnt/β-catenin signalling in endothelial cells has been shown to stimulate endothelial cell differentiation from embryonic stem cells (Wang *et al*., 2006), supports endothelial-mesenchymal transformation (Brade *et al*., 2006) and promotes endothelial cell proliferation (Masckauchan *et al*., 2005). Furthermore, in postnatal retina of mice loss of Lef or endothelial deletion of β-catenin leads to decreased vessel density and enhanced vessel regression indicative for a role of Wnt signalling for vessel stability (Phng *et al*., 2009). In addition, during early vascular development sustained β-catenin signalling prevented correct EC differentiation, altered vascular remodelling and caused a defect in arteriovenous

specification (Corada *et al*., 2010). Furthermore, during devlopment Wnt/β-catenin signalling in endothelial cells is essential for angiogenesis in the CNS (Stenman *et al*., 2008; Daneman *et al*., 2009).

Despite the well characterized role of Wnt signalling for tumor onset, progression and metastasis, there is only one study discussing the role of Wnt/β-catenin signalling in tumor angiogenesis. When glioma cells overexpressing Wnt-1 were grafted to nude mice reduced tumor angiogenesis, enhanced nuclear β-catenin and improved vessel coverage was observed. Furthermore, intracranial injection of glioma cells into mice expressing a constitutively active form of β-catenin in the endothelium resulted in reduced vessel density, more quiescent vessels and improved vessel coverage, overall indicating a role of Wnt/β-catenin signalling in vessel stability (Reis *et al*., 2012).

Therefore, we carefully need to analyse by further experiments: (i) whether indeed TNC promotes Wnt signalling in endothelial cells of the tumor endothelium (of RT2 mice), e.g. by staining for nuclear β-catenin in endothelial cells of RT2 tumors or use of Wnt reporter mice (*Axin2*$^{+/lacz}$ – generates β-gal in Axin2 expressing cells (Yu *et al*., 2005) or TCF/LEF-H2B-GFP^{Tr} – nuclear GFP in cells with nuclear β-catenin activity (Ferrer-Vaquer *et al*., 2010)), and if this is true (ii) what are the effects of TNC and activated Wnt signalling in endothelial cells on vessel formation, architecture, maturation and function.

In conclusion, these data suggest a potential role of TNC mediated DKK1 downregulation in tumor angiogenesis. In the future it needs to be addressed if non-canonical or canonical Wnt signalling pathways in endothelial cells are modulated downstream of TNC by reduced DKK1 levels.

Altogether, I have demonstrated that TNC enhances Wnt signalling in tumor cells by downregulation of DKK1. TNC also mediated DKK1 downregulation in stromal cells (endothelial cells, pericytes and CAFs), where recent studies (Min *et al.*, 2011, Ren *et al.*, 2013) suggest that DKK1 might exert mostly non-canonical signalling functions independent of β-catenin. As in a tumor context tumor and stromal cells are in close contact to each other and to a TNC rich matrix, TNC might modulate the crosstalk between stromal and tumor cells. This crosstalk should be addressed in the future in co-culture assays using CDM. DKK1 downregulation in stromal cells might have cell-autonomous but Wnt-signalling independent

consequences, and stromal cells might exert a differential cross-talk with endothelial cells affecting tumor angiogenesis or with tumor cells affecting their survival, proliferation and invasion (**Figure 34**).

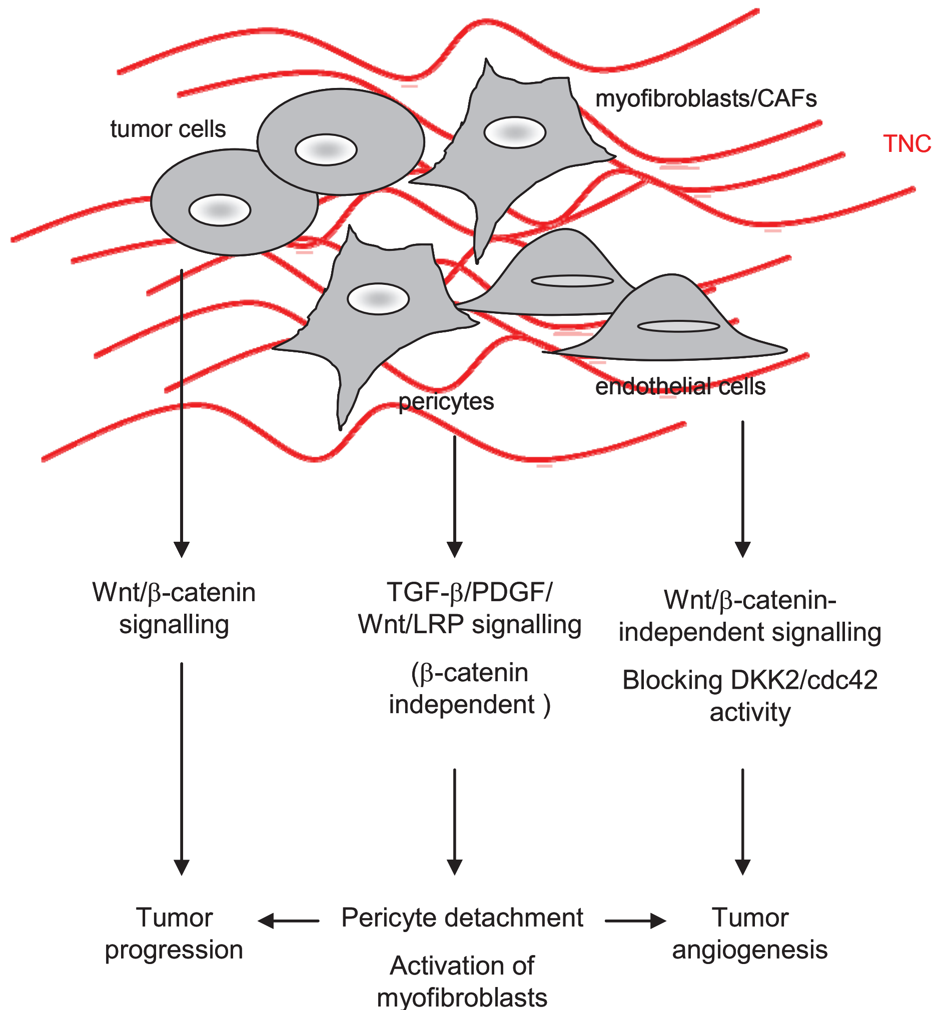


Figure 34. Summary and new working model – Consequences of DKK1 downregulation on tumor and stromal cells. I have demonstrated DKK1 downregulation in tumor and stromal cells by TNC. In tumor cells activation of Wnt signalling due to reduced DKK1 levels may promote tumor progression. In endothelial cells and pericytes DKK1 might modulate signalling pathways (TGF-β/PDGF/cdc42 signalling) independent of β-catenin signalling, driving tumor angiogenesis and tumor progression.

6.2 Regulation of *DKK1* gene expression by the actin cytoskeleton

One objective of this thesis was to elucidate the mechanism by which TNC regulates DKK1 gene expression. As reduced DKK1 levels were already observed shortly after seeding cells on a FN/TNC substratum where cells remained rounded with no actin stress fibres formed, I assumed that *DKK1* gene expression might be directly linked to the integrity of the actin cytoskeleton. Here I will discuss in more detail:

1. The role of the actin polymerisation status in regulating *DKK1* gene expression.

2. The potential role of RhoGTPases in regulating *DKK1* gene expression.

3. The potential role of actin-regulated co-transcription factors in regulating *DKK1* gene expression.

6.2.1 Induction of *DKK1* gene expression by stress fibres

I demonstrated that *DKK1* gene expression is affected by the stability of the actin cytoskeleton by experiments in which DKK1 expression was measured upon disruption and enforcement of actin stress fibres. Latrunculin B and Cytochalasin D (2 µM) that inhibit actin polymerisation or induce F-actin depolymerisation, respectively and therefore remove stress fibres from the cells, inhibited *DKK1* gene expression. Also knockdown of TPM1, an actin stress fibre stabilizing molecule, reduced *DKK1* gene expression. On the contrary, *DKK1* gene expression was induced by stabilization of actin stress fibres through treatment with LPA and overexpression of TPM1. Treatment with Jasplakinolide, an inducer of actin polymerisation, but not inducing actin stress fibres, downregulated DKK1 expression. In summary, these observations suggest that disruption of actin stress fibres by actin binding drugs or by TNC leads to downregulation of DKK1. Moreover, whereas actin polymerisation per se is not sufficient, signalling induced by stable actin stress fibres appears to be instrumental in induction of DKK1 expression.

Polymerized actin can form branched or unbranched filaments. In order to distinguish between the contribution of different forms of polymerised actin to DKK1 expression, it is possible to specifically target the formation of bundles of unbranched actin filaments, as

found in stress fibres, with the forming-inhibitor SMIFH2, while the Arp2/3 inhibitor CK666 would selectively target web-like F-actin structures (Blanchoin and Boujemaa-Paterski, 2009) as e.g. present in lamellipodia. It will be interesting to see whether manipulating specifically unbranched or branched actin filaments has an impact on DKK1 expression.

Expression of mutant actins would allow addressing further whether DKK1 is predominantly regulated by filamentous actin structures or if the concentration of monomeric G-actin in the cell also plays a role in regulating *DKK1* gene expression. The actin mutant R62D increases the concentration of monomeric actin, as it does not polymerise; in contrast the actin mutant V159N increases F-actin (Posern *et al*., 2002; Posern *et al*., 2004). In addition, overexpression of the formin mDia also leads to polymerisation of F-actin and to the formation of F-actin bundles (Copeland and Treisman *et al*., 2002). In the future it should be determined whether overexpression of V159N actin or mDia overexpression restores DKK1 expression on FN/TNC and whether DKK1 expression is affected by expression of R62D actin.

Since RhoA is a major regulator of stress fibre contractility through regulating ROCK activity (Levayer and Lecuit, 2011), but constitutively active RhoA did not induce DKK1 expression it is unlikely that ROCK and stress fibre contractility are major regulators of DKK1 expression. However, stress fibre contractility can be also regulated by RhoA-independent pathways, as cdc42 activity (Levayer and Lecuit, 2011). In order to address whether the presence of stress fibres per se or stress fibre contractility are involved in regulation of *DKK1* gene expression, one could either target ROCK that enhances stress fibre contractility by phosphorylation of MLC (Levayer and Lecuit, 2011), or target actomyosin directly. ROCK activity can be inhibited by the ROCK-inhibitor Y27632 (Uehata *et al*., 1997); or enhanced by overexpression of constitutive active ROCK (ROCKΔ4). Blebbistatin is an inhibitor of non-muscle myosin II ATPase activity (Straight *et al*., 2003; Kovacs *et al*., 2004) and calyculin A inhibits myosin light chain phosphatase and protein phosphatases PP1 and PP2A (Ishihara *et al*., 1989; Resjo *et al*., 1999).

Our results demonstrate that *DKK1* gene expression is inhibited by reduced stress fibre formation, which is accompanied by enhanced Wnt signalling activity on a TNC substratum in tumor cells. Of note, two recent publications demonstrated that reduced DKK1 expression and Wnt signalling activity are regulated by tissue stiffness. Tissue stiffness enhances actin stress fibre formation and contractility (Paszek *et al*., 2005) and integrin mediated focal adhesion signalling (Levental *et al*., 2009). Interestingly, increased matrix stiffness due to

enhanced concentrations of collagen or polyacrylamid caused reduced *DKK1* gene expression in epithelial ovarian carcinoma cells. Decreased DKK1 expression was accompanied by enhanced TOPFlash activity and nuclear β-catenin (Barbolino *et al*., 2013). Conditionally active ROCK expressed in mouse skin mediating cellular tension through actomyosin contractility, enhanced tissue stiffness by increasing collagen deposition, leading to stabilizing and nuclear localization of β-catenin. Inhibition of FAK, LIMK or myosin ATPase attenuated these responses (Samuel *et al*., 2011). Thus, interestingly, DKK1 appears to be downregulated and Wnt signalling to be enhanced by high tissue stiffness (enhancing actin contractility) and by low adhesion (reduced stress fibre formation) as on a TNC substratum. Different mechanisms might regulate DKK1 expression through their impact on the actin cytoskeleton and actin stress fibres.

Interestingly, TNC itself is induced by mechanical stress with RhoA/ROCK and MKL1 as central mediators (Sarasa-Renedo *et al*., 2006, Asparuhova *et al*., 2011). One could speculate for a role of TNC in counterbalancing tissue stiffness.

6.2.2 Is *DKK1* gene expression regulated by Rho GTPases activity?

As described above *DKK1* gene expression was regulated by LPA-induced stress fibre formation. RhoA is a central mediator of actin polymerisation and stress fibre formation downstream of LPA (Ridley and Hall, 1992; Amano *et al.*, 1997; Mills and Moolenaar, 2003); therefore I suggested that RhoA might be involved in regulation of *DKK1* gene expression. However, I did not observe induction of *DKK1* gene expression by overexpressing of wt RhoA or constitutively active (Q63L) RhoA. In addition, overexpression of this form of constitutively active RhoA, in contrast to LPA treatment, did not restore cell spreading and DKK1 expression on a FN/TNC substratum. As *SRF* gene expression and reporter activity were used to demonstrate that constitutively active RhoA indeed activates downstream signalling in transfected cells, these data unambiguously indicate that both TNC induced cell rounding and DKK1 downregulation are independent of RhoA activity.

Although RhoA is considered to be a main effector of LPA downstream signalling, LPA induces other signalling pathways besides RhoA signalling, which are implicated in the regulation of cell spreading, actin polymerisation and stress fibre formation, namely cdc42 (Ueda *et al*., 2001), Rac (Berrier *et al*., 2000; Ueda *et al*., 2001) and R-Ras (Barrier *et al*.,

2000; Mills and Moolenaar *et al*., 2003). Therefore, it is either possible that LPA regulates cell spreading on a FN/TNC substratum independent of RhoA signalling or that RhoA signalling alone is not sufficient to restore cell spreading on a FN/TNC substratum. Thus we need to examine further which specific Rho GTPases are implicated in the TNC mediated inhibition of cell spreading and DKK1 expression, by:

(i) Overexpression of constitutively active or dominant negative cdc42, Rac1 or Ras constructs and analyses of *DKK1* gene expression.

(ii) Treatment with selective Rho GTPase inhibitors that target several Rho GTPases at the same time. TAT-C3 inhibits RhoA, RhoB and RhoC by catalysing ADP-ribosylation (Sahai and Olson, 2006). TcdB inhibits Rho, cdc42 and Rac by glucosylation. TcdBF inhibits Rac and R-Ras by glucosylation. By this treatment it is possible to target the whole family of the indicated Rho GTPases simultaneously, which is not achieved by overexpression of constitutively active or dominant negative mutants.

My observation that RhoA is not sufficient to restore cell spreading in the presence of TNC in T98G cells is in contrast to the study of Wenk *et al*. (2000), where both LPA and constitutively active RhoA (RhoA G14V) restored NIH3T3 fibroblasts cell spreading when plated on a TNC containing FN-fibrin gel.

These contradictory results might be explained by the different experimental setup:

(i) The study of Wenk *et al*. (2000) was performed using NIH3T3 fibroblasts grown on fibrin gels, where FN or both FN and TNC were incorporated, while above described experiments were performed on plastic dishes coated with FN and TNC.

(ii) In addition, in the study of Wenk *et al*. (2000) a chimeric TNC molecule (70Ten) not corresponding to the full length TNC protein was used. This 70Ten chimeric TNC molecule contains the amino-terminal 70-kDa region of FN, promoting covalent cross-linking to the fibrin matrix, and the carboxy-terminal 150 kDa of TNC, containing all FN type III repeats and the fibrinogen globe domain. The authors have demonstrated beforehand that in NIH3T3 cells seeded on either TNC or 70Ten containing fibrin-FN gels under both conditions reduced stress fibre formation occurred.

(iii) In the study of Wenk *et al*. (2000) a constitutively active RhoA mutant harbouring the mutation G14V was used to restore cell spreading. In contrast we used RhoA Q63L. Both mutations lead to a constitutive activation of RhoA, shown by the ability to bind GTP but a lack of GTPase activity (Mayer *et al*., 1999). However, it is conceivable that these two mutants affect downstream pathways differently. While transfection of RhoA wt and RhoA G14V could not induce stress fibres and focal adhesions in RR1022 rat fibroblasts transformed by the Rous sarcoma virus, transfection with RhoA Q63L did induce stress fibres and focal adhesions (Mayer *et al*., 1999).

6.2.3 Transcriptions factors potentially involved in the regulation of *DKK1* gene expression by stress fibres

The activity of the SRF co-transcription factor MKL1 is controlled by the actin polymerisation status of the cell (Miralles *et al*., 2003). As I observed reduced SRF/MKL1 transcriptional activity in the presence of TNC, demonstrated that DKK1 expression is induced by enhanced actin stress fibre formation and showed that DKK1 expression is regulated by actin stress fibres that are blocked by TNC, I considered the possibility that MKL1 is regulating DKK1 expression.

Indeed I observed reduced *DKK1* gene expression and promoter activity, as well as lowered protein levels, when MKL1 expression was stably knocked down. In this experiment, shRNA off-target effects can be excluded as the same effects were observed with two different shRNAs, independently targeting MKL1 mRNA. However, the transient overexpression of wt or constitutively active MKL1 did not produce an effect on *DKK1* gene expression. Furthermore, the overexpression of constitutively active MKL1 did not restore DKK1 expression on a FN/TNC substratum. Together, these results suggest that while TNC regulates SRF/MKL1 target genes it does not downregulate DKK1 through this pathway.

From these experiments I do not fully understand yet why MKL1 knockdown affects *DKK1* gene expression while MKL1 overexpression does not. It is conceivable that the stable downregulation of MKL1 induces cellular changes that could not be observed with the short-term experiment relying on transient MKL1 overexpression, e.g. (i) that MKL1 downregulation induces reduced stress fibre formation which then changes the activity of

another transcription factor affecting DKK1; or (ii) that DKK1 is an indirect MKL1 target gene, i.e. DKK1 expression is regulated by a transcription factor that is itself a direct MKL1 target gene. It is possible that MKL1 might also interact with other transcription factors to regulate *DKK1* gene expression. Indeed, Myocardin and MKL1 have been shown to bind to other transcriptions factors than SRF, including Smad3 (Myocardin, smooth muscle actin promoter) (Qiu *et al*., 2005), Smad1/4 (MKL1, Id3 Promoter) (Iwasaki *et al*., 2008) or GATA4/6 (activation of smooth muscle myosin heavy chain and smooth muscle alpha actin promoter, or repression of telokin promoter) (Oh *et al*., 2004; Yin and Herring, 2005). It is also conceivable that repression of MKL1 activity leads to a higher availability of SRF to interact with another co-transcription factor that represses *DKK1* gene expression.

Treatment with the actin binding drugs Latrunculin B and Cytochalasin D demonstrated that *DKK1* expression is not regulated in the same manner as *SRF*, a prototypical SRF/MKL1 target gene. As additional control readout for MKL1 activity, the SRF/MKL1 luciferase reporter was used.

(i) Cytochalasin D induces F-actin depolymerisation, but also induces nuclear localization of MKL1 by sequestration of G-actin (Miralles *et al*., 2003) (**Figure 30**). As expected, I observed enhanced SRF/MKL1 activity induced by Cytochalasin D in a concentration-dependent manner. Indeed, upon treatment with low concentration of Cytochalasin D *DKK1* gene expression was induced, while at higher concentrations *DKK1* gene expression was repressed. It is conceivable that at low concentrations of Cytochalasin D *DKK1* gene expression is induced by the activity of SRF/MKL1 due to depletion of monomeric actin; however at a higher concentration of Cytochalasin D DKK1 is regulated by another factor the activity of which is reduced by disruption of stress fibres, finally resulting in lowered *DKK1* gene expression.

(ii) Latrunculin B cannot induce SRF-reporter activity alone, as in serum-starved cells usually all monomeric G-actin is retained in the cytosol and therefore Latrunculin B treatment cannot further reduce SRF/MKL1 activity (Miralles *et al*. 2003; Descot *et al*., 2009) (**Figure 30**). However, under the same conditions Latrunculin B could further decrease DKK1 expression. As I did not analyse if upon starvation monomeric actin is indeed fully depleted

from the cytosol one cannot exclude that this result rather indicates a different sensitivity of the different promoters to depletion of G-actin by Latrunculin B.

(iii) As shown by Miralles *et al.,* (2003) (**Figure 30**) Cytochalasin D-induced SRF luciferase activity is inhibited by pre-treatment with Latrunculin B. Of note, treatment with low concentrations of Cytochalasin D that induced *DKK1* gene expression was inhibited by pre-treatment with Latrunculin B, indicating that under those concentrations DKK1 is similarly regulated as SRF.

(iv) Jasplakinolide induces actin polymerisation and therefore depletes the pool of monomeric G-actin (Bubb *et al*., 1994; Bubb *et al*., 2000). *SRF* gene expression and luciferase activity were increased in a time- and concentration-dependent manner. In contrast, DKK1 expression levels were not changed after 1 hour of treatment and, after 3 hours of treatment *DKK1* gene expression was reduced in a concentration-dependent manner. This result could be interpreted in two ways. (a) First, it is possible that the reduced concentration of monomeric G-actin directly acts on DKK1 expression. (b) As Jasplakinolide induces the formation of polymerised F-actin, that is however not organized into stress fibres (Bubb *et al*., 2000), it is conceivable that due to the loss of the stress fibres *DKK1* gene expression is reduced. It needs to be analysed whether Jasplakinolide in our experiments differently affects stress fibres formation at the 1 and 3 hours timepoint of treatment. However, staining of F-actin with TRITC-phalloidin of Jasplakinolide treated cells is challenging as Jasplakinolide inhibits the binding of phalloidin to F-actin (Bubb *et al*., 1994; Holzinger, 2009). Other methods exist for staining of actin, such as immunofluorescence staining for actin with actin binding antibodies. However this method has the drawback that both monomeric as well as F-actin are stained, therefore resulting in a high background.

Together these results suggest that if DKK1 is regulated by MKL1, this regulation might be interfered by a second transcription factor which activity is also regulated by polymerised actin. Indeed there are other transcriptional regulators that are regulated by cytoskeletal dynamics, including NFkB, JMY or LIM-domain containing proteins (Olson and Nordheim, 2010); however the exact mechanisms of their regulation and/or their target genes are largely unknown.

The TEAD-co-transcription factors YAP and TAZ were lately added to the list of transcription factors regulated by the actin polymerisation state. However, the exact mechanism of their regulation by actin is not deciphered so far. Interestingly, some of the mechanistic insights achieved, suggest that YAP activity is maybe involved in regulating *DKK1* gene expression and is potentially blocked by TNC.

(i) YAP is retracted from the nucleus and inactive in detached, rounded Hela and MCF10A cells (Zhoa *et al*., 2012), which may also occur in cells that remain rounded on a FN/TNC substratum.

(ii) YAP/TAZ activity is inhibited by F-actin disruption upon Latrunculin A (Dupont *et al*., 2011; Wada *et al*., 2011), Latrunculin B (Zhao *et al*., 2012) and Cytochalasin D (Wada *et al*., 2011; Sansores-Garcia *et al*., 2011; Zhao *et al*., 2012) treatment. Similarly, treatment by Latrunculin B and Cytochalasin D (2 µM) reduced DKK1 expression.

(iii) Overall the ratio of monomeric G-actin versus filamentous F-actin does not play a role in regulation of YAP/TAZ, which is opposite to MKL1/2 that acts as a sensor of monomeric G-actin. In support, overexpression of mutant non-polymerisable G-actin did not change YAP/TAZ activity (Dupont *et al*., 2011). Overexpression of mDia, a nucleator of actin polymerisation and driver of stress fibre formation, leads to increased YAP/TAZ activity (Sansores-Garcia *et al*., 2011; Dupont *et al*., 2011).

(iv) YAP activity is induced by LPA treatment (Yu *et al*., 2012; Cai and Xu, 2013; Calvo *et al*., 2013). However, Cai *et al.* (2013) and Yu *et al*. (2012) showed that LPA-mediated YAP activation depends on RhoA signalling activating ROCK which would be in contrast to our results showing that RhoA is dispensable for induction of *DKK1* gene expression.

Most importantly, recently a study identified a functional TEAD binding site in the DKK1 promoter and demonstrated that *DKK1* gene expression is induced upon YAP overexpression and inhibited upon YAP knockdown (Seo *et al*., 2013).

Therefore, in the future, it should be analysed whether TNC regulates YAP/TAZ transcriptional activity. This could be accomplished by using a TEAD reporter construct (Mahoney *et al.*, 2005). If YAP regulates DKK1 in a TNC dependent manner, overexpression of constitutively active YAP (Lamar *et al*., 2012) may restore DKK1 expression on TNC.

In conclusion, it was demonstrated that TNC downregulates DKK1 on the promoter level by inhibiting stress fibre formation independent of RhoA signalling. For the future it will be interesting to analyse if the TEAD co-transcription factor YAP is involved in regulation of DKK1 gene expression downstream of TNC.

7 References

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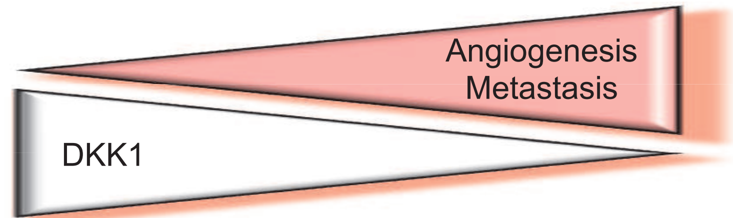
8 Annex

Manuscript 1: Tenascin-C promotes tumor angiogenesis and progression in a neuroendocrine tumor model by downregulation of Wnt inhibitor Dickkopf-1.

Cell Reports, *accepted*

Book: The extracellular matrix and cancer: regulation of tumor cell biology by tenascin-C. iConcept press, *provisionally accepted*

Rip1Tag2
TNCKO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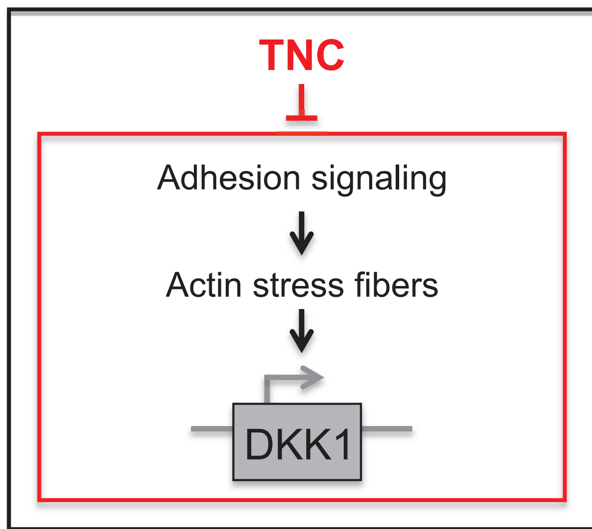
TNC
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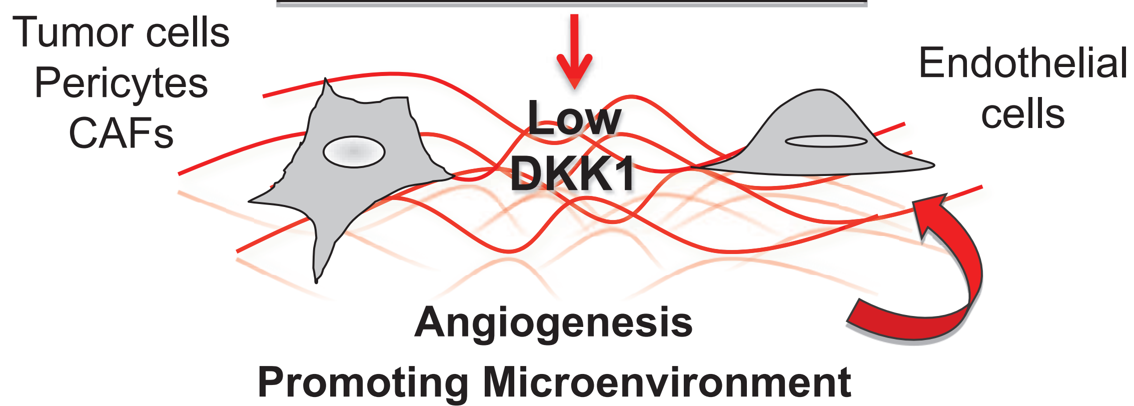
Tumor cells
Pericytes
CAFs
Low
DKK1
Endothelial
cells
Angiogenesis
Promoting Microenvironment

Highlights

First immune competent stochastic tumor model with high TNC as in human cancer.

Decisive role of TNC in tumor progression by promoting early and late events.

Increased tumor cell survival, proliferation, invasion, angiogenesis and metastasis.

TNC promotes pro-tumorigenic microenvironment by downregulation of DKK1.

TNC downregulates DKK1 promoter through disruption of actin stress fibers.

TNC transgenic tumor mice as model for human insulinoma progression and TNC targeting.

*Manuscript
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Tenascin-C promotes tumor angiogenesis and progression in a neuroendocrine tumor model by downregulation of Wnt inhibitor Dickkopf-1

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Abstract

The extracellular matrix molecule tenascin-C (TNC) is a major component of cancer-specific matrix, and high TNC expression is linked to poor prognosis in several cancers. To provide a comprehensive understanding of TNC's functions in cancer we established an immune-competent transgenic mouse model of pancreatic β-cell carcinogenesis with varying levels of TNC expression and compared stochastic neuroendocrine tumor formation in abundance or absence of TNC. We show that TNC promotes tumor cell survival, the angiogenic switch, more and leaky vessels, carcinoma progression and lung micrometastasis. TNC downregulates Dickkopf-1 (DKK1) promoter activity through blocking actin stress fiber formation, activates Wnt signaling and induces Wnt target genes in tumor and endothelial cells. Our results implicate DKK1 downregulation as an important mechanism underlying TNC-enhanced tumor progression by providing a pro-angiogenic tumor microenvironment.

\body

Introduction

Manifestation of cancer requires many steps in which the microenvironment plays an essential role (Bissell and Labarge, 2005). A group of tumor cells with oncogenic mutations does not readily cause cancer, a phenomenon known as tumor dormancy (Aguirre-Ghiso, 2007). Angiogenesis presents an important step in awakening quiescent tumors and in driving their development into metastatic cancer (Almog, 2010). Tumor cells secrete soluble factors that attract endothelial cells (Kerbel, 2008). In addition, the extracellular matrix (ECM) constitutes a major fraction of cancer tissue and contributes to tumor angiogenesis and metastasis (Lu et al., 2012). An important component of the tumor-specific ECM is tenascin-C (TNC). TNC is known to promote malignant tumor progression and lung metastasis, yet the underlying mechanisms are poorly understood (Midwood et al., 2011).

Since no stochastic and immune-competent *in vivo* model existed that would recapitulate the roles of TNC in tumor progression we generated mouse lines with different expression levels of TNC (overexpression, wildtype, knock-out) in the Rip1Tag2 (RT2) model of pancreatic β-cell carcinogenesis (Hanahan, 1985). This model recapitulates multistage tumorigenesis as observed in most human cancers (Nevins, 2001; Pipas and Levine, 2001).

Here, we demonstrate that TNC promotes several steps in RT2 tumorigenesis including the angiogenic switch and lung micrometastasis. We provide a mechanistic basis showing that TNC downregulates expression of the soluble Wnt inhibitor Dickkopf1 (DKK1) (Glinka et al., 1998) by blocking actin stress fiber formation and, induces canonical Wnt signaling in tumor and endothelial cells. Our data suggest that DKK1 downregulation by TNC on tumor and stromal cells may provide a tumorigenesis signaling promoting microenvironment. Given that Wnt signaling is a crucial pathway driving angiogenesis and is activated by TNC this pathway may play an important role in promoting tumor angiogenesis and metastasis

by TNC. Thus, targeting TNC or its associated signaling pathways may represent a strategy to counteract tumor progression.

Results

Tenascin-C promotes tumor cell survival, proliferation and invasiveness

To address whether TNC potentially plays a role in the RT2 model (Hanahan, 1985), we determined TNC expression during RT2 tumorigenesis by immunofluorescence microscopy analysis (IF). In normal pancreatic islets TNC expression was undetectable, whereas a large fraction of hyperplastic and almost all angiogenic and tumorigenic islets expressed TNC (**Fig. S1A**), suggesting a potential role of TNC during RT2 tumor progression. Therefore, we generated RT2 mice with overexpression of TNC (RT2/TNC) and a lack of TNC (RT2/TNCKO) (**Fig. S1B-E**).

We performed tissue analysis to address whether ectopically expressed TNC had an effect on cell proliferation. We quantified the proportion of cells positive for phospho-histone-H3 by IF (**Fig. S2A**) and observed that tumors of RT2/TNC mice exhibited 1.4-fold more proliferating cells than those from RT2 mice (**Fig. 1A**) with a significant difference in hyperplastic islets (**Fig. S2C**). Surprisingly, a similar difference was also seen in RT2/TNCKO tumors (**Fig. 1B, Fig. S2D**). We also investigated a potential impact of ectopically expressed TNC on apoptosis by staining for cleaved caspase-3 (**Fig. S2B**). RT2/TNC tumors exhibited 2.8-fold less apoptotic cells than RT2 wildtype tumors (**Fig. 1C, Fig. S2E**). In contrast apoptosis was unchanged in RT2/TNCKO tumors in comparison to RT2 controls (**Fig. 1D, Fig. S2F**). However, no difference was seen in tumor multiplicity nor tumor volume between genotypes (**Fig. S2G, H**). Interestingly, upon tumor grading we observed that the frequency of carcinomas and the ratio of carcinomas over adenomas was higher in RT2/TNC mice (1.8) than in RT2 controls (0.8) (**Fig. 1E, Table S1**). We conclude that transgenic TNC increases proliferation and survival in RT2/TNC mice and more importantly, promotes tumor progression.

Tenascin-C promotes the angiogenic switch and the formation of leaky and abnormal tumor vessels

To address whether TNC has an effect on RT2 tumor angiogenesis we isolated islets at the age of 8 weeks when the angiogenic switch takes place in a subset of neoplastic islets (Hanahan et al., 1996; Parangi et al., 1996) (**Fig. S2I**). We noticed that the number of angiogenic islets was 2.4-fold higher in RT2/TNC and 2.9-fold lower in RT2/TNCKO mice in comparison to RT2 littermates (**Fig. 1F, G, Table S2**). By quantification of CD31-positive endothelial cells (**Fig. S2J**) in tumor sections of 12 week old RT2 mice we observed that the abundance of blood vessels was 2.6-fold higher and 1.6-fold lower in tumors of RT2/TNC and RT2/TNCKO mice, respectively, than in RT2 control tumors (**Fig. 1H, I**).

We next addressed the question of a potential impact of TNC on vessel anatomy by scanning electron microscopy in Mercox corrosion casts of the tumor vasculature of multiple tumors of RT2 and RT2/TNC mice. Using this descriptive approach, we observed a highly aberrant vessel phenotype in some RT2/TNC tumors that has not been seen in RT2 tumors. These vessels were irregularly shaped, wider, discontinued and bifurcated (see arrows), reminiscent of high vessel branching and/or leakage (**Fig. 1J, Fig. S2K**). Since this approach is not suitable for quantitative determinations, we then studied vessel lining by pericytes using NG2 staining as readout for vessel functionality and maturation (**Fig. S2L**). Despite more abundant pericytes in RT2/TNC tumors (**Fig. S2M**) quantification of combined NG2 and CD31 staining signals revealed a 23.7% reduced ratio of NG2 over CD31 in RT2/TNC tumors (**Fig. 1K**) which is indicative of a reduced pericyte coverage of vessels (Song et al., 2005). Finally, we assessed vessel functionality by analyzing fibrinogen (FBG) leakage in tumors upon PBS perfusion of tumor vessels followed by FBG staining (Huijbers et al., 2010) **(Fig. S2N).** Whereas FBG leakage was slightly increased (close to significance, $p = 0.064$) in RT2/TNC over control tumors (**Fig. 1L, Fig. S2O**), this analysis revealed a 1.7-fold significantly reduced FBG staining in RT2/TNCKO tumors over RT2 wildtype tumors (**Fig. 1M, Fig. S2P**).

Altogether, our results suggest that, while TNC promotes the angiogenic switch and increases tumor blood vessel density, it decreases vessel coverage by pericytes and increases leakage, thus perturbing tumor vessel functionality.

Tenascin-C increases lung micrometastasis

In a C57Bl/6 background, RT2 mice do not exhibit macroscopically visible metastasis. To address whether TNC had an effect on micrometastasis formation we determined expression of insulin (as tumor cell specific marker) in liver and lung tissue of tumor-bearing mice. Upon tissue staining we detected cohorts of insulinoma cells within liver and lung tissue confirming their metastatic nature (**Fig. 2A, Fig. S3A**). Staining with H&E revealed their parenchymal localisation. In a subset of mice we compared quantification of insulin by immunostaining versus qRTPCR. This showed a good correlation between both methods and indicates that quantification by qRTPCR reflects parenchymal localisation of micrometastasis rather than circulating tumor cells. We then analyzed a larger sample size of liver and lung tissue by qRTPCR. Although we did not observe differences in liver tissue between genotypes (**Fig. S3B, C**), insulin mRNA levels in lungs of RT2/TNC mice were 5.4-fold higher in comparison to lungs of RT2 controls (**Fig. 2B**). Moreover, we observed 28.3-fold lower insulin mRNA levels in lungs of mice lacking TNC in comparison to control littermates carrying one TNC allele (**Fig. 2C**). Our results suggest that in the RT2 model TNC does not affect liver metastasis, but increases lung micrometastasis formation.

TNC expression correlates with low Dkk1 levels and increases Wnt target gene expression

Since we had noticed downregulation of the Wnt pathway inhibitor DKK1 in T98G glioblastoma (GBM) cells cultivated on a TNC-containing substratum (Ruiz et al., 2004), we assessed a potential impact of TNC on Dkk1 expression in tumors of the different RT2 genotypes. By qRTPCR we noticed that 12-times more RT2/TNC tumors (46.1%) lacked Dkk1 expression as compared to RT2 controls (3.7%) (**Fig. 3A**). In RT2/TNC tumors with detectable Dkk1 expression, the levels were 16.1-fold reduced in comparison to RT2 controls (**Fig. 3B**). In contrast, Dkk1 levels were 2.6-fold higher in tumors lacking TNC as

compared to control tumors with one TNC allele (**Fig. 3C**). These observations demonstrate an inverse correlation between TNC and Dkk1 expression and suggest that TNC may activate Wnt signaling through Dkk1 repression. To address this possibility we determined the expression of Wnt target genes by qRTPCR. We observed an increased expression of the bona fide Wnt signaling target Axin2 (1.35-fold) in RT2/TNC tumors (**Fig. 3B**), while its expression was unchanged in RT2/TNCKO tumors (**Fig. 3C**). This result suggested that ectopic TNC expression induced Wnt signaling prompting us to analyze expression of other Wnt target genes. Indeed, other Wnt targets such as Cyclin D1 (2.01-fold), CD44 (2.06-fold) and Slug (1.84-fold) were upregulated in small differentiated tumors of RT2/TNC mice (**Fig. 3D, Table S3**). These results suggest that TNC may contribute to Wnt signaling activation in RT2/TNC tumors through downregulation of the inhibitor Dkk1.

Wnt activation and DKK1 inhibition by TNC in cultured tumor and stromal cells

We then designed *in vitro* experiments to evaluate a potential Wnt activation by TNC involving DKK1. We used a Wnt reporter (TOPFlash) assay where the expression of the luciferase gene is driven by a promoter containing TCF/LEF binding sites. Upon growth of Wnt-3A stimulated osteosarcoma KRIB cells on a TNC-containing substratum, we observed a 3.5-fold increased Wnt reporter activity (**Fig. 4A**) and a 2.0-fold increased expression of Axin2 (**Fig. 4B**), demonstrating that TNC activates the Wnt pathway.

Next, we determined whether TNC affects secretion of soluble factors regulating Wnt signaling in KRIB cells. Therefore we measured Wnt reporter activity of Wnt-3A-stimulated KRIB cells upon incubation with conditioned medium (CM) from the same cells previously grown on FN/TNC or FN and observed that, indeed, Wnt activity was higher with CM from cells cultured in the presence of TNC (**Fig. 4C**). These results suggest that TNC activates Wnt signaling through modulating the secretion of activators or inhibitors of the Wnt pathway.

To address whether Wnt inhibitors are regulated by TNC we investigated their expression by qRTPCR in cells grown on FN/TNC and FN. While some inhibitors (DKK4, SFRP2) were not expressed no consistent effect of TNC was observed on the expression of other analyzed Wnt inhibitors (DKK2, DKK3, SFRP1, SFRP3, SFRP4) in KRIB, T98G and MDA-MB435 cells (**Fig. S4A**). In contrast, we observed a robust downregulation of DKK1 in all 5 analyzed tumor cell lines of different origin after 24h on the TNC-containing substratum (**Fig. 4D, Fig. S4A**). DKK1 downregulation was observed at both RNA and protein levels, with a fast (5h, RNA) and long lasting (up to 12 days, protein) effect in T98G cells (**Fig. S4B,C**).

To determine whether modulation of DKK1 expression contributes to TNC-dependent Wnt signaling in KRIB cells, TOPFlash activity was measured upon overexpression and knockdown of DKK1, respectively (**Fig. S4D, E, F, G**). Indeed, activity of the Wnt signaling reporter was DKK1-dependent as it was increased upon DKK1 knockdown and decreased upon DKK1 overexpression (**Fig. 4E**) and, was repressed by DKK1 containing CM in a dose-dependent manner (**Fig. S4H**). When KRIB cells were incubated with DKK1 CM on FN/TNC and FN, Wnt reporter activity was largely reduced (**Fig. 4F**) suggesting that TNC-induced repression of DKK1 facilitates Wnt pathway activation.

Next we determined whether stromal cells also downregulated DKK1 on a TNC substratum. Therefore, DKK1 expression was determined in two monocytic/macrophage cell lines, primary human brain pericytes, two colo-rectal cancer derived carcinoma associated fibroblasts (ABD CT5.1, ABD CT 14) and HUVEC upon growth on FN/TNC and FN. We noticed that in contrast to the two macrophage lines that did not at all express DKK1, pericytes (5-fold), CAFs (3.0- and 1.6-fold) and HUVEC (2.2-fold) significantly downregulated DKK1 mRNA (**Fig. 4G, I, J**) and protein (**Fig. 4K**) on a TNC substratum. Whereas Axin2 expression was not affected in pericytes (**Fig. 4H**) Axin2 mRNA was 2.3-fold increased in HUVEC on FN/TNC in comparison to FN (**Fig. 4L**). Altogether, our results show

that TNC induces downregulation of DKK1 in tumor and stromal cells and, activates Wnt signaling in tumor and endothelial cells.

Mechanism of DKK1 downregulation by TNC

First we determined whether DKK1 mRNA stability is substratum dependent. Therefore, T98G cells were treated with the Pol II inhibitor Actinomycin D, but DKK1 mRNA levels were equally low in cells on FN and FN/TNC suggesting that DKK1 is not regulated by mRNA stabilization (**Fig. S5A**). Next we addressed whether TNC downregulates DKK1 at transcriptional level. Therefore we performed reporter assays by measuring luciferase activity under control of a 3.2 kb DKK1 promoter sequence. Indeed, we observed a 2.5-fold reduced DKK1 promoter activity in cells grown for 48h on a TNC containing substratum (**Fig. 5A**).

Since TNC blocks actin stress fiber formation (Huang et al., 2001; Midwood et al., 2004; Murphy-Ullrich et al., 1991; Orend et al., 2003) we investigated whether disruption of the actin cytoskeleton has an impact on DKK1 mRNA levels. Treatment with Latrunculin B (LB) and Cytochalasin D (CD) disrupted actin stress fibers, focal adhesions and, importantly reduced DKK1 expression (**Fig. 5B - D**). To address the converse whether more actin stress fibers stimulate DKK1 expression we treated KRIB and T98G cell with LPA and observed an increased and dose-dependent DKK1 mRNA expression similar to SRF, a known actin stress fiber regulated gene (**Fig. S5B-F**). Moreover, LPA (30 μM) restored cell spreading, actin stress fibers and focal adhesions in T98G cells on a FN/TNC substratum and most importantly, largely restored DKK1 levels on this substratum to that on FN (**Fig. 5E, F**). Since LPA can trigger RhoA signaling (Mills and Moolenaar, 2003) and, RhoA expression (Lange et al., 2007) and function (Wenk et al., 2000) are impaired by TNC, we determined whether overexpression of a constitutively active (CA) RhoA molecule impacts on DKK1 expression. Whereas, CA-RhoA increased SRF target gene expression (**Fig. S5G-J**) it did not alter DKK1 expression (**Fig. S5K, L**) suggesting that LPA triggers DKK1 expression by a RhoA independent pathway.

Since TPM1 and syndesmos overexpression bypass the cell adhesion blocking and actin stress fiber disrupting effect of TNC on a FN/TNC substratum (Lange et al., 2008), we determined whether ectopic expression of syndesmos and TPM1 have an impact on DKK1 expression. Whereas shTPM1 blocked DKK1 expression, overexpression of syndesmos and TPM1 increased DKK1 mRNA levels to 4.7- and 3.6-fold, respectively (**Fig. 5H-J, Fig. S5M-P**).

Altogether these results demonstrated that DKK1 expression is regulated at promoter level and that actin stress fibers and focal adhesion signaling drives DKK1 transcription independently of RhoA. We conclude that TNC downregulates DKK1 transcription by blocking focal adhesion and actin stress fiber formation.

Repression of tumor angiogenesis by DKK1

As we observed that TNC promotes tumor angiogenesis and downregulates DKK1 expression, we addressed whether DKK1 impacts on tumor angiogenesis in xenografted tumors of KRIB cells with different DKK1 levels. We found that upon DKK1 overexpression (**Fig. S6A**) tumors were significantly smaller (**Fig. 6A**) and pale (**Fig. 6B**). Quantification of microvessel density upon CD31 staining revealed that Dkk1 overexpressing tumors were less vascularized (**Fig. 6C**) suggesting that Dkk1 overexpression impaired tumor angiogenesis. In addition, conditioned medium from KRIB cells overexpressing mDkk1 inhibited HUVEC tubulogenesis on matrigel *in vitro* (**Fig. S6D**). We addressed whether Dkk1 potentially had an impact on tumor growth through inhibiting tumor cell proliferation and found no statistically significant difference in proliferation in cultured cells nor in the tumors with elevated Dkk1 levels (**Fig. S6B, C**). As Dkk1 neither influenced proliferation of tumor cells *in vitro* nor *in vivo* our data suggest that Dkk1 overexpression impairs angiogenesis and thereby inhibits KRIB tumor growth. Since DKK1 blocks angiogenesis in a VEGFA context (Min et al., 2011) we investigated whether full length TNC binds VEGFA. Indeed by surface plasmon resonance we observed a dose-dependent binding of VEGF to TNC (**Fig. S7**), extending data on binding of VEGFA to the 5th FNIII domain in TNC (De

Laporte et al., 2013) by providing a KD of 2.7 x 10^{-7} M which is in the range of a VEGFA/glycosaminoglycan interaction (2.4 x 10^{-8} M) (Wu et al., 2009).

TNC expression in human insulinomas

As we demonstrated a tumor-promoting effect of TNC in the murine RT2 insulinoma model, we assessed a potential clinical relevance by determining TNC expression in human insulinomas using qRTPCR and immunohistochemical staining of patient tumor tissue. Of note, insulinomas are rare and most are benign, yet a few (10-15%) metastasize to lymph nodes and liver (Metz and Jensen, 2008). At RNA level, we found that TNC expression was detectable in all analyzed human insulinomas (**Fig. 7A**). Most importantly, we observed the highest TNC expression levels (3/14) in tumors from patients with metastasis to liver or lymph nodes (**Fig. 7A, B**), suggesting that a high TNC expression correlates with metastasis formation in human insulinomas.

Discussion

We have used the RT2 model of multistage pancreatic β-cell tumorigenesis with abundant and no TNC expression to obtain a better understanding of TNC contribution to tumor progression and we have observed multiple effects. Enhanced TNC levels in TNC transgenic RT2 mice correlate with an increase in tumor cell proliferation and survival, carcinoma formation, angiogenesis and lung micrometastasis. On the contrary, the absence of TNC results in reduced angiogenesis and lung micrometastasis. These results confirm a crucial role of TNC in tumor progression as has been suspected in human cancer.

There is multiple evidence for an important role of TNC in promoting tumor angiogenesis (Midwood et al., 2011; Orend, 2013). However, despite TNC has been extensively investigated since almost 3 decades (Chiquet-Ehrismann et al., 1986) it is not resolved how TNC impacts on tumor angiogenesis at the molecular level. Whereas TNC can have stimulatory effects on endothelial cell migration, conflicting reports exist concerning its impact on tubulogenesis (Orend, 2013). A pro-angiogenic effect of TNC linked to VEGFA expression was seen in human melanoma xenografts implanted into immune compromised mice lacking TNC (Tanaka et al., 2004). Of note, in the RT2/TNC tumors we did not observe an increased VEGFA expression (data not shown). Our study is the first to address the role of TNC on tumor angiogenesis systematically by using a stochastic genetic tumor model with an intact immune system. Here, we investigated the angiogenic switch, tumor blood vessels and their functionality. Most importantly, our study shows that TNC promotes the angiogenic switch, a rate-limiting step along tumor progression (Hanahan and Folkman, 1996), and the abundance of endothelial cells. However TNC seems to impair vessel functionality since tumor vessels of RT2/TNC mice are morphologically aberrant and less covered by pericytes. Moreover, vessels in RT2 tumors lacking TNC are less leaky than those with TNC suggesting a role of TNC in the formation of more but less functional tumor vessels.

We have identified DKK1 as an important TNC target in RT2 tumors. Our *in vivo* and *in vitro* results suggest that TNC promotes tumor progression involving DKK1 downregulation and activation of Wnt signaling. First, the TNC copy number inversely correlates with DKK1 expression in RT2 tumors and, a TNC substratum downregulates DKK1 expression in tumor and several stromal cell types (CAFs, pericytes, endothelial cells). Second, Wnt signaling is increased by TNC in the RT2 model and in cultured endothelial and tumor cells. Third, TNC-induced Wnt activation is reduced in tumor cells by DKK1. Finally, downregulation of DKK1 by TNC may be a key event since no other major Wnt inhibitor is consistently regulated by a TNC containing substratum (our data and (Ruiz et al., 2004)).

Several transcriptional regulators, epigenetic silencing as well as tissue tension were shown to regulate DKK1 expression (Aguilera et al., 2006; Barbolina et al., 2013; Liao et al., 2008; Menezes et al., 2012; Pendas-Franco et al., 2008; Zhou et al., 2012). Here, we demonstrate that TNC downregulates DKK1 expression by promoter inhibition. Since TNC blocks actin stress fiber formation (Huang et al., 2001; Midwood et al., 2004; Murphy-Ullrich et al., 1991; Orend et al., 2003) we investigated whether DKK1 expression is regulated by the actin polymerization state and demonstrated that disruption of the actin cytoskeleton by LB and CD as well as by shTPM1 reduced DKK1 mRNA levels. On the contrary enforcing actin polymerization and stress fiber formation by overexpression of syndesmos, bridging integrin $\alpha5\beta1$ and syndecan-4 in focal adhesions (Bass and Humphries, 2002) largely increased DKK1 expression. We further showed that LPA rescued focal adhesion and actin stress fiber formation and, cell spreading on FN/TNC which we linked to restored DKK1 expression in a RhoA-independent manner. How TNC downregulates DKK1 expression at promoter level is currently unknown and requires further investigation, but it does not appear to involve the SRF cotranscription factor MKL1 that is regulated by actin polymerization (Miralles et al., 2003), (Schwenzer and Orend, unpublished). Previously it was shown that a stiffened collagen substratum, implicating integrin adhesion signaling (Levental et al., 2009) induces DKK1 downregulation in several cell types including

endothelial cells (Barbolina et al., 2013). Here we report a novel mechanism that TNC blocks DKK1 transcription through its actin stress fiber disrupting activity.

The role of DKK1 in developmental and tumor angiogenesis appears to be context-dependent, as DKK1 can produce pro- and anti-angiogenic effects (24-31). Interestingly, the growth factor context seems to be particularly critical for the outcome, as for example DKK1 promotes bFGF-induced (24, 25, 31) but blocks VEGFA-induced (27) angiogenesis in Matrigel plug assays *in vivo*. We here confirm that DKK1 inhibits HUVEC tubulogenesis *in vitro* (Min et al., 2011) and tumor angiogenesis in an osteosarcoma xenograft model *in vivo*.

Employing the RT2 model we show that TNC promotes metastasis formation to the lung but not to the liver. This is reminiscent of breast cancer where, TNC is part of a gene expression signature specifically associated with lung but not bone metastasis (Minn et al., 2005), an initial observation that has been subsequently confirmed and functionally validated using xenograft models (Oskarsson et al., 2011; Tavazoie et al., 2008). Mechanistically, TNC expression was linked to an increased tumor cell survival and activation of Wnt and Notch signaling, as revealed by increased expression of Lgr5 and Msi1, respectively (Oskarsson et al., 2011). Although we have shown that Wnt signaling is activated in TNC-overexpressing RT2 tumors and in cellular models comprising tumor and endothelial cells *in vitro*, the expression of Lgr5 and of several Notch pathway members are unaffected in the *in vivo* and *in vitro* models we used (**Table S3** and data not shown). Multiple explanations for these differences may exist, such as difference in model systems and in organ and tissue context. We have shown that the ectopic expression of TNC leads to DKK1 downregulation and Wnt signaling activation in RT2/TNC tumors as revealed by the upregulation of other Wnt target genes, including the prototypical Wnt target Axin2. Conversely, in RT2/TNCKO tumors DKK1 levels were increased but Axin2 expression was unchanged. This result is in line with a previous report showing that the Wnt pathway has minimal basal activity in pancreatic beta tumor cells and is dispensable for RT2 tumor progression (Herzig et al., 2007). In addition to canonical Wnt signaling, the DKK1 receptor

LRP6 was shown to promote PDGF-BB, TGF-β and CTGF signaling in pericytes and fibroblasts. Importantly, these signaling actvitities were blocked by DKK1 through binding to LRP6 (Ren et al., 2013). We suggest that a TNC-rich matrix induces a microenvironment with low DKK1 levels that is susceptible to angiogenic signaling from Wnt and other pathways regulated by DKK1. This possibility is supported by our results that have shown an inverse correlation of TNC and DKK1 expression, promotion of the angiogenic switch by TNC and a strong downregulation of DKK1 by TNC in tumor and several stromal cell types.

In the TNC transgenic RT2 model we observed that TNC promotes multiple early events such as proliferation and survival in hyperplastic islets, Wnt target upregulation in small differentiated tumors and the angiogenic switch. A major role of TNC early in tumorigenesis combined with a less functional vasculature may explain why macroscopically visible RT2 tumors of the different genotypes did not differ in size. A potential early role of TNC in tumorigenesis has not got much attention since patient data with a correlation of high TNC expression and malignancy (Orend, 2013; Oskarsson et al., 2011) rather suggested a major role of TNC in late events. In human cancer tissue early events cannot be easily addressed which might explain why we did not see a correlation of TNC and DKK1 mRNA expression levels in human cancer tissues. TNC promotes metastasis (Minn et al., 2005; Oskarsson et al., 2011; Tavazoie et al., 2008) which has also been recapitulated here in the RT2 model and, in human insulinomas where the highest TNC expression levels were observed in the few available metastatic insulinomas.

In summary, we have shown that DKK1 expression is dependent on actin stress fibers that are disrupted by TNC. We have established a transgenic immune-competent tumor mouse model that mimics the high expression of TNC observed in human cancer. Our results prove that TNC plays crucial roles along tumor progression by promoting early and late events. We demonstrate that TNC levels determine the extent of tumor cell survival, invasion, tumor angiogenesis and metastasis. These phenotypes appear to be linked to DKK1 downregulation creating a pro-angiogenic tumor microenvironment. Finally, our human

TNC-expressing transgenic tumor mice offer a model for human insulinoma progression and for the preclinical evaluation of drugs that target human TNC.

Material and Methods

Detailed information are provided in the **SI Material and Methods** section. ***Human insulinoma patient material*** was obtained from the Klinikum rechts der Isar (Munich, Germany) or the Hôpital de Hautepierre (Strasbourg, France). Samples were obtained after patient informed written consent and upon approval by an ethics committee. Analysis by qRTPCR, IF and IHC was performed as described in **SI Material and Methods**. ***Experiments comprising animals*** were performed according to the guidelines of INSERM and the Swiss Federal Veterinary Office. RT2 mice developing pancreatic neuroendocrine tumors (Hanahan, 1985) were crossed with RipTNC (this study) or TNCKO (Forsberg et al., 1996) mice to generate double-transgenic mice with forced expression of TNC (RT2/TNC) or lacking TNC expression (RT2/TNCKO). Generation of transgenic RipTNC mice, breeding, genotyping, xenograft experiments and analysis of tumor material are specified in the **SI Material and Methods** section. ***In vitro experiments*.** Coating of cell culture dishes with FN and TNC was performed as described earlier (Huang et al., 2001; Lange et al., 2007). Cells were seeded on the coated surfaces and analyzed using standard protocols as described in **SI Material and Methods**.

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Figure legends

Fig. 1 TNC enhances proliferation, survival and tumor progression in RT2 tumors. **(A, B)** Quantification of proliferating cells in tumor sections as PH3 positive nuclei in 12 week old RT2 **(A)** (N = 13 mice, n = 199 sections) and **(B)** RT2 (N = 6 mice, n = 131 islets) and RT2/TNCKO (N = 6, n = 137) mice. **(C, D)** Quantification of apoptotic cells as cleaved caspase-3 positive cells in tumor sections of 12 week old **(C)** RT2 (N = 6 mice, n = 122 tumors) and RT2/TNC (N = 8, n = 181) mice. **(D)** RT2 (N = 4 mice, n = 95 islets) and RT2/TNCKO (N = 4, n = 83) mice. **(E)** Tumor grading into adenoma or invasive carcinoma (H&E stained tumor sections) of RT2 tumors (N = 26 mice, 78 adenomas, 79 carcinomas) and RT2/TNC (N = 22, 44 adenomas, 76 carcinomas). See **Table S1**. **(F, G)** Number of angiogenic islets per mouse normalized to RT2 controls. See **Table S2**. **(H, I)** Tumor blood vessel quantification upon CD31 staining of tumor sections from 12 week old mice as CD31 positive area fraction per tumor normalized to RT2 controls. **(H)** RT2 (N = 6 mice, n = 34 tumors, 203 images), RT2/TNC (N = 4, n = 17, 106 images), **(I)** RT2 (N = 3, n = 71), RT2/TNCKO (N = 3, n = 111). **(J)** Morphology of the tumor vasculature in Mercox perfusion casts from 12 week old RT2 and RT2/TNC mice. Arrows point at break point, branching and constriction. Scale bars, 50 μm. **(K)** Pericyte coverage of tumor blood vessels upon quantification of the ratio of NG2 over CD31 staining signals. RT2 (N = 6 mice, n = 155 tumors), RT2/TNC (N = 8, n = 204). **(L, M)** Tumor blood vessel leakage quantification upon Fibrinogen staining of tumor sections from 12 week old mice as Fibrinogen positive area fraction per tumor. **(L)** RT2 (N = 5 mice, n = 94 tumors), RT2/TNC (N = 3, n = 84), **(M)** RT2 (N = 4, n = 60), RT2/TNCKO (N = 5, n = 125). Error bars represent SEM and asterisks (*) indicate p-values < 0.05.

Fig. 2 Lung micrometastasis in RT2 mice. Insulin expression in a lung RT2 micrometastasis **(A)** and quantification by qRTPCR **(B, C)**. **(A)** Detection of metastasized insulin positive tumor cells in lung parenchyma (RT2 mouse) by immunostaining (upper panel) and H&E staining (adjacent section, lower panel). Scale bar 50 μm. Detection of insulin expression in RT2 (9/24) and RT2/TNC mice (11/24), **(B)** and, in RT2/TNC+/-

(8/13) and RT2/TNCKO littermates (4/13) **(C)**. Error bars represent SEM and asterisks (*) indicate p-values < 0.05.

Fig. 3 Dkk1 expression in RT2 tumors. (A – C) DKK1 and Axin2 expression was analyzed by qRTPCR. **(A)** Tumors were stratified according to Dkk1 levels, as expressing (Dkk1 +) or not expressing (Dkk1 -). Dkk1 was found to be expressed in 26 of 27 RT2 tumors and in 7 of 13 RT2/TNC tumors. Difference between genotypes, p < 0.05. **(B)** DKK1 expression was largely reduced in those RT2/TNC tumors with detectable Dkk1 expression. Axin2 expression was enhanced in RT2/TNC tumors. **(C)** In RT2/TNCKO tumors (15 of 24 tumors were Dkk1 positive) Dkk1 expression was higher compared to RT2/TNC+/- tumors (16 of 23 tumors were Dkk1 positive). Axin2 expression was not changed. **(B, C, D)** Wnt target gene expression in all RT2/TNC and RT2/TNCKO tumors **(B)** or in small differentiated tumors **(D),** see **Table S3**. Error bars represent SEM and asterisks (*) indicate p-values < 0.05.

Fig. 4 TNC leads to DKK1 downregulation and Wnt signaling activation in tumor cells and endothelial cells. (A-C) Enhanced Wnt signaling in Wnt-3A treated KRIB cells by TNC. TOPFlash activity of cells grown on FN or FN/TNC for 48h **(A)** or treated for 48h with Wnt-3A conditioned medium (CM) and CM of cells grown on FN or FN/TNC **(C). (B)** Axin2 mRNA expression (qRTPCR, 5h). **(D)** DKK1 expression (qRTPCR, 24h) in the indicated tumor cell lines (KRIB, T98G, MDA-MB-435 (MDA), MCF-7 and Caco2) on FN/TNC is represented relative to its expression on FN. **(E)** Cell autonomous impact of low (knockdown) and high (overexpression) DKK1 on Wnt signaling as analyzed by TOPFlash assay after 48h. **(F)** Repression of TNC mediated Wnt signaling activation by Dkk1. TOPFlash luciferase activity was performed as in **(A)** except the addition of CM from KRIB control or mDkk1 overexpressing cells after 5h of cell seeding on the indicated substrata. Note that the TNC-containing substratum still induced Wnt signaling activity in presence of Dkk1 containing CM, but to a lesser extent (1.8 fold) than in the control conditions (3.1 fold). **(G-I)** DKK1 and Axin2 mRNA expression in pericytes **(G, H)** and two human colo-

rectal cancer derived CAF primary lines **(I)** seeded on FN or FN/TNC (5h). TNC leads to downregulation of DKK1 in pericytes and CAFs (**G, I**), but Axin2 expression remains unchanged in pericytes **(H)**. **(J-L)** Enhanced Wnt signaling by TNC in HUVEC. qRTPCR for DKK1 and Axin2 (5h) **(J, L)** and DKK1 immunoblotting (24h) **(K)**. Data from 3 independent experiments (except **(D)**: MCF-7 and Caco2 cell lines, 1 and 2 experiments, respectively) and **(I)** 2 experiments, are shown as mean ± SEM, * $p < 0.05$.

Fig 5. Mechanism of DKK1 downregulation

(A) Reduced DKK1 promoter activity by TNC. DKK1 promoter driven luciferase activity in T98G cells is shown upon growth for 48h on the indicated substrata. **(B, E)** Phalloidin (red) and vinculin (green) stainings of T98G cells upon CTR, CD (2 μM) or LB (5 μM) treatments for 3 hours **(B)**. Nuclei are stained in blue (DAPI). Scale bar 20 μm. **(C, D)** DKK1 mRNA expression in serum starved T98G upon LB (5 μM, 3 hours) **(C)** or CD treatment (2 μM, 3 hours) **(D). (E)** IF staining of T98G cells upon control or LPA (30 μM) treatment. Serum starved T98G were plated on fibronectin (FN) or fibronectin/tenascin-C (FN/TNC) and, after 1 hour LPA was added for 4 hours. While cells are poorly spread under control conditions on FN/TNC (no actin stress fibers, few focal adhesions), LPA treatment restored cell spreading associated with the formation of focal adhesions and actin stress fibers. Scale bar 20 μm. **(F)** DKK1 mRNA expression determined by qRTPCR upon treatment with 30 μM LPA. LPA restores DKK1 expression on FN/TNC. **(H-J)**. DKK1 mRNA expression determined by qRTPCR upon ectopic expression of chicken syndesmos, a focal adhesion component **(H)**, and mouse tropomyosin1 (TPM1), a F-actin stabilizing protein **(I)**, or upon knockdown of TPM1. Syndesmos or TPM1 overexpression induce DKK1 mRNA expression while TPM1 knockdown leads to DKK1 downregulation. Data are shown as mean ± SEM, * $p < 0.05$

Fig. 6 Dkk1 overexpression inhibits osteosarcoma growth and angiogenesis. (A) Mean tumor volume (±SEM) of control (CTR, n = 10) and mDkk1 overexpressing (n = 9) KRIB tumors upon subcutaneous injection of the corresponding cells into nude mice. **(B)** Representative tumor images. **(C)** Tumor microvessel density, as determined by CD31

staining and quantification, was 2.2-fold reduced in KRIB:mDkk1 tumors (n = 8) as compared to control KRIB tumors (n = 10). Error bars represent SEM and asterisks (*) indicate p-values < 0.05.

Fig. 7 TNC expression correlates with metastasis formation in human insulinomas. (A) TNC mRNA expression was determined by qRTPCR in two patient groups (Group 1, Munich cohort; Group 2, Strasbourg cohort) and is displayed as relative expression upon normalization to GAPDH. Combining data of the two groups, TNC expression in patients with metastasis is increased over that in patients without metastasis ($p < 0.05$). **(B)** TNC expression was determined by IHC in all tumors of the two groups of insulinomas. Representative pictures of the 3 metastastic and 3 non-metastatic insulinomas are shown. Scale bar, 100 µm.

Fig. 1

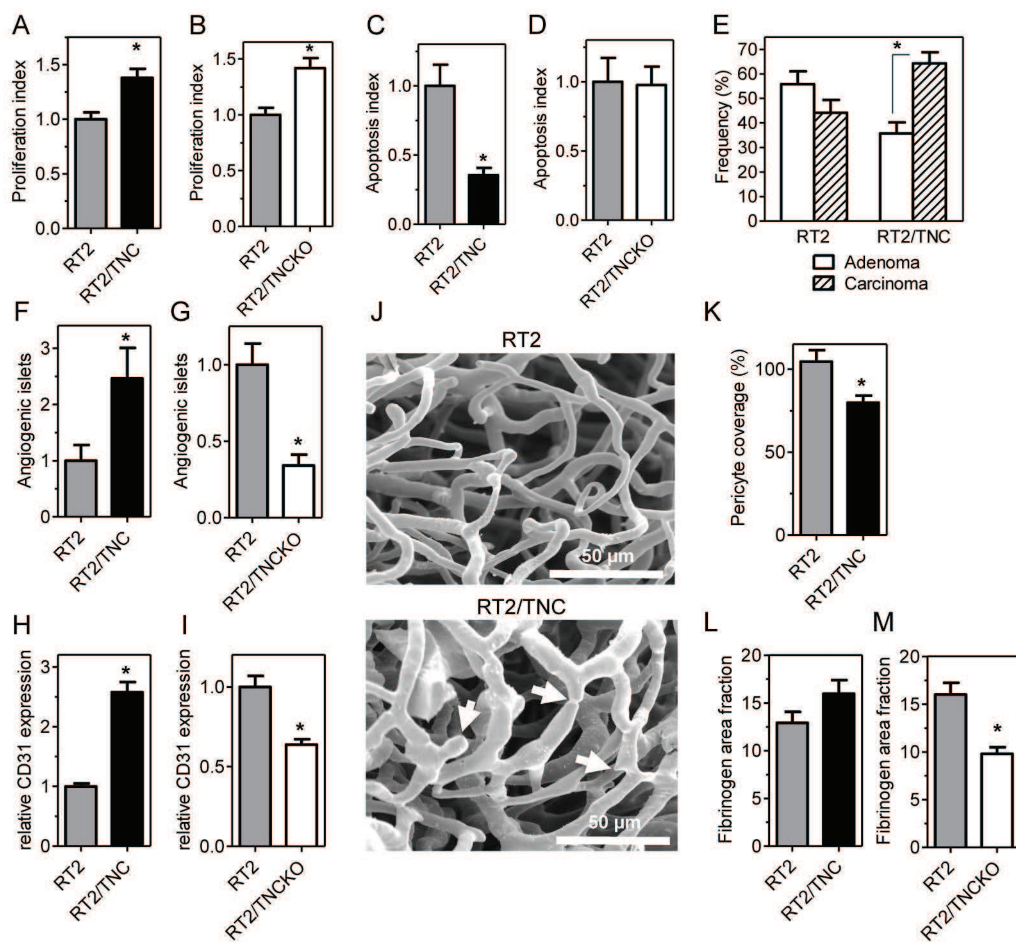


Fig. 2

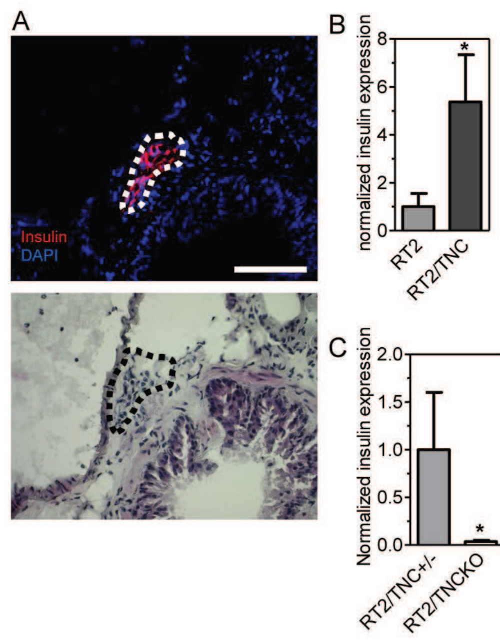


Fig. 3

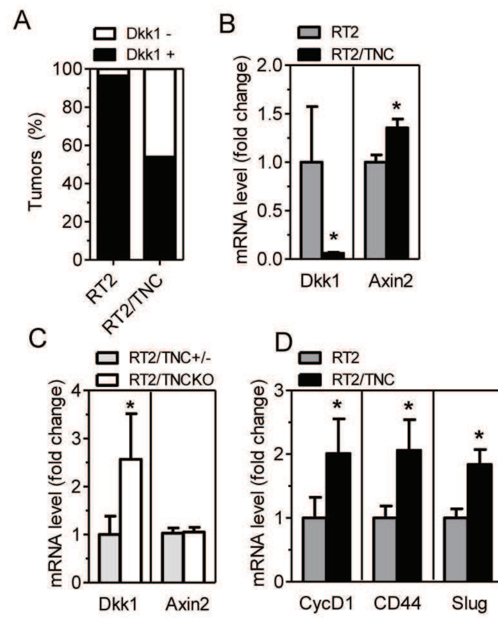


Fig. 4

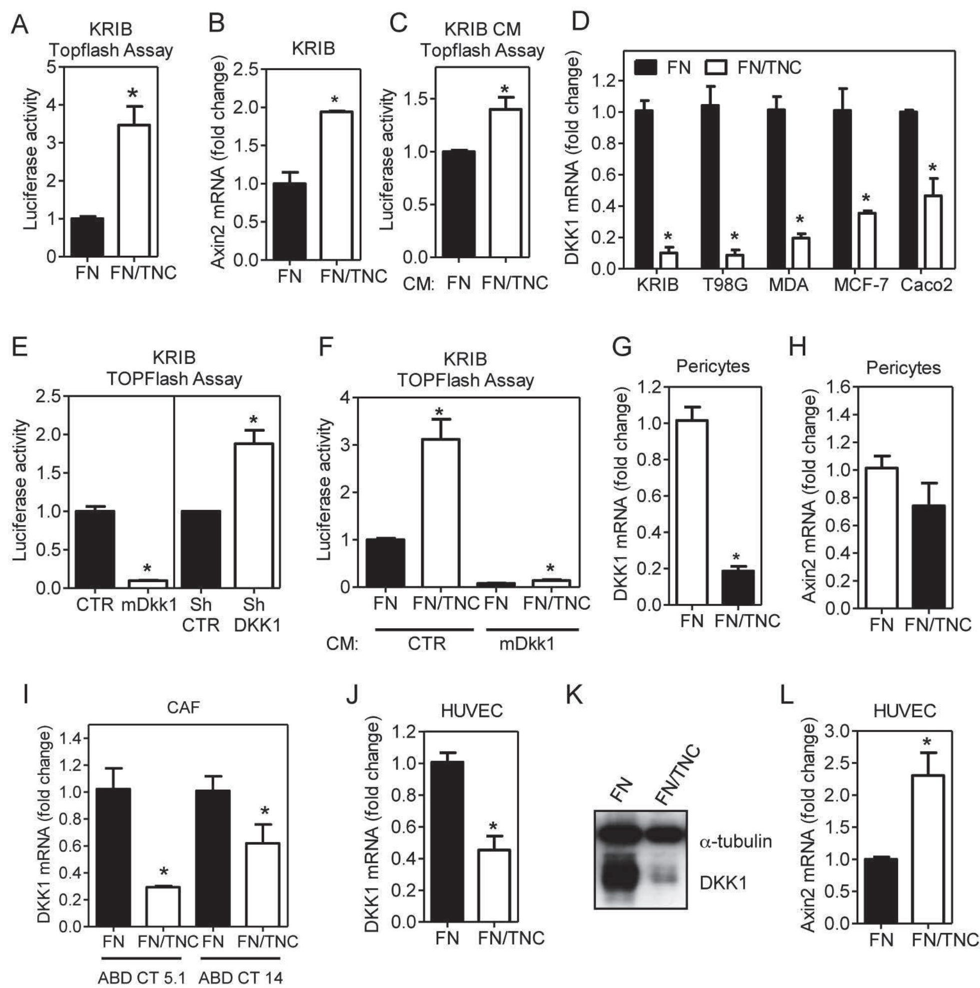


Fig. 5

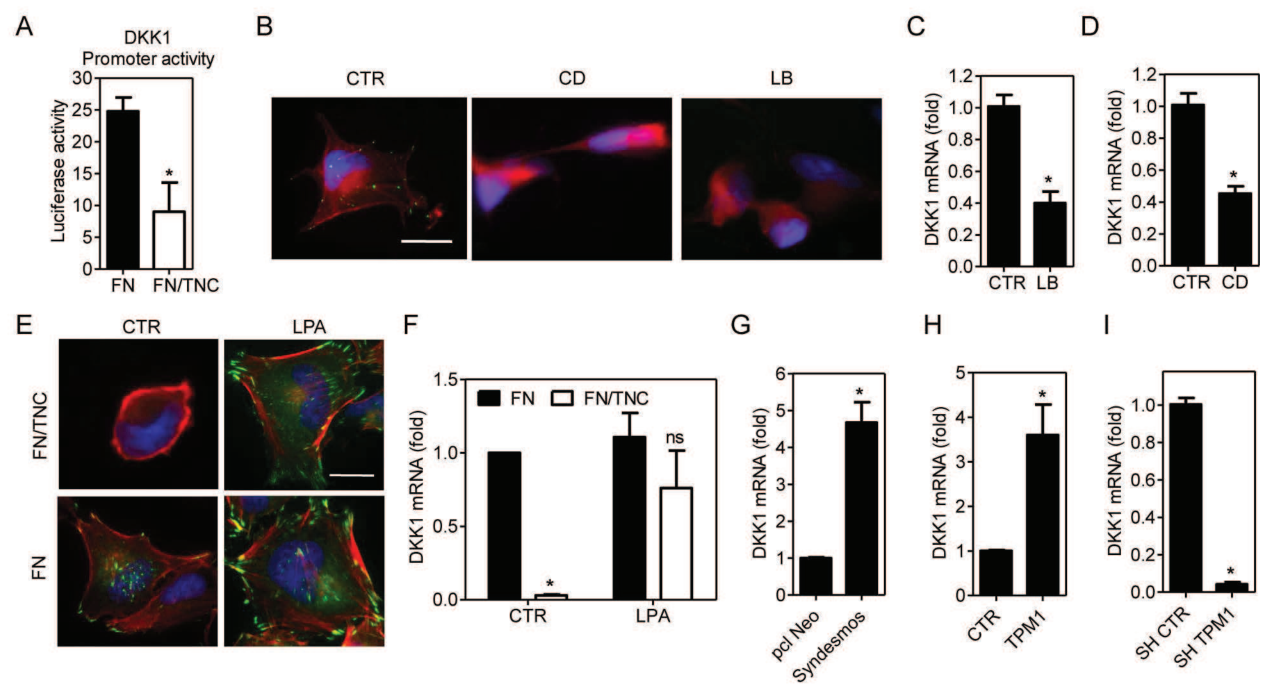


Fig. 6

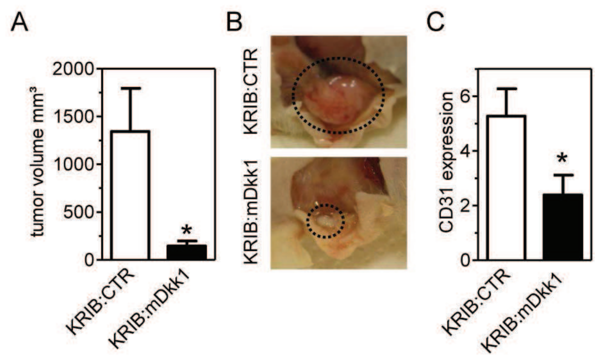
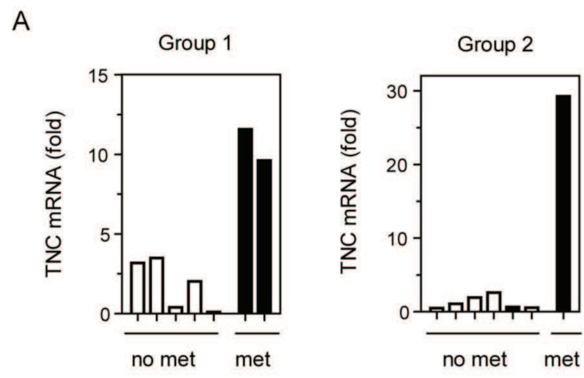
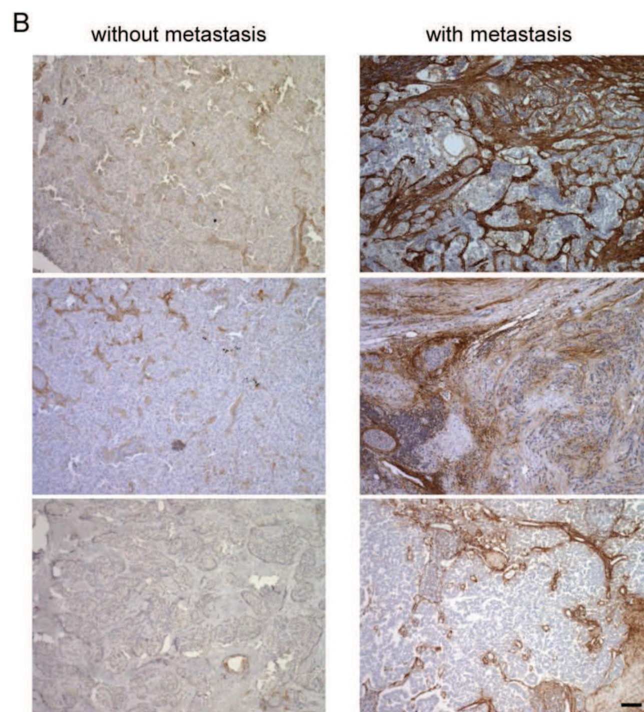


Fig. 7

A



B



Supplemental Material and Methods

Generation of transgenic RipTNC mice

The human TNC cDNA sequence (GenBank X78565.1) comprising all but AD1 and AD2 extra domains was removed from the HxBL.pBS plasmid (Aukhil et al., 1993) and cloned into the Rip1 expression vector (Hanahan, 1985) for insulin promoter driven expression by using the intermediate pcDNA3.1/Hygro(-) vector (**Fig. S1B**). Successful cloning was confirmed by restriction enzyme analysis and partial sequencing. Expression and secretion of TNC was determined in a RT2 cell line by immunostaining and sandwich ELISA. The TNC expression vector was injected into the pronucleus of fertilized oocytes giving rise to transgenic RipTNC mice with stable transmission and expression of the transgene. Transgenic mice were healthy and fertile and did not exhibit any detectable alterations in tissue morphology (**Fig. S1F**) nor blood glucose homeostasis (**Fig. S1G**). All experimental procedures involving mice were done according to the guidelines of INSERM and the Swiss Federal Veterinary Office.

Generation of tumor mice with different TNC expression levels

RT2 mice (Hanahan, 1985) were bred with RipTNC mice (three lines) or TNCKO mice (Forsberg et al., 1996) to generate RT2/TNC or RT2/TNCKO mice respectively, with different TNC expression levels (**Fig. S1D, E**). TNC expression analysis confirmed that tumors of RT2/TNC mice expressed transgenic human TNC (**Fig. S1D**), whereas those from RT2/TNCKO mice lacked the TNC protein (**Fig. S1E).** Starting at the age of 10 weeks the drinking water was supplemented with 5% (w/v) glucose (FLUKA). Most data were obtained from mice in a C57Bl6 background except results in **Fig. 2C, Fig. 3C** and **Fig. S3C** that were derived from RT2/TNCKO mice and littermates with one TNC copy (RT2/TNC+/-) in a mixed 129/Sv-C57Bl6 background. For genotyping by PCR the following primers were used, RipTNC (Fwd: 5′-TAA TGG GAC AAA CAG CAA AG-3′, Rev: 5′-GAA AGA CAC CTG CCA ACA GC-3′), SV40 Tag (Fwd: 5′-GGA CAA ACC ACA ACT AGA ATG CAG-3′, Rev: 5′-CAG AGC AGA ATT GTG GAG TGG-3′) and TNCKO (Fwd wt: 5′-CTG CCA GGC ATC TTT CTA GC-3′, Fwd TNCKO: 5′-CTG CTC TTT ACT GAA GGC TC-3′, Rev: 5′-TTC TGC AGG TTG GAG GCA AC-3′).

Oral glucose tolerance test

12 week old mice (14 RipTNC and 15 wildtype) were starved overnight in clean cages with free access to water. Tail vein blood glucose concentration was measured at time = 0 using Glucofix sensor for Glucofix mio (A. Menarini Diagnostics). Glucose (2 mg per g of mouse) was orally administered by gavage and blood glucose levels (mg/dL) were measured every 15 minutes for 1.5 hours.

Histopathological analysis

For RT2 mice, tumor incidence per mouse was determined as the number of all visible tumors with a diameter of 1 mm. Tumor volume was calculated assuming a spherical shape with formula $V=1/6*\pi*d^3$ (d = tumor diameter). Pancreata, liver and lung tissue were isolated and either fixed in 4% paraformaldehyde (PFA) overnight followed by embedding in paraffin or were fixed for 2h in 4% PFA, immersed in 20% sucrose overnight and embedded in Tissue-Tek O.C.T. (Sakura Finetek) or were freshly embedded in O.C.T. and frozen on dry ice.

Histological analysis was performed on tissue sections (5 µm thick paraffin, 7 µm thick cryosections) by direct staining with hematoxylin and eosin (H&E) or by immunostaining. Primary antibodies were incubated overnight at 4°C. Immunohistochemical (IHC) detection was performed on paraffin embedded tissue using Vectastain developing system (Vector labs), followed by staining with hematoxilin. Detection by immunofluorescence (IF) was performed on fixed or fresh frozen tissue using FITC- or Cy3-coupled secondary antibodies (Jackson ImmunoResearch); cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). The primary antibodies detecting the following molecules were used: phospho-histone H3 (PH3;1:200, Upstate 06-570), cleaved caspase-3 (1:50, Cell Signaling 9661), CD31 (1:50, BD Pharmingen 550274, Acris BM4086), NG2 (1:200, Millipore AB5320), Insulin (1:200, DakoCytomation A0564), Glucagon (1:1000, Sigma G2654), KI67 (1:200,

clone SP6, Thermo Scientific, RM-9106-S1), human TNC BC-24 (1:3000, Sigma T2551), fibrinogen (1:500, Dako A0080). The polyclonal rabbit antiserum TNC 1.2, recognizing human and murine TNC, was derived upon injection of recombinant human TNC protein (Lange et al., 2007). Anti-mouse TNC MTn12 (Aufderheide and Ekblom, 1988) and anti-human TNC B28.13 antibodies (Schenk et al., 1995) were purified from hybridoma culture supernatants.

Quantification of IF microscopic pictures was done by using ImageJ (National Institutes of Health, USA) and ZEN Blue (Carl Zeiss) softwares. The staining protocol (fixation, blocking, antibody dilution), image acquisition setting (microscope, magnification, light exposure, acquisition time) were always kept constant per experiment. Data were quantified as counted events over analyzed tumor area (PH3) as area fraction over analyzed DAPI positive cell area (cleaved caspase 3, PH3, KI67) or as area fraction over analyzed tumor area (CD31, NG2, fibrinogen).

Determination of adenomas (differentiated tumor cells, encapsulated tumors) and carcinomas (Grade 1, differentiated tumor cells, one invasive tumor front; Grade 2, partially dedifferentiated tumor cells, more than one invasive tumor front; Grade 3, heterogeneous appearance and loss of differentiated tumor cells, many invasion sites) was performed on H&E stained paraffin sections.

Gene expression analysis in RT2 and KRIB tumors and in lung and liver tissue

Tissue from isolated tumors, liver and lung was snap frozen in liquid nitrogen. Total RNA (extracted with NucleoSpin RNA II kit (Macherey-Nagel)) from liver, KRIB tumors (1 µg), RT2 tumors or lung (2 µg) was treated with DNase I (Invitrogen) and reverse transcribed (MultiScribe reverse transcriptase (Applied Biosystems)). qRTPCR was done on cDNA diluted 1:5 (liver, lung) or 1:10 (tumors) with specific primers (Roche Probefinder v2.45, **Table S4**) on a 7500 Real Time PCR machine (Applied Biosystems) using SYBR green or Taqman reaction mixtures (Applied Biosystems). Data were normalized versus TBP (liver, lung),

HMBS (KRIB tumors), GAPDH (human insulinoma) or a combined value of RPL19, TBP and GAPDH (RT2 tumors). Relative expression levels ($2^{-\Delta\Delta ct}$) were calculated for each individual sample.

Isolation of pancreatic islets

Langerhans islets were isolated from 8 week old RT2 mice by using Liberase RI (Roche) (RT2/TNC and RT2) and Liberase TL (Roche) (RT2/TNCKO and RT2). The pancreas was perfused via the bile duct with 2 ml Liberase solution (0.82 (RI), 1 (TL) Wünsch units/ml), collected and digested at 37°C (24 minutes (Liberase RI), 17 minutes (Liberase TL)). Upon recovery from the interphase of a Histopaque 1077 (Sigma)/DMEM centrifugation gradient (30 minutes, 1500 x g) intact islets were handpicked under a stereomicroscope and quantified as non-angiogenic (white) or angiogenic (red).

Methylmethacrylate (Mercox) casting and SEM analysis

Anaesthetized mice were perfused through the thoracic aorta with 0.9% NaCl/1% heparin/1% procaine followed by freshly prepared Mercox solution (Vilene Japan Hospital Co. Ltd.) containing 0.1 ml accelerator per 5 ml resin. After solidification pancreata were excised and kept for 3 weeks in 7.5 % KOH for tissue dissolution. Casts were dehydrated in ethanol and vacuum dried. Samples were mounted on aluminum stubs, sputtered with gold and examined in a Philips XL-30 SFEG scan electron microscope.

Cell culture, gene expression and immunoblotting

Human brain vasculature pericytes (ScienCell 1200), tumor cell lines, monocyte/macrophage cell lines (J774.A1 and RAW264.7) and L cells (fibroblasts, control and overexpression of Wnt-3A) (American Type Culture Collection, Rockville, MD, (ATCC)) were maintained in DMEM/4.5g/l Glucose/10% FCS. Cancer-associated fibroblasts (CAF) ABD CT5.1 and ABD CT14 were cultured in DMEM/1g/l Glucose/10% FCS. BOSC cells (ATCC) were maintained in DMEM/10% FCS supplemented with 1mM NaPyruvate and 10 mM Hepes and HUVEC (Promo cell, C-12203) were maintained in Endothelial Cell Growth

Medium (PromoCell, C-22010). T98G:TPM1, T98G:Syndesmos and T98G:shTPM1 (Lange et al., 2008; Ruiz et al., 2004) were cultured in DMEM/10%FCS with 400 µg/ml G418.

Human carcinoma-associated fibroblasts (CAF) were isolated from colo-rectal adenocarcinoma resection specimens from 2 patients that were obtained in accordance with the local ethics committee (Ghent University Hospital) (De Boeck et al., 2013; De Wever et al., 2004). Tissue fragments were cut in small pieces 1-2mm3) and transferred into a pre-scratched 6-well plate with 100microl. of FCS supplemented with antibiotics added on top of each fragment. Cultures were incubated at 37°C with 10% CO2 in air for 24 hours. DMEM containing 10% FCS was added into each well. Cell outgrowth was observed after 3-6 days. After 15 days, adherent cells were transferred to 25 cm2 tissue culture flasks. CAF cultures were characterized by qRTPCR expression analysis for vimentin, α-smooth muscle actin and cytokeratin.

Conditioned medium containing Wnt-3A or mDKK1 was collected from L cells overexpressing Wnt-3A and from KRIB cells overexpressing mDKK1, respectively after 3 to 4 days of culture. Medium was filter-sterilized and stored at -20°C. Cells starved in DMEM/1% FCS (tumor cells, pericytes, CAF) or M199/1% FCS/1µg/ml Hydrocortison/10 ng/ml Heparin/10 ng/ml mEGF/10 ng/ml bFGF (HUVEC) were seeded onto matrix coated dishes as published (Huang et al., 2001; Lange et al., 2007). Briefly, FN and TNC were sequentially coated in PBS/0.01% Tween-20 at 1 µg/cm^2 before saturation of the non-coated surface with 10 mg/ml BSA/PBS. RNA was isolated (NucleoSpin RNA extraction kit, Macherey-Nagel or Trizol, Life Technologies) according to the manufacturer's instructions. RNA was reverse transribed (MultiScribe reverse transcriptase, Applied Biosystems) and qRTPCR was done on cDNA diluted 1:2.5 in water with specific primers (**Table S5**) on a 7500 Real Time PCR machine (Applied Biosystems) using SYBR green reaction mixture (Applied Biosystems). Data were normalized versus ß2-microglobulin expression and relative expression levels was calculated ($2^{-\Delta\Delta ct}$).

Cells were starved overnight before treatments with 1, 10 or 30 µM LPA for 3 hours or 4 hours on FN or FN/TNC substrata (Santa Cruz, H_2O), 5 µg/ml Actinomycin D for 30, 60 or

90 minutes (Sigma-Aldrich, DMSO), 5 μg/ml Latrunculin B for 3 hours (Calbiochem, DMSO) and 2 μg/ml Cytochalasin D four 3 hours (Calbiochem, DMSO).

For immunoblotting cells were lysed in Laemmli buffer (Laemmli, 1970). The following antibodies were used: Anti-DKK1 n-terminal (Sigma-Aldrich, D3195), 1:1500, Anti-DKK1 (R&D, AF1096) 1:500, Anti-6xHis tag (Abcam, ab18184), Anti-RhoA (Santa-Cruz, sc-418) 1:5000, Anti-α-tubulin (CP06, Oncogene, Boston, MA, USA), 1:2000. Secondary antibodies were ECL Anti-Rabbit IgG (NA934V) and ECL Anti-Mouse IgG (NXA931) Horseradish Peroxidase linked whole antibodies (GE Healthcare, Buckinghamshire, UK) and donkey anti-goat IgG-HRP (sc-2020, Santa Cruz, Biotechnology). Amersham ECL Western Blotting detection reagent (RPN2106) or Amersham ECL Plus Western Blotting Detection System (RPN2132) (GE Healthcare, Buckinghamshire, UK) were used.

Immunofluorescence staining of cultured cells

Cells were fixed in 1% PFA for 10 minutes and permeabilized in PBS-Triton 0.1% for 10 minutes. Cells were stained with Anti-Vinculin (Abcam; 1/50; 2 hours) and anti-mouse Alexa-488 (Jackson ImmunoResearch; 1/800; 1 hour). For phalloidin staining cells were incubated for 20 minutes with Phalloidin-Tetramethylrhodamine B isothiocyanate (Sigma P1951; 1/200). Nuclei were stained with Dapi (Sigma D9542).

Retrovirus construction and infection, plasmid transfection and reporter assays

For generating mDKK1 cDNA with a V5-His-tag the mDKK1 cDNA (Ruiz et al., 2004) was cloned into the pcDNA3.1 mDKK1 V5-His-TOPO vector (Invitrogen) according to the manufacturer's guidelines. For generating the pQCXIP-mDKK1 V5 His vector a BamHI and an EcoRI site were added in the pcDNA3.1 mDKK1 V5-His-TOPO before the ATG or the stop codon of the mouse DKK1 cDNA respectively, using the GeneEditor™ in vitro Site-Directed Mutagenesis System, with the primers 5'-P-GGT GGA ATT GCC CTT GGA TCC ACA TGA TGG TTG TGT-3' and 5'-P-ACC ATC ACC ATT GAG AAT TCA CCC GCT GAT CAG CC-3'. Upon BamHI-EcoRI restriction digest, the mDKK1 V5 His fragment was gel purified (NucleoSpin®

Extract II, Machery-Nagel, France) and cloned in the BamHI-EcoRI site of the pQCXIP retroviral vector (Clontech, Ozyme, France) generating the pQCXIP-mDKK1 V5 His vector. BOSC cells were transfected with the pQCXIP-mDKK1 V5 His vector or empty control (CTR) vector to obtain retroviruses for transduction of KRIB cells followed by selection with Puromycin (2.5 µg/ml). Expression of mDKK1 was determined by qRTPCR and immunoblotting.

T98G were transiently transfected (JetPEI, Polyplus, Strasbourg, France) with plasmids encoding RhoA wt (Addgene Plasmid 12962: pRK5-myc-RhoA-wt), RhoA-Q63L (Addgene Plasmid 12964: pRK5-myc-RhoA-Q63L) and RhoA-T19N (Addgene Plasmid 12963: pRK5-myc-RhoA-T19N) (all RhoA plasmids deposited at Addgene by Gary Bokoch). Empty pCB6 was used for control transfection.

For the β-catenin Luciferase Reporter Assay cells were transiently transfected (JetPEI, Polyplus, Strasbourg, France) with the Super 8x TOPFlash or control Super 8x FOPFlash (mutant TCF/LEF binding sites) (Veeman et al., 2003) (obtained from Addgene, plasmids 12456 and 12457). Upon seeding for 5h on matrix coated dishes, CM containing Wnt-3A, mDKK1 or CTR medium was added for a total of 48h. TOPFlash Luciferase activity was calculated after normalization to Renilla and FOPFlash activity (Dual-Luciferase Reporter Assay System, Promega, Madison, WI, USA).

For SRF luciferase reporter assays T98G cells were transfected with the 3DA.Luc plasmid (provided by Guido Posern) encoding c-fos derived SRF binding sites and a pRL-TK plasmid for normalization of the luciferase signal.

A 3177 bp human DKK1 promoter sequence was cloned from HCT116 genomic DNA into the multiple cloning site of the pGL3-basic Luciferase reporter vector (Promega). Cells were transiently transfected with the pGL3-DKK1 promoter construct or empty pGL3-basic vector

for 40 hours. Luciferase activity normalized to Renilla activity is presented as the ratio of pGL3-DKK1 to pGL3-basic.

For generation of KRIB shDKK1 cells, KRIB cells were infected with MISSION Lentiviral Transduction Particles (SHCLNV, clone ID TRCN0000033386, Sigmal-Aldrich) or MISSION Non-Target shRNA Control Transduction Particles (SHC002V, Sigma-Aldrich) with a MOI=10, transduced cells were selected with 10 µg/ml Puromycin.

Cell proliferation assay

To determine cell proliferation, cells were plated into 96-well plates ($5x10^3$ cells/well in quadruplicate for each time point). A MTS assay was performed following manufacturer's instructions (CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay, Promega) after 24h (day 1), 48h (day 2) and 72h (day 3). Measures were normalized to the relative cell number on day 1.

HUVEC tubulogenesis assay on matrigel

Tubulogenesis assessment was done in 15 well dishes (µ-Slide Angiogenesis, Ibidi LLC), using growth factor-reduced matrigel (BD Bioscience). The matrix was prepared by loading 10 µl of matrigel in each well and allowing it to solidify for 45-60 minutes at 37°C in a humidified incubator. HUVEC (Promocell) were trypsinized and resuspended at 100,000 cell/ml in conditioned medium (CM) obtained from KRIB cells overexpressing mouse DKK1 or its respective control (CM was collected after 2 days in confluent layer of both KRIB cell type). $5 X 10^3$ cells/well (50µl) were loaded on top of the solidified matrigel and was incubated for 6 hours at 37°C in a humidified incubator in a 5% CO_2 atmosphere. Bright field mosaic pictures were taken using an Zeiss Imager Z2 inverted microscope and AxioVision software (Carl Zeiss) at 5X magnification, which allowed imaging of the whole well in 9 pictures. Tube-like structures (defined by the numbers of closed loops) were counted using the ZEN Blue software (Carl Zeiss). 3 independent experiments were performed with 3-5 replicates per experiment, and measures subsequently averaged.

Perfusion, fibrinogen staining and quantification of tumor vessel leakiness

Twelve weeks old mice were anesthesized by i.p. injection of pentobarbital (5%, 4µL/g body weight). The chest was opened and the right atrium was cut. The left heart ventricle was perfused with 10mL of 4% paraformaldehyde (wt/vol) followed by 10mL of PBS through a 23G syringe connected to a peristaltic pump at constant pressure. The pancreas was dissected, incubated overnight in 20% sucrose at 4°C and frozen in OCT. Seven µm sections were processed for fibrinogen and CD31 immunofluorescent staining as described above. Fibrinogen immunoreactive areas were measured by using the ImageJ software and were expressed as percentage of tumor/islet total area (area fraction).

Tumor xenograft experiments

$4x10^6$ KRIB CTR or mDKK1 overexpressing cells were injected subcutaneously in the left upper back of nude mice (Charles River). After 3.5 weeks, mice were sacrificed, the tumor size was measured with a caliper and the tumor volume was calculated using formula $V=(a^2*b)/2$, where b is the longest axis and a is the perpendicular axis to b. Tumor tissue was snap frozen in liquid nitrogen or directly embedded in O.C.T. and further analyzed by qRTPCR and immunostaining as described above.

Human insulinomas

Analysis of the human insulinomas had been approved by the ethics committee of the Klinikum rechts der Isar and the Hospital Hautepierre. All samples were obtained after prior informed written consent. Tumor tissue was obtained from 14 patients with endocrine pancreatic cancer and was histopathologically confirmed as insulinoma by an experienced pathologist. Patients underwent surgical resection at the Department of Surgery, Klinikum rechts der Isar, Munich, Germany (between 1991 and 2011) (Group 1) and at the Hospital Hautepierre, Strasbourg (between 1994 and 2007) (Group 2). Tissue specimens were transferred into liquid nitrogen and stored until further processing for mRNA extraction,

embedded in Tissue-Tek (Sakura, Labonord) and stored at -80°C, or fixed in formalin and embedded in paraffin. The median age of patients from Group 1 was 52 years (35 to 82 years, 5 male and 2 female patients) and of patients from Group 2 was 46 years (13 to 69 years, 2 male and 5 female patients). Presence of metastasis was diagnosed in three patients (liver or lymph node n=2, Group 1; liver and lymph node n=1, Group 2).

VEGFA/TNC binding study

Surface Plasmon Resonance Binding experiments were performed on a Biacore 2000 instrument (Biacore Inc.) at 25 °C. VEGFA165 (Millipore) or TNC (Huang et al., 2001) were immobilized at high surface density (around 7000 resonance units) on an activated CM5 chip (Biacore Inc.) using a standard amine-coupling procedure according to the manufacturer's instruction. Soluble molecules were added at a concentration of 10 µg/ml in 10 mm sodium acetate, pH 5.0, and at a flow rate of 5 µl/min for 20 min before addition of 1 M ethanolamine. Soluble TNC (5 - 20µg in 200µl) or VEGFA165 was added to the chip in 10 mm MES, pH 6.0, 150 mm NaCl, 0.005% (v/v) surfactant P20, at a flow rate of 10 µl/min. A blank CM5 chip was used for background correction. 10 mm glycine, pH 2.0, at 100 µl/min for 1 min was used to regenerate the chip surface between two binding experiments. A steady state condition was used to determine the affinity of VEGFA165 for TNC and the affinity of TNC for VEGFA165. The Dissociation constant (Kd) was determined using the 1:1 Langmuir association model as described by the manufacturer.

Statistical analysis and graphical representation

Statistical analysis was performed using GraphPad Prism. For significance of an association (contingency) Fisher's exact test was applied (tumor staging, gene expression, metastasis incidence). Statistical differences were analyzed by unpaired t-test (Gaussian distribution) or nonparametric Mann-Whitney test (no Gaussian distribution). Gaussian data sets with different variances were analyzed by unpaired t-test with Welch's correction. Gaussian distribution was tested by the Shapiro-Wilk normality test. p-values < 0.05 were considered as statistically significant.

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Legends to Supplemental Figures

Fig. S1 TNC expression in RT2 mice, TNC expression vector and impact of transgenic TNC on pancreatic tissue function (A) TNC expression in RT2 islets determined by IF analysis (MTn12 antibody) in tissue sections of 12 week old RT2 mice. In contrast to the absence of TNC from normal islets (N < 0.2 mm diameter), TNC is expressed in 50%, 80% and 100% of hyperplastic (H, 0.2 – 0.5 mm diameter), angiogenic (A, > 0.5 – 1 mm diameter) and tumorigenic islets (T, diameter above 1 mm), respectively. Right panels, dotted lines delineate the islet circumferences. 82 islets (N = 26, H = 34, A = 14, T = 8) of 3 RT2 mice were analyzed. Scale bar, 100 μm. **(B)** Strategy for the generation of the TNC expression vector. The human cDNA (Gherzi et al., 1995) was removed from the HxBL-pBS plasmid (Aukhil et al., 1993) and cloned into the Rip1 expression vector (Hanahan, 1985) for insulin-promoter driven expression of the transgene by using the pcDNA3.1./Hygro(-) plasmid as intermediate vector. The inserted human cDNA sequence comprises 45 nucleotides upstream of the start site and 639 nucleotides downstream of the stop signal. **(C, D, E)** TNC expression analysis in RipTNC **(C),** RT2/TNC **(D)** and RT2/TNCKO pancreatic tissue **(E)** by IHC **(C, D)**, IF **(E)**, scale bar 100 μm. **(F)** No sorting difference of α-glucagon and insulin positive α- and β-cells in pancreatic tissue of wildtype and RipTNC mice was observed as determined by IF. **(G)** Determination of blood glucose levels after an oral glucose tolerance test in 14 RipTNC and 15 wildtype mice. Average ±SEM is presented for each time point.

Fig. S2 Expression analysis, islet quantification, tumor incidence and burden (A, B, J, L, N) Expression of the indicated molecules in RT2 tumor tissue upon IF analysis. Scale bar 100 μm. **(C, D)** Quantification of proliferation according to tumor stage in 12 weeks old mice, **(C)** RT2, N = 9 mice, 99 hyperplastic (H), 37 angiogenic (A) and 14 tumorigenic (T) islets; RT2/TNC, N = 8, H = 71, A = 47, T = 22, **(D)** RT2, N = 6, H = 73, A = 38, T = 20; RT2/TNCKO, N = 6, H = 72, A = 40, T = 25. **(C)** In RT2/TNC mice a significant 1.5 - fold increase in proliferation in hyperplastic islets is observed. **(D)** a

significant increase in proliferation in hyperplastic (1.4 - fold) and angiogenic (1.6 - fold) islets from RT2/TNCKO mice is observed. **(E, F)** Quantification of apoptosis in 12 weeks old mice, **(E)** RT2, FALK: N = 6 mice, H = 58, A = 19, T = 10; RT2/TNC, N = 8, H = 59, A = 43, T = 21, **(F)** RT2, N = 4, H = 56, A = 26, T = 13; RT2/TNCKO, N = 4, H = 72, A = 40, T = 25. In hyperplastic islets of RT2/TNC mice a significant 2.9-fold decrease in apoptosis is observed, while no significant differences were seen in RT2/TNCKO mice. **(G, H)** Tumor incidence and burden, **(G)** RT2, N = 33 mice, RT2/TNC, N = 26, **(H)** RT2, N = 28, RT2/TNCKO, N = 31. Differences were not statistically significant. **(J)** Image of isolated angiogenic and non-angiogenic islets of an 8 week old RT2 mouse. Scale bar 500 µm. Red arrows: angiogenic islets, blue arrows: non-angiogenic islets. **(K)** Representative SEM pictures from RT2 (N = 5 mice) and RT2/TNC tumors (N = 3). **(M)** Quantification of NG2, a marker for pericytes; area fraction in 12 weeks old RT2 (N = 6 mice, n = 155 islets) and RT2/TNC (N = 8 mice, n = 204 islets) mice. A significant 1.2 - fold increase of NG2 area fraction is observed in RT2/TNC islets. **(O, P)** Quantification of fibrinogen, a marker of vessel leakiness; area fraction according to tumor stage in 12 weeks old mice, **(O)** RT2, N = 5 mice, H = 36, A = 18, T = 8; RT2/TNC, N = 3, H = 26, A = 20, T = 4; **(P)** RT2, N = 4, H = 35, A = 18, T = 7; RT2/TNCKO, N = 5, H = 59, A = 47, T = 9. **(O)** In hyperplastic islets of RT2/TNC mice an increased fibrinogen leakiness is observed (1.5 - fold, p = 0.06). **(P)** In hyperplastic (1.75 - fold) and angiogenic islets (1.45 - fold) of RT2/TNCKO mice, a significant decrease of fibrinogen area fraction is observed. Error bars represent SEM and asterisks (*) indicate p values < 0.05.

Fig. S3 Liver micrometastasis in RT2 mice **(A)** Detection of insulin expressing tumor cells in liver tissue of a RT2 mouse by IF (upper panel) and H&E of a neighboring section (lower panel). Scale bar, 50 µm. **(B, C)** Quantification of insulin expression in liver tissue of RT2 mice. Insulin expression was detected in RT2/TNC (7/24) and RT2 (6/24) **(B)** and RT2/TNCKO (5/10) and RT2/TNC+/- littermates (6/8) **(C)**. Differences were not statistically significant. Error bars represent SEM.

Fig. S4 Impact of TNC on the expression of Wnt inhibitors, DKK1 downregulation and activation of Wnt signaling in tumor cells **(A)** Expression of Wnt inhibitors in T98G, KRIB and MDAMB-435 (MDA) cells as determined by qRTPCR upon plating on the indicated substrata for 24h (KRIB, MDAMB-435) or 48h (T98G). There was no expression of SFRP2 nor DKK4 detectable in any of the cell lines and conditions tested. **(B)** DKK1 expression (qRTPCR) upon plating T98G cells on FN/TNC and FN for the indicated time. DKK1 expression on FN/TNC is represented relative to its expression on FN. **(C)** Reduced DKK1 protein levels by TNC in T98G or KRIB cells upon plating for the indicated time as determined by immunoblotting. **(D, E)** Reduced DKK1 expression upon shRNA mediated DKK1 knockdown as determined by qRTPCR **(D)** and immunoblotting **(E)** in KRIB Sh-DKK1 cells in comparison to KRIB Sh-control (CTR) cells. **(F)** Expression of murine DKK1 in control (CTR) and mDKK1 overexpressing KRIB cells. Overexpression of murine DKK1 in KRIB:mDKK1 cells. Lysates of KRIB cells expressing His-tagged mDKK1 or empty vector control were analyzed by immunoblotting with antibodies against DKK1 or the His-tag. (**G)** Expression of murine DKK1 in the conditioned media (CM) from control (CTR) and mDKK1 overexpressing KRIB cells. Supernatants from the KRIB cells expressing His-tagged mDKK1 or empty vector control were analyzed by immunoblotting. **(H)** Addition of DKK1 containing CM assessed by immunoblotting inhibits TOPFlash activity in KRIB cells in a dose dependent manner. KRIB cells were plated for 48h and treated with increasing dilutions of CM from KRIB:mDKK1 cells. Data are from at least 3 independent experiments, except for (**H**). Error bars represent SEM and asterisks (*) indicate p values < 0.05.

Fig. S5 Mechansim of DKK1 downregulation

(A) Serum starved T98G cells were seeded on FN or FN/TNC, after 30 minutes 5 µg/ml Actinomycin D was added and cells were lysed after 30, 60 or 90 minutes of treatment for analysis of DKK1 mRNA expression. **(B)** Serum starved T98G were treated with the indicated concentrations of LPA. IF staining of vincullin (green) and phalloidin (red). **(C, D)** SRF and **(E, F)** DKK1 mRNA expression of serum starved T98G and KRIB cells treated with the indicated concentrations of LPA. **(G-L)** T98G cells were transfected with RhoA wt, RhoA

Q63L (CA) or RhoA T19N (DN). **(G, H)** Overexpression of RhoA was validated by immunoblotting **(G)** and qRTPCR **(H)**. **(I, J)** SRF mRNA expression **(I)** and SRF Luciferase activity of T98G cells **(J)**. **(K, L)** DKK1 mRNA expression analysed by qRTPCR of cells seeded on uncoated **(K)** or FN and FN/TNC coated dishes **(L)**. **(M)** RTPCR for chicken syndesmos of T98G CTR and T98G:syndesmos cells. **(N, O)** TPM1 mRNA levels analysed by qRTPCR in T98G:TPM1 **(N),** T98G:shTPM1 **(O)** and control cells. **(P)** IF of vinculin (green) and phalloidin (red) in T98G control, T98G:TPM1, T98G:syndesmos and T98G:shTPM1 cells. Error bars represent SEM and asterisks (*) indicate p values < 0.05.

Fig. S6 DKK1 expression in KRIB:mDKK1 tumors and impact of DKK1 overexpression on tumor cell proliferation and migration **(A)** Quantification of murine DKK1 gene expression by qRTPCR in control or mDKK1 overexpressing KRIB derived tumors. **(B)** DKK1 does not change tumor cell proliferation *in vitro*. Proliferation of KRIB (parental) and KRIB:mDKK1 cells was analyzed with a MTS Assay. Data are normalized in each group to values of day 1. **(C)** DKK1 does not change tumor cell proliferation *in vivo*. Proliferating cells were quantified in tumors derived from KRIB control or KRIB:mDKK1 cells. Ki67 positive areas were determined using ImageJ software upon staining for Ki67 and reported to the DAPI positive areas per tumor. No significant (ns) difference was observed (n = 5 per group). **(D)** HUVEC tubulogenesis on matrigel upon addition of CM derived from KRIB control or DKK1 overexpressing cells. N = 5. Phase contrast picture. Error bars represent SEM and asterisks (*) indicate p values < 0.05.

Fig. S7 Binding of TNC to VEGFA Binding of VEGFA to TNC was determined by Biacore including normalization to a blank surface. Binding of TNC and VEGFA to a sensorchip adsorbed with VEGFA **(A)** and TNC **(B),** respectively is shown. We observed that VEGFA and TNC bind to each other in a dose dependent manner with a Kd of 2.7×10^{-7} M (TNC binding to VEGFA) and 1.5×10^{-9} M (VEGFA binding to TNC) which is lower than VEGFA

binding to its receptor (3.3×10^{-11} M) but in the range of a VEGFA/glycosaminogycan interaction (2.4×10^{-8} M) (Wu et al., 2009).

Fig. S1

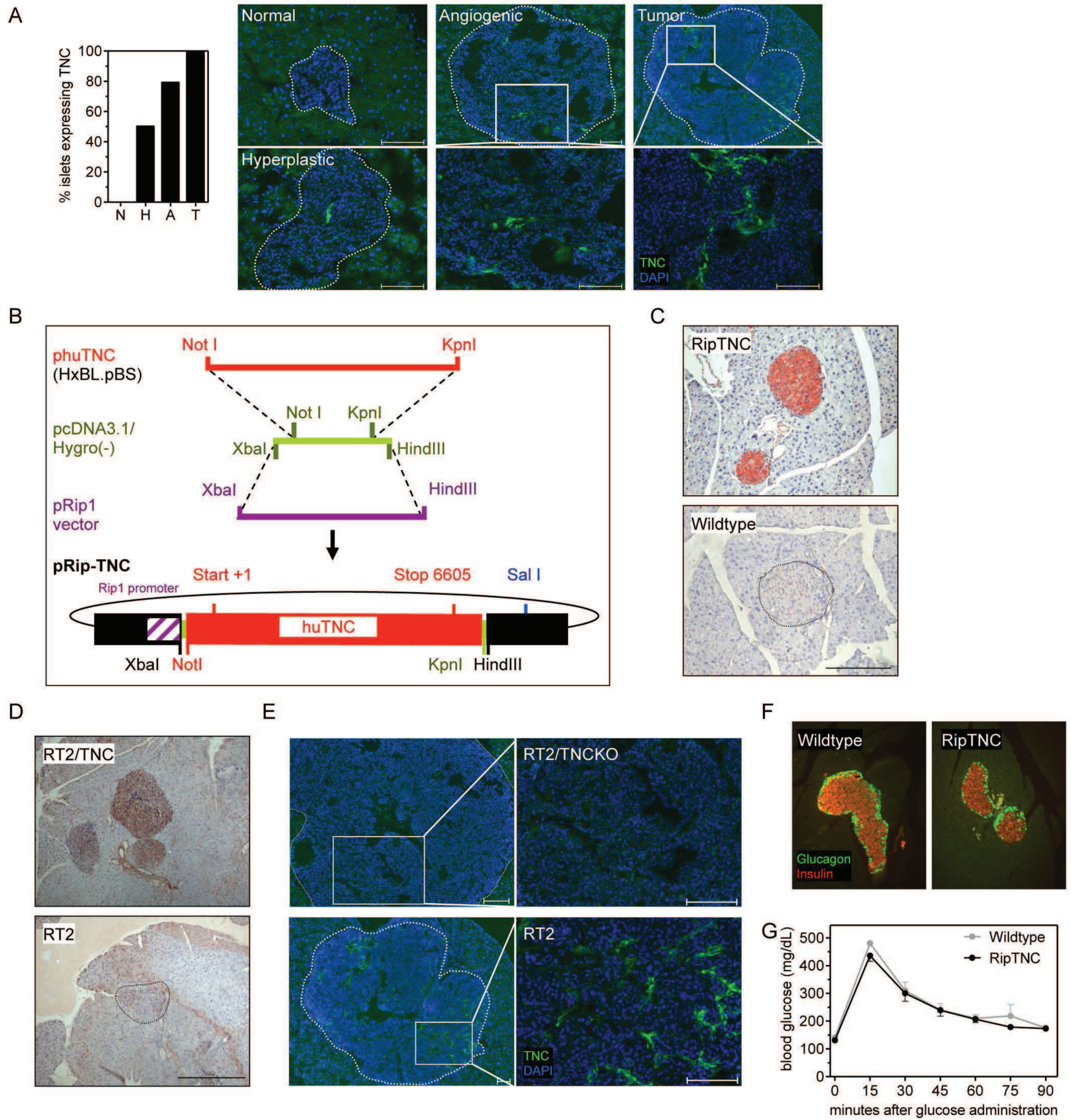


Fig. S2

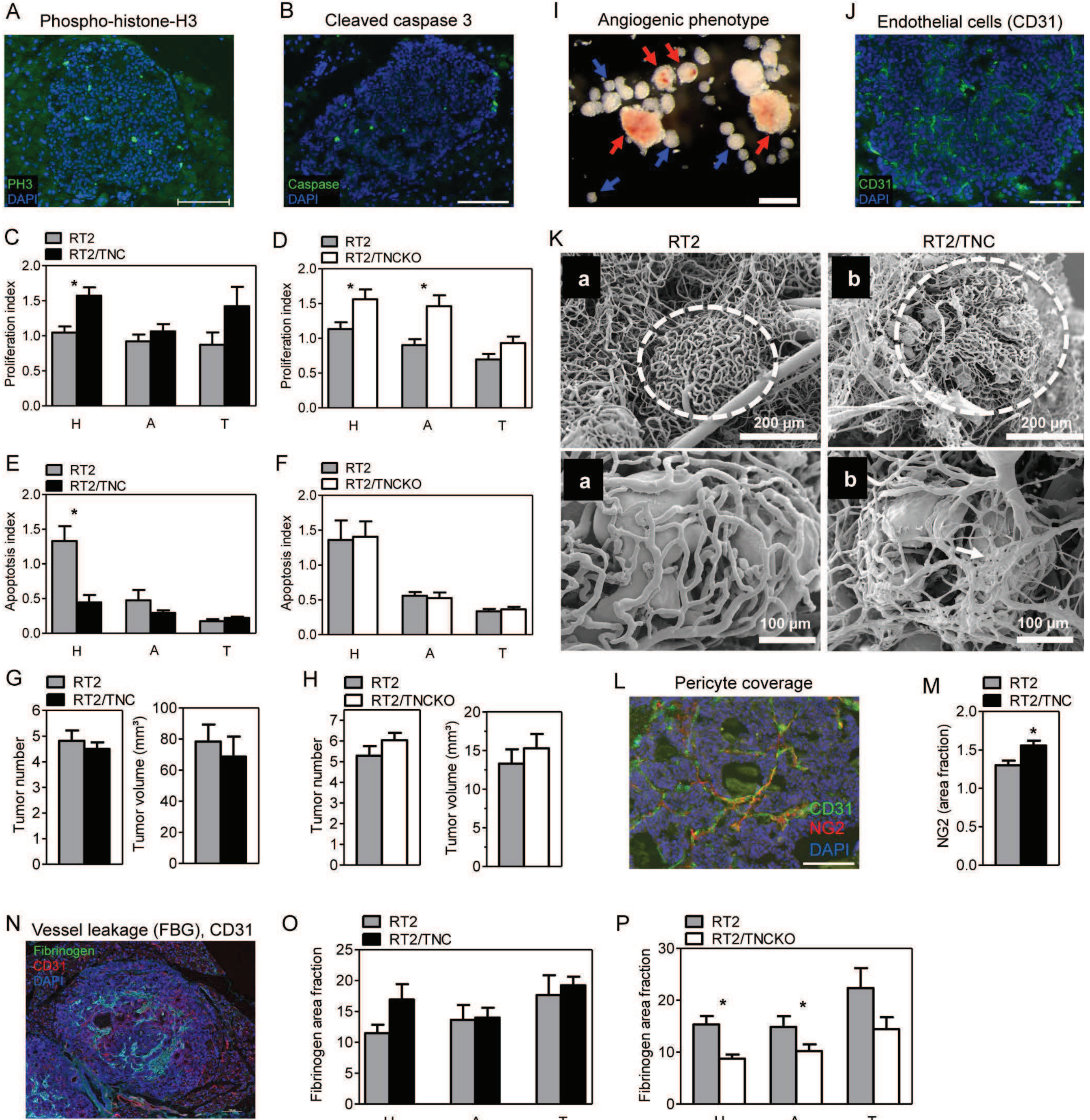


Fig. S3

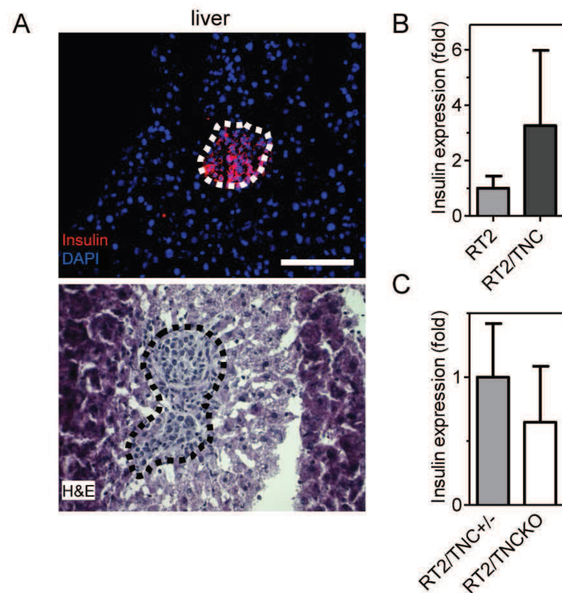
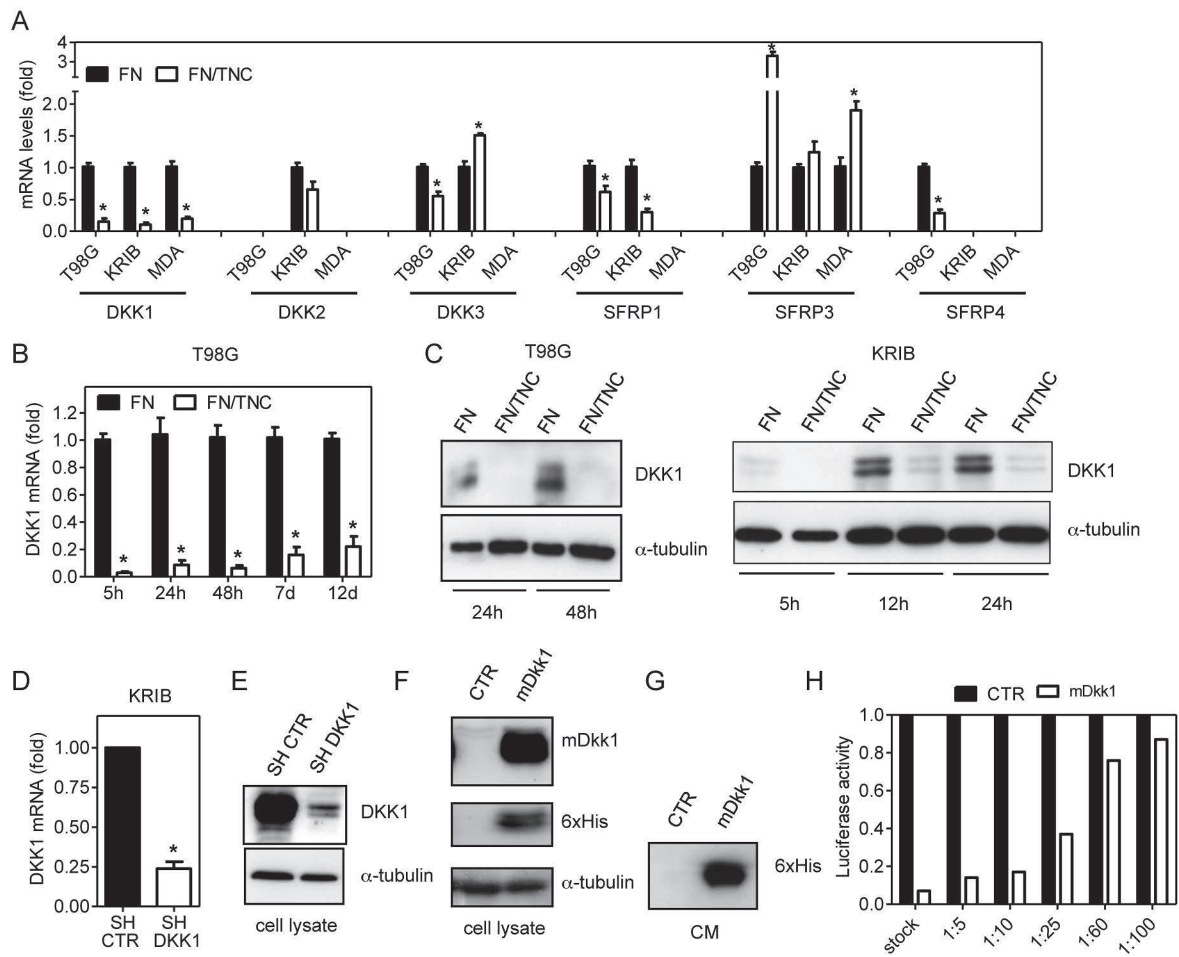


Fig. S4



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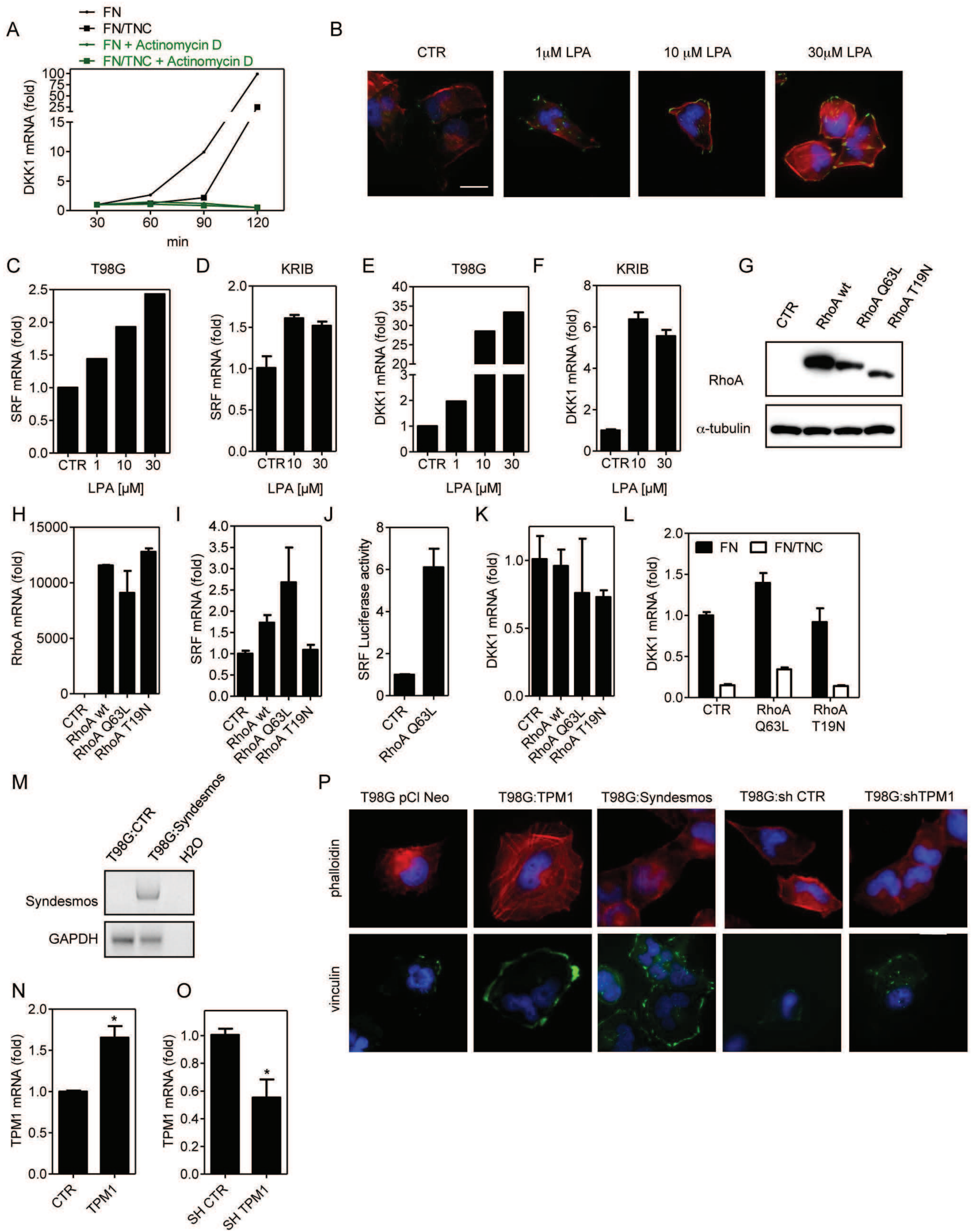

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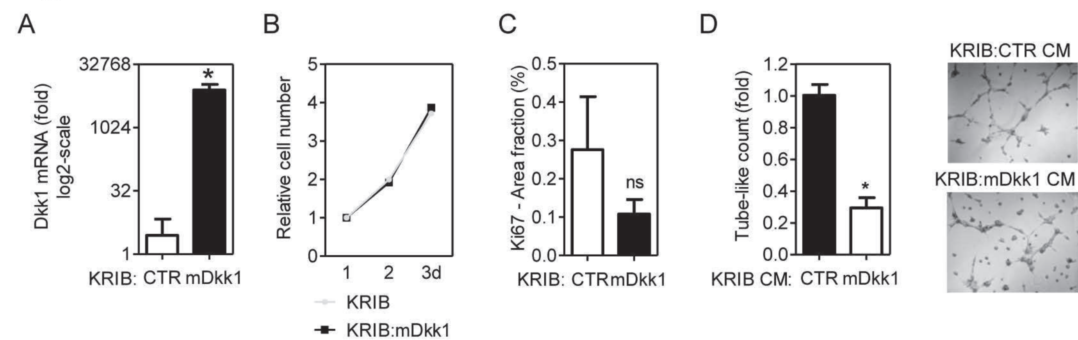

Fig. S7

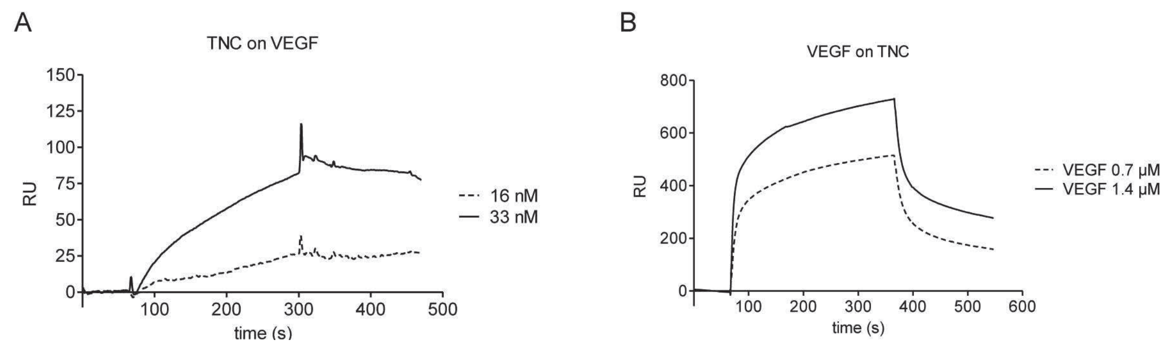

Table S1 Carcinoma progression by TNC

Genotype	Adenoma (%)	Carcinoma (%)				
		Grade 1	Grade 2	Grade 3	Grade 1 - 3	Ca/Ad
RT2	55.8	32.7	10.5	0.9	44.2	0.8
RT2/TNC	35.7	39.9	21.3	3.2	64.3	1.8

Numbers of adenomas and carcinomas Grade 1 to 3 in 12 week old mice. Average frequency of each tumor grade per mouse is displayed; 26 RT2 mice (78 adenomas, 79 carcinomas) and 22 RT2/TNC mice (44 adenomas, 76 carcinomas). p = 0.0378, Fisher‘s exact test. RT2/TNC mice developed 1.8-fold more carcinomas than adenomas (p = 0.001, Student’s t-test) compared to RT2 mice (0.8-fold, p = 0.9454, Student’s t-test).

Table S2 TNC dependent angiogenic switch

Genotype	All islets			Islets per mouse	
	A plus NA	A	NA	A	NA
RT2 (n = 9)	826	71	755	7.9 (±2.2)	83.9 (±7.4)
RT2/TNC (n = 7)	810	136	674	19.4 (±4.3)	96.3 (±10.0)
RT2/TNC versus RT2 (fold)				2.46	1.15
p-value		< 0.0001 [a]		0.0226 [b]	0.3248 [b]
RT2 (n = 7)	809	255	554	36.4 (±5.0)	79.1 (±8.6)
RT2/TNCKO (n = 7)	840	87	753	12.4 (±2.6)	107.6 (±11.8)
RT2/TNCKO versus RT2 (fold)				- 2.94	1.36
p-value		< 0.0001 [a]		0.0011 [c]	0.2008 [b]

Angiogenic (A) and non-angiogenic (NA) islets were isolated from 8 week old RT2 mice with the indicated genotypes and were quantified (average number (± SEM) per mouse). Islets from RT2 littermates were prepared independently in both series of experiments. (a) Fisher's exact test, (b) Student's t-test, (c) Mann Whitney test. Note that differences for RT2 controls originate from inherent experimental conditions (e.g. efficiency of collagenase treatment).

Table S3 Gene expression analysis of RT2 and RT2/TNC tumors

Gene	Tumor class	Relative expression	p-value	Gene	Tumor class	Relative expression	p-value
Axin2	All	**1.35**	0.008	**Dll4**	All	1.66	0.153
	Small	**1.30**	0.034		Small	1.55	0.186
	Small+Diff	1.27	0.055		Small+Diff	1.43	0.258
	Big	1.49	0.194		Big	1.93	0.376
	Big+Diff	1.18	0.571		Big+Diff	1.61	0.786
	Diff	**1.25**	0.037		Diff	1.45	0.194
CD44	All	1.64	0.225	**Hey-1**	All	-1.27	0.134
	Small	1.72	0.077		Small	-1.02	0.911
	Small+Diff	**2.06**	0.029		Small+Diff	1.03	0.843
	Big	-1.63	0.133		Big	**-3.08**	0.019
	Big+Diff	-1.58	0.143		Big+Diff	**-2.67**	0.036
	Diff	1.85	0.157		Diff	-1.16	0.343
CyclinD1	All	1.45	0.166	**Lgr5**	All	-6.81	0.931
	Small	1.45	0.113		Small	-1.72	0.477
	Small+Diff	**2.01**	0.037		Small+Diff	-1.73	0.340
	Big	1.17	0.776		Big	-23.06	0.279
	Big+Diff	-1.02	0.571		Big+Diff	-6.74	0.786
	Diff	1.78	0.112		Diff	-2.65	0.528
DKK1	All	**-16.07**	0.035	**Slug**	All	1.28	0.220
	Small	-16.34	0.062		Small	**1.67**	0.010
	Small+Diff	**-4.13**	0.043		Small+Diff	**1.84**	0.004
	Big	-15.49	n.a.		Big	-2.56	0.081
	Big+Diff	-3.19	n.a.		Big+Diff	-2.20	0.294
	Diff	**-3.90**	0.044		Diff	1.46	0.063
DKK2	All	-1.85	0.204				
	Small	-1.46	0.551				
	Small+Diff	-1.35	0.841				
	Big	**-3.69**	0.032				
	Big+Diff	**-4.67**	0.036				
	Diff	-1.85	0.366				

Relative gene expression in RT2/TNC versus RT2 tumors as determined by qRTPCR. RNA was isolated from tumors of 14 week old RT2 (N = 11 mice, n = 27 tumors) and RT2/TNC mice (N = 3, n = 13). Data are presented for all tumors (All) and subgroups : small tumors (Small : 1 – 3 mm in diameter, RT2 (n = 19), RT2/TNC (n = 10)), big tumors (Big : > 3 mm, RT2 (n = 8), RT2/TNC (n = 3)), differentiated tumors (Diff. : high

expression of insulin and E-cadherin, RT2 (n = 22), RT2/TNC (n = 13)), small and differentiated tumors (Small + Diff : RT2 (n = 17), RT2/TNC (n = 10)) and big and differentiated tumors (Big + Diff, RT2 (n = 5), RT2/TNC (n = 3)). Bold numbers represent statistically significant changes in relative expression, n.a., not applicable due to low sample number.

Table S4 Primer list for qRTPCR on tumor, liver and lung tissue

Gene	Species	Forward primer	Reverse primer
Axin2	mouse	CTGCTGGTCAGGCAGGAG	TGCCAGTTTCTTTGGCTCTT
CD44	mouse	GTCTGCATCGCGGTCAATAG	GGTCTCTGATGGTTCCTTGTTC
CyclinD1	mouse	CGCACTTTCTTTCCAGAGTCA	AAGGGCTTCAATCTGTTCCTG
DKK1	mouse	Taqman (ABI) Mm00438422_m1	
DKK1	mouse	CCGGGAACTACTGCAAAAAT	CCAAGGTTTTCAATGATGCTT
DKK2	mouse	GCCAAACTCAACTCCATCAAG	TCACTGCTGCAAGGGTAGG
Dll4	mouse	AGGTGCCACTTCGGTTACAC	GGGAGAGCAAATGGCTGATA
E-Cadherin	mouse	CAGCCTTCTTTTCGGAAGACT	GGTAGACAGCTCCCTATGACTG
GAPDH	mouse	Taqman (ABI) Mm99999915_g1	
GAPDH	human	ATCTTCTTTTGCGTCGCCAG	AATCCGTTGACTCCGACCTTC
Hey-1	mouse	CATGAAGAGAGCTCACCCAGA	TTGGGGACATGGAACACAG
HMBS	human	Qiagen QT00494130 (for Sybr green)	
Insulin	mouse	TGGCTTCTTCTACACACCCAAG	ACAATGCCACGCTTCTGCC
Insulin	mouse	Taqman mIns1 Mm01259683_g1	
Lgr5	mouse	GGAAAGAAATGCTTTGATGGAC	AGTGGGGAATTCATCAAGGTT
RPL19	mouse	ACCCTGGCCCGACGG	TACCCTTCCTCTTCCCTATGCC
Slug	mouse	GAAAAGCACATTGCATCTTTTCT	TGTTCCTTTGGTTGAAATGGT
TBP	mouse	CCCCACAACTCTTCCATTCT	GCAGGAGTGATAGGGGTCAT
TNC	human	GTCACCGTGTCAACCTGATG	GTTAACGCCCTGACTGTGGT

Table S5 Primer list for qRTPCR on cultured cells

Gene	Forward primer	Reverse primer
Axin2	CCACACCCTTCTCCAATCC	TGCCAGTTTCTTTGGCTCTT
DKK-1	GACCATTGACAACTACCAGCCG	TACTCATCAGTGCCGCACTCCT
DKK-2	GGCAGTAAGAAGGGCAAAAA	CCTCCCAACTTCACACTCCT
DKK-3	GAGGACACGCAGCACAAA	TGCCAGGTTCACTTCTGATG
DKK-4	AGGAGGTGCCAGCGAGAT	CATCTTCCATCGTAGTACAAACATC
SFRP1	GCTGGAGCACGAGACCAT	TGGCAGTTCTTGTTGAGCA
SFRP2	GCTTGAGTGCGACCGTTT	CAGGCTTCACATACCTTTGGA
SFRP3/FRZB	GGGCTATGAAGATGAGGAACG	CTGAGTCCAAGATGACGAAGC
SFRP4	CGATCGGTGCAAGTGTAAAA	ACCACCGTTGTGACCTCATT
SRF	AGACGGGCATCATGAAGAAG	TGATCATGGGCTGCAGTTT
Tropomyosin 1	CCCGTAAGCTGGTCATCATC	CTTGTGTGCTCATCATTCCGA
RhoA (Sauzeau et al., 2003)	GCAGGTAGAGTTGGCTTTATGG	CTTGTGTGCTCATCATTCCGA
β2-Microglobulin	GTGGGATCGAGACATGTAAGCA	AATGCGGCATCTTCAAACCT

The extracellular matrix and cancer: regulation of tumor cell biology by tenascin-C

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Running Head: Tenascin-C and Cancer Cell Biology

1 Introduction

The environment within which cancer cells exist is a key determinant of tumor survival and growth. The extracellular matrix that comprises the tumor stroma provides vital cues that control cell phenotype and enable the tumor to thrive. Moreover, emerging evidence reveals the importance of the extracellular matrix in permitting colonization of secondary tumor sites by creating a specific environmental niche tailored to enhance metastatic cell survival. Tenascin-C is an extracellular matrix glycoprotein whose expression is specifically induced in many different types of cancer and where it drives processes such as tumor cell growth, angiogenesis, immune modulation and metastatic fitness. Here, we review the molecular mechanisms underlying the pleiotrophic role of tenascin-C in disease pathology and highlight the impact of these data on the development of new strategies to diagnose and treat cancer.

The extracellular matrix (ECM) is a complex 3D network of secreted molecules that provides structural support to tissues and which dynamically and reciprocally communicates with cells in order to regulate cell behaviour. Established during embryonic development to guide morphogenesis and maintained in adults to define tissue architecture and to support tissue homeostasis, it has long been established that the ECM undergoes profound changes during tumorigenesis. More recently, the idea that the expression, or re-expression, of specific matrix molecules in cancer forms a tumorigenic niche creating an environment that supports tumor growth has emerged. This environment impacts the phenotype of both tumor cells and stromal cells enabling survival and expansion of the primary tumor, as well as mediating the escape of metastatic tumor cells and their colonization of new sites at distinct and specific tissue locations. The ECM does this by virtue of a number of functions; it serves to anchor cells, it can provide either a migration barrier or a migration track to control cell movement, it directly interacts with the cell to provide external cues, it acts as a reservoir for soluble signaling molecules controlling their localization and concentration, often additionally serving as a co-receptor or presenter of these molecules at the cell surface, and finally it defines the physical properties of the tissue providing biomechanical cues to modulate cell behavior (reviewed by (Lu et al., 2012).

Tenascin-C is a large hexameric extracellular glycoprotein. It was discovered nearly 30 years ago by a number of independent labs (Bourdon et al., 1983; Chiquet and Fambrough, 1984; Erickson and Inglesias, 1984; Grumet et al., 1985). Identified as the antigen to monoclonal antibody 81C6, raised against glial fibrillary acidic protein (GFAP)-positive glioma cells (Erickson and Taylor, 1987), high levels of tenascin-C were detected in the tumor stroma and around the vasculature of glioma tissue (Bourdon et al., 1983). Indeed, tenascin-C can be

purified from human glioblastoma cultures, with yields reported up to 10 mg from 1 L of conditioned medium (Aukhil et al., 1990). The expression of tenascin-C in glioma, but not in normal brain, highlighted its potential use as a much needed tumor specific marker and only 5 years later, the antibody 81C6 was shown to specifically deliver cytotoxic radioisotopes to subcutaneous human glioma xenografts in mice or intracranial human glioma xenografts in rats with significant therapeutic effect (Lee et al., 1988a; Lee et al., 1988b). Since then, the distinct pattern of expression of tenascin-C has become increasingly well characterized and its use both as a diagnostic and prognostic marker for a number of different cancers has become increasingly sophisticated. Moreover, promising results in phase II clinical trials targeting tenascin-C as a unique therapeutic modality to treat some types of tumor have been reported. Here we review the recent advances in this field, highlighting new ideas that have emerged about the expression of tenascin-C in cancer, in addition to what is known about how this protein contributes to tumorigenesis from within the ECM.

1.1 Tenascin-C Structure

The human *tenascin*-C gene is located on the antisense strand of chromosome 9q33.1, flanked by the *DEC1* and *TNFSF8* genes, separated by ~23 kb and ~90 kb of intergenic sequences, respectively. The *tenascin*-C gene is relatively large, spanning 97,680 nucleotides and comprising 30 exons (Gherzi et al., 1995; Mighell et al., 1997; Sriramarao and Bourdon, 1993). The first exon is untranslated, with translation starting in exon 2. The exons range in size from 90 to 1410 nucleotides and the introns from 578 to 26,827 nucleotides. The mature mRNA transcript is 8150 nucleotides long, and the entire protein-coding region comprises 7500 nucleotides (Goh et al., 2010). This RNA encodes a protein of a maximal length of 2385 amino acids (Hancox et al., 2009; Jones et al., 1989; Pas et al., 2006).

The tenascin-C protein comprises a number of distinct structural domains. The N-terminal stretch of 110 residues is unique to tenascin and is followed by a short heptad repeat region. The adjacent epidermal growth factor (EGF)-like repeats, of which there are 14.5 in human tenascin-C, are 30 to 50 amino acids long and each contain six cysteine residues that mediate intrachain disulfide bonds. Next to these modules lie up to 17 fibronectin type III like domains (TNIII), which are ~90 amino acids long each and form two sheets of seven antiparallel β-strands. The number of TNIII domains is generated by alternative splicing; at least nine different TNIII domains are differentially included or excluded by RNA splicing. Finally, the C-terminal fibrinogen-like globular (FBG) domain is 210 amino acids long and forms intrachain disulfide bonds (figure 1A). Tenascin-C possesses 23 potential glycosylation sites, two in the heptad region, two in the EGF repeats, 18 in the TNIII repeats and one in the FBG domain. Tenascin-C purified from human glioma cells is glycosylated (Taylor et al., 1989) and this modification, together with alternative splicing, determines the molecular weight of the tenascin-C monomer that can range between 190 and 330 kDa.

Tenascin-C monomers are assembled into a hexameric form commonly called a hexabrachion (figure 1B). The molecular weight of the hexamer was calculated to be 1.9×10^6 Da by sedimentation equilibrium analysis and by electrophoresis on non-reducing agarose gels (Taylor et al., 1989). Pulse chase data indicate that nascent tenascin-C polypeptides rapidly assemble into hexamers intracellularly, even prior to completion of translation, and that this is followed by slower transport to the Golgi before secretion (Redick and Schwarzbauer, 1995). Hexamer assembly comprises a sequential two-step process. Trimerization is first mediated by the formation of a parallel three stranded α-helical coiled coil by sequences in the heptad repeats between alanine 114 and glutamine 139. This is followed by the connection of two trimers by sequences N-terminal to the heptad repeats (Kammerer et al., 1998). Cysteine residues flanking this region are also important in tenascin-C multimerization: mutation of cysteine 64 abolishes trimer dimerization (Luczak et al., 1998) and cysteines 111 and 113 are thought to further stabilize native tenascin-C by mediating interchain disulfide bonds (Kammerer et al., 1998). Secreted hexameric tenascin-C can then be further assembled into a 3D matrix at the cell surface, creating a dense pericellular localization of fibrillar tenascin-C (Chung and Erickson, 1997; Chung et al., 1995).

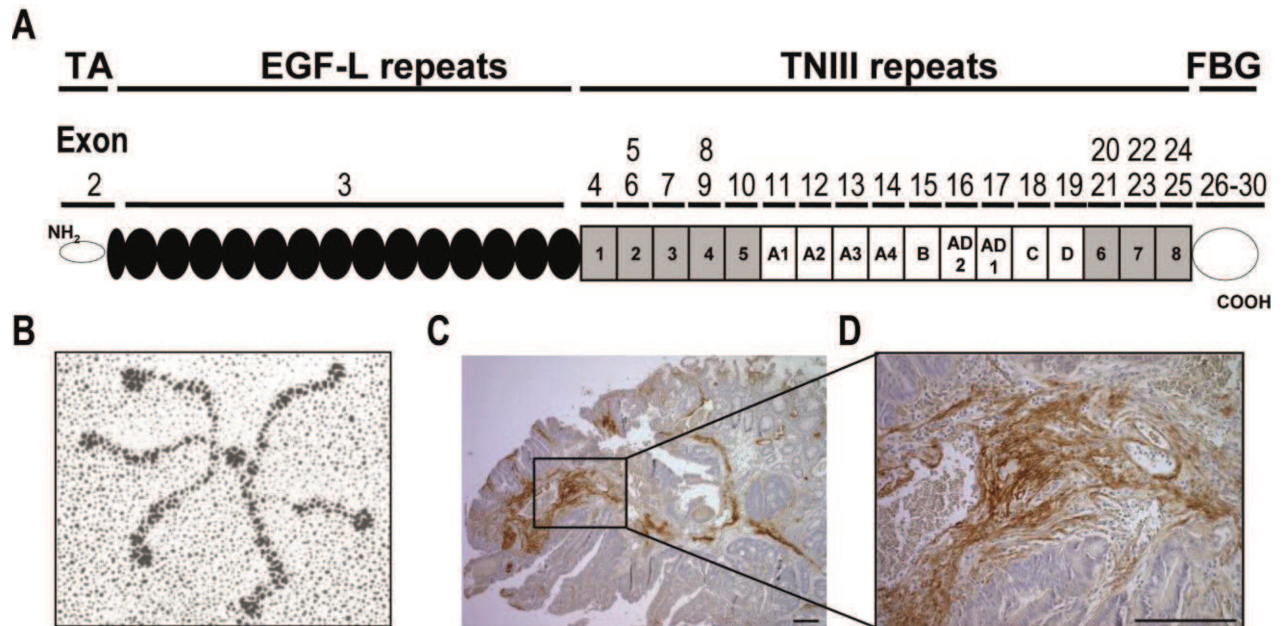

Figure 1. Tenascin-C structure and expression in the tumor stroma. (A) The exon/intron structure of the human tenascin-C gene and the organization into the different protein domains is depicted schematically. Information on the exon/intron organization is based on analysis of sequence entries in the EMBL sequence data bank. The multimodular organization of tenascin-C protein is shown comprising four distinct domains: an assembly domain (TA), a series of epidermal growth factor-like repeats (EGF-L), a series of fibronectin type III-like repeats (TNIII), and a C-terminal fibrinogen-like globe (FBG). Constitutively expressed TNIII repeats (1-8) are shown in grey and alternatively spliced TNIII repeats (A1-D) in white. (B) The appearance of purified tenascin-C protein as a hexamer upon electron microscopy. Image modified from (Midwood and Orend, 2009). (C, D) Tenascin-C in human colorectal cancer tissue visualized by immunohistochemistry using antibody B28-13. Scale bars = 200 µm.

1.2 Tenascin-C Expression in Tumors

Highly expressed during development, tenascin-C synthesis is down regulated postnatally and little or no tenascin-C is detected in most healthy adult tissues. It is transiently re-expressed upon tissue injury, but only for the duration of tissue repair (reviewed in (Midwood and Orend, 2009; Udalova et al., 2011). Tenascin-C is also highly expressed in glioma tissue, as originally observed in 1983, as well as in other types of cancer, but here expression persists throughout disease. Most malignant solid tumors exhibit high levels of tenascin-C including brain, breast, uterus, ovaries, prostate, pancreas, colon, stomach, mouth, larynx, lung, liver, kidney, bladder, skin and bone cancers, as well as cancers of soft tissues, and lymphomas. These data were extensively reviewed in 2006 and a detailed list of the expression of tenascin-C in tumors up to that point can be found in (Orend and Chiquet-Ehrismann, 2006). The majority of these reports, and further studies in the last 6 years, have examined tenascin-C protein expression histologically. In many cases increased tenascin-C in the ECM of the tumor was observed (for example figure 1C, D). Moreover, in cancers such as glioma, lung carcinoma and osteosarcoma, high tenascin-C expression correlated with low survival rates. Table 1 highlights cancers for which high stromal tenascin-C expression correlates with advanced disease activity or poor patient prognosis.

Tumor type	Correlation of tenascin-C expression	Reference
Adrenal pheochromocytoma	Prediction of aggressiveness	(Salmenkivi et al., 2001)
Astrocytoma	Correlation with higher tumor grade and proliferation.	(Zagzag et al., 1995)
	Correlation with degree of histological malignancy	(Higuchi et al., 1993)
Astrocytoma, oligodendrocytoma, gliobalstoma	Elevated compared to normal tissue	(Brellier et al., 2011b)
Bladder carcinoma	Correlation with shorter survival	(Brunner et al., 2004)
Breast carcinoma	Associated with malignancy	(Mackie et al., 1987)
	Correlation with vascular grade and early invasion	(Tokes et al., 1999), (Jahkola et al., 1998a)
	In axillary node negative cases expression at invasion boarder serves as prognostic factor for local recurrence	(Jahkola et al., 1998b)
	Correlation with lymph node metastasis and poor outcome	(Ishihara et al., 1995)
	Inverse correlation with receptor status, correlation with lymph node status and tumor grade, marker for negative prognostic value in invasive cancer	(Ioachim et al., 2002)
	Correlation with shorter survival of tamoxifen-treated patients with estrogen-positive breast cancer	(Helleman et al., 2008)
	Correlates with aggressiveness of lung metastases	(Oskarsson et al., 2011)
Breast carcinoma, adenoses, fibroadenoma	Diagnosis of malignant disease and prediction of invasive potential of premalignant lesions	(Goepel et al., 2000)
Colorectal carcinoma	Correlation with advanced stage and shorter survival	(Sis et al., 2004)
	Correlation with progression and metastatic spread	(Emoto et al., 2001)
	Correlation with lymph node metastasis	(Riedl et al., 1992)
	Correlation with poor prognosis	(Sugawara et al., 1991)
Ependymoma	Prediction of recurrence	(Korshunov et al., 2000)
	Correlation with decreased progression-free survival	(Zamecnik et al., 2004)
Endometrial carcinoma	Correlation with metastasis, muscle and vascular invasion	(Doi et al., 1996)
Giant cell tumors of bone	Predictive of local recurrence and metastasis	(Pazzaglia et al., 2010)
Glioma	Correlation with shorter survival	(Leins et al., 2003)
	Prognostic for early tumor recurrence	(Herold-Mende et al., 2002)
	Correlation with macrophagic/microglial infiltration	(Kulla et al., 2000)
	Correlation with vascular proliferation and malignancy	(Oz et al., 2000)
Laryngeal and hypopharyngeal carcinoma	Correlation with metastases, tumor recurrence and lethality	(Juhasz et al., 2000)
Medulloblastoma	Correlation with poor prognosis	(Korshunov et al., 1999)

Non-small cell lung carcinoma	Correlates with microvessel density and shorter survival	(Ishiwata et al., 2005)
	Predicts malignancy	(Kahn et al., 2012)
Oral squamous carcinoma	Indicative of poor prognosis	(Lyons and Jones, 2007)
	Predicts poor prognosis and survival	(Wang et al., 2010b)
Osteosarcoma	Correlation with metastasis and poor survival	(Tanaka et al., 2000)
Papilla Vater carcinoma	Correlation with poor prognosis	(Vaidya et al., 1996)
Peripheral nerve sheath tumor	Distinguishes malignant from benign tumors	(Dugu et al., 2010)
Pleural mesothelioma	Correlation with malignancy and shorter survival	(Kaarteenaho-Wiik et al., 2003)
Primary melanoma	Absence of tenascin-C at invasion front correlates with lower risk for metastasis	(Ilmonen et al., 2004)
Prostate carcinoma	Little staining in high grade tumors but in well and moderately differentiated staining correlates with tumor progression	(Xue et al., 1998a; Xue et al., 1998b)
Renal cell carcinoma (clear cell)	Associated with higher stage and nuclear grade and lower survival and predictor of metastatic potential	(Ohno et al., 2008)
Salivary gland tumors	Correlation with metastasis	(Felix et al., 2004)
	Marker for recurrent disease in benign tumors	(Karja et al., 1995)
Urothelial carcinoma	Correlation with grade, stage and proliferative activity and shorter survival	(Ioachim et al., 2005)
	Expression higher in more agressive micropapillary variant	(Ishii et al., 2011)
Vulvar carcinoma	Marker of malignancy and correlation with inflammation	(Goepel et al., 2003)

Table 1. Stromal expression of tenascin-C protein as a diagnostic or prognostic marker of cancer

However, expression of tenascin-C is not universally associated with poor prognosis. For example, no correlation with clinical history or neurological function in pediatric supratentorial glioblastoma multiforme could be found (Germano et al., 2000). Likewise there appears to be no correlation between stromal expression of tenascin-C and tumor stage or prognosis in stomach adenocarcinoma (Zirbes et al., 1999), nor with invasion, metastasis or survival in gastric carcinoma ((Ikeda et al., 1995); Ilunga and Iriyama, 1995). Tenascin-C is not a predictor for survival in oral and pharyngeal squamous cell carcinomas (Atula et al., 2003), nor does it correlate with survival, clinical stage or metastasis in pancreatic carcinoma (Juuti et al., 2004). Furthermore, poor prognosis has been reported for cases of cervical carcinoma that exhibited no staining of stromal tenascin-C (Pilch et al., 1999). Moreover, opposing data have emerged from independent studies of the same type of cancer. In contrast to studies that report high tenascin-C expression correlating with a low survival prognosis in breast cell carcinoma (see table 1), other studies found no correlation between tenascin-C expression and prognostic factors such as p53, Ki-67 and estrogen receptor status (Tokes et al., 1999), nor with long term survival (Iskaros et al., 1998; Melis et al., 1997). Some studies have even reported a positive correlation of tenascin-C expression with relapse free and overall survival (Suwiwat et al., 2004) and better prognosis (Shoji et al., 1993) in breast cancer patients. The expression and role of tenascin-C in breast cancer has recently been reviewed (Guttery et

al., 2010b). Likewise whilst some data show tenascin-C to be predictive of poor prognosis and shorter survival in colorectal cancer (Sis et al., 2004; Sugawara et al., 1991), other data show tenascin-C correlates with better long-term survival (Iskaros et al., 1997). The reason for these differences is not clear and may depend on patient sampling and methods of disease assessment. What is clear is that the link between tenascin-C expression and each individual cancer is not always a straightforward or linear relationship. Indeed, in some cancers, whilst tenascin-C expression does not correlate with any clinical parameter, it is significantly linked to a specific aspect of tumor cell biology. For example, in condyloma and intraepithelial neoplasias, there is no link between tenascin-C expression in the tumor stroma and hyperplasia but it was positively associated with inflammation (Pollanen et al., 1996). Similarly, tenascin-C has been shown to be a reliable marker for epithelial proliferation in endometrial adenocarcinoma (Vollmer et al., 1990) and to associate with tumor cell proliferation in adenoid cystic carcinoma (Shintani et al., 1997).

Interestingly, in squamous cell lung carcinoma, in addition to tenascin-C staining in the ECM of the tumor stroma, intracellular tenascin-C was also reported (Soini et al., 1993) suggesting that tumor cells constitute a cellular source of tenascin-C. In fibrohistiocytic tumors, an increase in cytoplasmic staining in tumor cells and a decrease in ECM staining was observed in malignant disease (Franchi and Santucci, 1996). The significance of this intracellular accumulation of tenascin-C is not clear, nor is it known whether this effect is specific to tenascin-C or whether it reflects a global defect in tumor cell secretion with approaching malignancy.

In addition to the elevated tenascin-C protein expression reported in the tumor stroma, high levels of tenascin-C have been detected in the serum of patients with cancers including stage IV melanoma (Burchardt et al., 2003), soft tissue carcinoma and squamous cell carcinoma. In these patients tenascin-C levels did not correlate with tumor burden (Schenk et al., 1999; Schenk et al., 1995). However, serum levels of tenascin-C were specifically elevated in head and neck squamous cell carcinoma at higher tumor stages and in recurrent disease (Pauli et al., 2002) and in colorectal cancer serum levels did correlate with total tumor burden and metastatic disease (Riedl et al., 1995). Moreover, serum tenascin-C levels were elevated in the cerebrospinal fluid of patients with astrocytic cancer, but not with any other type of brain cancer, and correlated with both the tumor grade and tumor dissemination (Yoshida et al., 1994). These data suggest, as one might expect, that circulating or soluble tenascin-C may provide some information about the ability of certain tumors to spread, rather than revealing the status of the primary tumor.

In addition to examination of tenascin-C protein in cancer, investigation of tenascin-C mRNA has highlighted a number of interesting points. Firstly, these data reveal that the tenascin-C within a tumor may derive from either the tumor cell, the neighbouring stromal cell or from both cell types. For example, in malignant and fibroadenomatosus tumors, elevated tenascin-C transcripts were observed in both tumor and epithelial cells (Lightner et al., 1994). In chondyloma and intraepithelial neoplasias mRNA was found in basal epithelium and in fibroblasts (Pollanen et al., 1996). In breast ductal carcinoma mRNA was detected in cancer cells and stromal cells but in scirrhous carcinoma tenascin-C mRNA was only expressed by stromal cells (Yoshida et al., 1997). Secondly, tenascin-C transcript levels have also been shown to correlate with clinical outcome. For example, in oral squamous cell carcinoma high transcript levels correlated with lymph node metastasis (Nagata et al., 2003), in colon adenoma and carcinoma a correlation between transcript levels and the depth of invasion and frequency of metastasis to the lymph node was noted (Hanamura et al., 1997) and in oral tongue squamous cell carcinoma, RNA extracted from paraffin embedded biopsy tissue was predictive of poor prognosis (Wang et al., 2010b). Furthermore, tenascin-C expression is not limited to cells within the primary tumor site; RT-PCR of RNA isolated from circulating tumor cells in patients with grade I and II colorectal cancer shows a positive correlation between tenascin-C and disease prognosis (Gazzaniga et al., 2005).

More recently, array technology has been used to define tumor specific gene signatures, which frequently contain tenascin-C. Table 2 lists the tumors for which this type of analysis has been performed. In some cases total gene expression in biopsies of the primary tumor was assessed and a unique cluster of genes differentially expressed in the tumor compared to normal tissue was identified. These genes, unrelated by function, family or pathway, were used to reliably predict disease stage or patient survival in giant tumors of

bone (Pazzaglia et al., 2010) or glioblastoma (Colman et al., 2010). Profiling of total gene expression in the endobronchial epithelial lining fluid of non-small cell lung carcinoma also identified a gene signature including tenascin-C that was up-regulated in malignancy compared to normal or benign samples (Kahn et al., 2012). In addition to serving as a marker for disease outcome, transcriptional analyses have also revealed that tenascin-C expression in the primary tumor may be indicative of how patients will respond to treatment. Helleman *et al.* (2008) showed that high tenascin-C expression in patients with estrogen receptor positive breast cancer correlated to shorter survival upon treatment with tamoxifen (Helleman et al., 2008). This study provides the first hint that tenascin-C may be useful defining the most effective treatment for individual patients, as well as in disease diagnosis.

Tumor type	**Gene signature**	**Clinical use**	**Reference**
Astrocytoma	Of 26 ECM specific genes assayed, tenascin-C, brevican, neurocan and phosphoglycan were elevated	Correlated with an invasive phenotype in grade II tumors	(Varga et al., 2012)
Breast cancer	One of 95 genes linked to lung metastasis (48 overexpressed and 47 underexpressed), tenascin-C was elevated	Contributes to metastasis to the lung after xenograft in nude mice	(Minn et al., 2005)
	One of 6 ECM related genes upregulated: collagen 1A1, fibronectin, lysyl oxidase, SPARC, TIMP3 and tenascin-C	Associated with resistance to tamoxifen	(Helleman et al., 2008)
Giant cell tumors of bone	Of 109 differentially expressed genes that correlated with prognosis, tenascin-C was the most significant prognostic biological marker	Predictive for local recurrence and metastasis (4- and 8 fold increased risk respectively)	(Pazzaglia et al., 2010)
Glioblastoma	One of a 38 gene consensus profile: 31 associated with poor prognosis (including tenascin-C) and 7 associated with better prognosis	Predictive for malignancy, associated with mesenchymal differentiation of tumor cells and angiogenesis. Associated with poor prognosis	(Colman et al., 2010)
	One of 31 validated genes	Associated with malignant glioma	(Persson et al., 2007)
Non-small cell lung carcinoma (endobronchial epithelial lining fluid)	Tenascin-C, [C-X-C motif] ligand 14, S100A9, and keratin 17 were upregulated compared to normal subjects and benign samples	Together with nodule size, tenascin-C positivity improved the prediction of malingancy	(Kahn et al., 2012)
Pulmonary adenocarcinoma	up-regulated genes: cyclin B1, polo-like kinase 1, tenascin-C, keratin 8, keratin 19, DAN topoisomerase 2A. down-regulated genes: caveolin 1 and 2, TIMP3, SOCS2 and 3, DOC2 and gravin	Present in tumor tissue but not normal lung tissue	(Wikman et al., 2002)

Table 2. Gene expression signature of tenascin-C+ tumors; relation to diagnosis, prognosis and/or treatment response

Global transcriptomic profiling has also been applied to stromal fibroblasts from areas of prostate cancer or from areas with benign hyperplasia either alone or co-cultured with the human prostate cancer cell line PC-3. Amongst the genes identified, tenascin-C was shown to be down-regulated in tumor cells co-cultured with non-cancerous fibroblasts compared to fibroblasts from the tumor stroma (Reinertsen et al., 2012). These data highlight the importance of the environment on tumor cell behaviour.

Further studies have focused their analysis on specific subsets of genes, comprising only those that code for ECM components, providing a more detailed descriptor of the tumor cell environment. For example, tenascin-C was one of four genes specifically up-regulated in the astrocytoma ECM (Varga et al., 2012). It was also one of 6 ECM genes uniquely expressed in the stroma of breast cancers (Helleman et al., 2008). In both cases the expression of these gene sets were shown to be associated with an invasive cell phenotype and thus reveal the content of an ECM that is particularly permissive to tumor metastasis. These data also emphasize the fact that no ECM protein exists in isolation and suggest that, for those tumors where no clear association with a single molecule has been demonstrated, analysis of a number of molecules may be a more reliable indicator of disease activity or outcome. It would be interesting to perform a global assessment of these ECM signatures in further cancer types to define if a single unifying environment promotes the metastasis of distinct tumor types or if different tumors require tailor made ECMs in order to disseminate.

An elegant series of recent papers has begun to address this question. Their data reveal that metastasis of the same tumor type to different secondary sites is driven by very different molecular mechanisms. Using whole genome derived primer sets, 95 genes were identified that were specifically associated with breast cancer cells that possessed high potential to metastasize to the lungs in nude mice, compared to cells with a low metastatic potential (Minn et al., 2005). This gene set was largely distinct from the gene-expression signature of the same parental breast cancer cell lines that exhibit high potential to metastasize to the brain (Bos et al., 2009)or to bone (Kang et al., 2003) in nude mice. Amongst these gene sets, tenascin-C was specifically enriched in those breast cancer cells that spread to the lungs, but not to either the brain or bone. Most recently, Okarsson *et al.* (2011) confirmed the relevance of these data in human breast cancer. They showed that a high level of tenascin-C in the primary breast tumor was specifically associated with a shorter time to lung relapse (median time from primary tumor diagnosis to lung metastasis was 24 months in cases with high expression of tenascin-C versus 56 months in cases with low tenascin-C). They also showed that tenascin-C expression was particularly high at the invasive edge of metastatic lung nodules and that high tenascin-C expression at this site predicted poor overall survival (median time from metastasis diagnosis to death was 7 months in cases with high tenascin-C expression and 34 months for cases with low tenascin-C). These data highlight the importance of the ECM not only in the growth of the primary tumor but also in driving tumor cell metastasis to specific secondary sites. This paper went on to demonstrate that cancer cell derived tenascin-C was required for survival of the lung metastasis, until stromal tenascin-C could be synthesized as the major source in the secondary lung site (Oskarsson et al., 2011). The mechanism by which tenascin-C promotes tumor cell survival is discussed further in section 3.3 below.

Global analysis of tumor cell attributes has also extended to other ‘omic approaches. miRNA profiling revealed lost expression of miR355 in aggressive breast cancer that metastasizes to the lung. Tenascin-C was identified as one of the genes down regulated by this tumor suppressor miR, providing some mechanistic insight into why high levels of tenascin-C are observed in malignant breast cancers (Tavazoie et al., 2008). Proteomic analysis of normal and transformed mammary epithelia cells grown in 3D culture systems also identified tenascin-C as a mediator of tumor progression (O'Brien et al., 2011). Laser capture micro-dissection was used to isolate vessels from clinical samples of invasive ductal carcinoma and the proteomic profile of this tissue compared to patient matched adjacent non-malignant breast tissue. These data show elevated tenascin-C in malignant vessels, amongst a list of 29 differentially expressed genes that could predict survival in independent sets of microarray data from breast cancer patients (Hill et al., 2011). Furthermore, high throughput screening of 204 proteins using multiplexed immunoassays identified 11 analytes in the blood, including tenascin-C, that could distinguish women with ovarian cancer from those with benign conditions with 90% specificity and

sensitivity (Amonkar et al., 2009). These data may offer a non-invasive means to screen for malignant ovarian cancer, if validated in further blind studies.

Finally, investigation into the genetic contribution to the risk of developing breast cancer, using a wistar rat model, has identified a mammary cancer susceptibilty locus. Fine mapping pinpointed a region of ~176 kb on chromosome 5 showed that the WKy allele at locus Mcs5c reduces carcinoma multiplicity after 7,12-dimethylben-[α]anthracene exposure. This region itself is gene poor and thought to mediate this effect by regulation of genes outside the locus. One candidate found within this locus is tenascin-C; tenascin-C expression is reduced in the thymus and ovarian tissues of Mcs5c WKy homozygous rats compared to controls (Veillet et al., 2011).

1.3 The Impact of Tenascin-C Splicing in Tumors

In addition to the reported increase in the overall level of tenascin-C in tumor tissues, changes in the way that tenascin-C is spliced also occur during tumorigenesis. Upon western blotting of basal cell carcinoma lysates, two distinct tenascin-C variants of 210 and 300 kDa were detected, in contrast to detection of only the larger variant in normal cultured human skin fibroblasts (Verstraeten et al., 1992). Conversely, western, as well as northern, blotting showed that a high MW tenascin-C variant was more strongly expressed in hyperplastic and neoplastic breast compared to normal breast tissue (Borsi et al., 1992). Interestingly, normal human articular chondrocytes exhibited a high ratio of small to large splice tenascin-C whereas chondrosarcoma cell lines possessed a low small to large splice ratio. Clinical chondrosarcoma specimens with a lower small to large splice ratio showed a trend towards decreased survival suggesting that the prognosis of these patients may be predicted by assessing the ratio of tenascin-C variants (Ghert et al., 2001).

A number of studies have more precisely identified alternatively spliced TNIII repeats that are expressed in a cancer tissue-specific manner and these are summarized in figure 2. Hindermann *et al.* (1999) used a probe spanning TNIII repeats A3, A4 and B to localize expression of tenascin-C variants containing these alternatively spliced domains by *in situ* hybridization in oral squamous cell carcinoma. No reactivity was observed in normal non-keratinized buccal mucosa but transcripts containing these repeats were found in carcinoma and moreover correlated with malignancy (Hindermann et al., 1999). The same probe was used to demonstrate that transcripts with domains A3, A4 and B are specifically found at the invasive front in prostatic adenocarcinoma, despite uniform staining of the tumor stroma with antibodies recognising repeats A1-A4 (Katenkamp et al., 2004). These data imply that specific subsets of tumor cells synthesize cancer specific tenascin-C variants.

Specific TNIII repeats have also been found to delineate malignancy or predict potential disease recurrence in a tumor specific manner. In ovarian cancer, larger transcripts containing TNIII A4, B and C are limited to malignant tumors (Wilson et al., 1996). Repeat AD2 is not found in normal or premalignant mucosal samples but is found in a subset of patients with malignant oral mucosa (Mighell et al., 1997). In non-small cell lung carcinoma, RT-PCR analysis with primers for TNIIIA1 demonstrated that expression of isoforms containing this repeat correlated with tumor recurrence: patients with recurrent disease had an 18-fold increase in the expression of A1 positive isoforms compared to patients with non-recurrent disease (Parekh et al., 2005). In colorectal cancer, extra repeat D is underrepresented in metastasizing cancers, whilst repeats A1, 2 and 4 are over expressed (Dueck et al., 1999). The presence of extra repeat B and D correlates with the invasive phenotype of breast carcinoma by *in situ* (Adams et al., 2002), and extra repeat B with Ki-67 levels in intraductal breast carcinoma and found at the invasive front (Tsunoda et al., 2003). In astrocytoma, repeat C is highly expressed in grade III tumors and in glioblastoma but not in breast, lung, gastric carcinoma or low grade astrocytoma (Carnemolla et al., 1999). The presence of repeat C is also a marker of vascular proliferation in cavernoma (Viale et al., 2002). An up-regulation of repeats B and D in breast cancer (Adams et al., 2002) and in ovarian cancer (Wilson et al., 1996) has been reported.

The existence of tumor specific tenascin-C isoforms may explain why differences in tenascin-C expression have been observed in the same tumor types by different groups; data may be dependent on which

probe or method of detection was used in analysis. Furthermore, these data have also provided functional insight into the role of tenascin-C in different tumors.

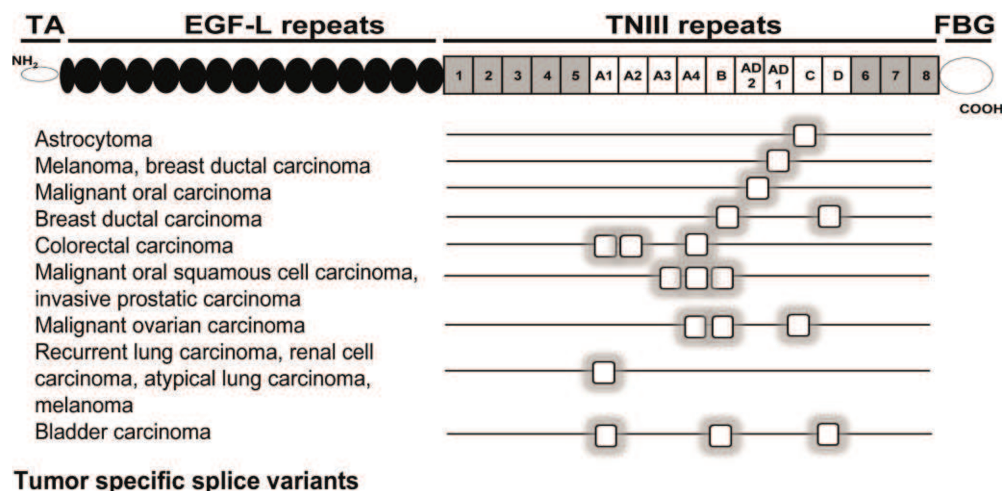

Figure 2. Tumor specific TNIII domains. Alternatively spliced repeats of tenascin-C that are reported to be up-regulated or over-represented in specific tumor types are shown.

Hancox *et al.* identified how the presence of these extra repeats causes distinct cellular responses. Overexpression of tenascin-C variants containing either domain B or domains B plus D in breast cancer cell lines or normal fibroblasts promoted tumor cell proliferation and invasion compared to expression of small spliced variants. Overexpression of all types of tenascin-C stimulated MMP-13 expression (Hancox et al., 2009). These data demonstrate how specific tumor supporting functions can be assigned to individual spliced domains. This group also showed that, whilst not tumor specific, repeats AD1 and AD2 are over represented in invasive breast carcinoma more frequently in women younger than 40 years old and that AD1 expression correlated with estrogen receptor negative, grade 3 tumors. Moreover, expression of variants containing domains B, D and AD1 stimulated tumor cell invasion and growth *in vitro* (Guttery et al., 2010a). The effect of alternatively spliced TNIII domains on resistance to cytotoxic agents has also been examined. Using pancreatic cell cultures, Gong *et al.* (2010) showed that addition of a recombinant protein comprising TNIII repeats A-D suppressed gemcitabine induced cell toxicity via interaction with cell surface annexin A2. Ligation of annexin A2 by repeats A-D activated phosphatidylinositol 3-kinase, Akt and nuclear factor-kappaB signaling (Gong et al., 2010). These data imply that alternatively spliced regions of tenascin-C may confer tumor cell survival upon delivery of cytotoxic drugs and provide a potential point of therapeutic manipulation.

Indeed targeting tumors using antibodies raised against specific tenascin-C splice variants has been validated in a number of types of tumor. The distribution of TNIII domains A1-D in urinary bladder carcinoma was assessed by immunohistochemical staining using domain specific antibodies and RT-PCR. Whilst a universal increase in large variant tenascin-C was observed in tumors of a higher grade, domains A1, B and / or D were restricted to invasive tumors and tumor vessels; repeats AD1 and C were rarely expressed (Berndt et al., 2006). Phage display was subsequently used to identify antibodies specific for domains A1 and D. Both antibodies selectively accumulated at tumor sites in a U87 glioblastoma murine xenograft model but were rapidly cleared from other organs. Accumulation of antibodies to domain D was lower than that of antibodies to domain A1 and the former exhibited some non-specific localization to the intestine (Brack et al., 2006). Based on these data, antibodies recognizing domain A1 have been further characterized; they are able to selectively

stain tumor tissue, for example most Hodgkin and non-Hodgkin lymphomas (Schliemann et al., 2009). The same authors report expression of A1 in renal cell carcinoma and atypical carcinoid of the lung (Berndt et al., 2010), as well as in primary cutaneous melanoma lesions, particularly in the basal lamina at the interface between epidermis and dermis (Frey et al., 2011). In addition, vascular expression of A1, as well as domain C, has been reported in renal cell carcinoma (Galler et al., 2012). Given the tumor specific pattern of tenascin-C splicing, antibodies targeting alternatively spliced tenascin-C domains, particularly repeat A1, are currently being used in clinical trials for the delivery of cytokines or therapeutic radionuclides to tumor sites in patients with cancer, whilst sparing unaffected organs. These studies are discussed in section 4 below.

1.4 Tenascin-C Form Versus Function in Tumors

Tenascin-C is assembled into a pericellular fibrillar matrix within the ECM. This 3D structure has quite different properties to soluble tenascin-C in terms of binding to other ECM proteins, affecting cell behaviour and defining the mechanical properties of the tissue (To and Midwood, 2011). The impact of the structural organization of tenascin-C within the tumor ECM has been investigated by Chen *et al.* (2009). The assembly of tenascin-C into a dense fibrillar matrix was specifically observed in metastatic pancreatic cancers, compared to a less well organized matrix in benign tumors or healthy pancreatic tissue. Deposition of a fibrillar tenascin-C matrix by pancreatic tumor cells required co-culture of these cells with stromal fibroblasts, and purified fibrillar, but not soluble, tenascin-C reduced adhesion and promoted migration of tumor cell lines (Chen et al., 2009). These data highlight the importance of the structural organization of the ECM in defining cell phenotype.

Tenascin-C in the ECM is also dynamically turned over. This can be mediated by degradation via the activity of numerous proteases including MMPs-1, -2, -3, -7, -9 and -19, or serine proteases; (reviewed in (Udalova et al., 2011). Indeed in tumors, tenascin-C degradation products have been detected. Western blotting of tenascin-C purified from invasive colonic carcinomas displayed several low MW degradation products compared to that extracted from a human umbilical cord fibroblast cell line (Sakai et al., 1993). Subsets of patients with lung cancer demonstrated degradation of tenascin-C; and the presence of tenascin-C breakdown significantly correlated with metastasis to the lymph node and was thus proposed as a marker for metastatic potential (Kusagawa et al., 1998). Furthermore, in non-small lung cell carcinoma the presence of tenascin-C fragments was detected by western blotting tumor extracts and found in a subset of patients that exhibited recurrence of stage 1 disease. The absence of tenascin-C fragments was predictive of a good prognosis for lack of recurrence at 4 and 10 years (Cai et al., 2002). Finally, levels of tenascin-C isoforms containing TNIII domains B and C was analysed by ELISA in the urine of patients with urothelial carcinoma. Levels of both were increased as tumors progressed as did the presence of tenascin-C fragments (Richter et al., 2009). Proteolytic fragments of tenascin-C can be biologically active, often containing cryptic functions not present in full-length tenascin-C (To and Midwood, 2010). However, the identity of the fragments observed in different tumors is not known; it will be interesting to define the sequence of these molecules and to examine if they actively contribute to tumor cell biology or simply represent a marker of tumor ECM turnover.

In summary, existing data demonstrate a consistent up-regulation of tenascin-C in a wide variety of tumors. However, these studies also highlight the complexities of associating tenascin-C expression with disease activity and prognosis. For some tumor types corroborative, independent data indicate that tenascin-C expression alone, or as part of a wider gene signature, may constitute a reliable marker for disease diagnosis and prognosis, most notably in glioblastomas. Further studies reveal that analysis of tenascin-C expression may indicate the most effective method of treating patients, for example subsets of breast cancer patients. Tenascin-C also appears to constitute a specific marker of tumor cells likely to metastasize to distinct secondary sites. Moreover, the precise form of tenascin-C expressed in each type of cancer may have a profound impact on tumor cell phenotype; the pattern of tenascin-C splicing, and the supramolecular assembly or degradation of tenascin-C may each contribute to disease progression. Next we discuss functional data that reveal what the purpose of tenascin-C in tumors may be; focusing on studies that have examined what this ECM glycoprotein is doing in the tumor stroma as well as at sites of metastatic colonization.

2 The Function of Tenascin-C in the Tumor Stroma

Tenascin-C is an extraordinarily pleiotrophic molecule. This functional plasticity is made possible by the multimodular structure of the molecule. Each domain of tenascin-C interacts with a number of specific matrix molecules and cell surface receptors (table 3). In this way, tenascin-C can influence tissue structure, modulate signals derived from other extracellular components, and signal directly to cells to affect cell phenotype. Below we summarize established data that document the role of tumor derived tenascin-C and discuss in more detail newer emerging data that provide further insight into the role of this molecule within the tumor ECM.

Domain	Ligand	References
Full length tenascin-C	Fibronectin	(Chiquet-Ehrismann et al., 1991)
	Collagen I – VI, IX	(Faissner et al., 1990)
	Von Willebrand Factor	(Schaff et al., 2010)
	SMOC1	(Brellier et al., 2011b)
	Periostin	(Kii et al., 2010)
	Nidogen-2	(Brellier et al., 2011b)
	Fibrillin-2	(Brinckmann et al., 2010)
	Phosphoglycerate kinase 1	(Brellier et al., 2011b)
	Streptococcus	(Vollmer et al., 2010)
	Clusterin	(Brellier et al., 2011b)
TA	Tenascin-C	(Kammerer et al., 1998; Luczak et al., 1998)
EGF-L 1-2	EGFR	(Swindle et al., 2001)
EGF-L 11-14	EGFR	(Swindle et al., 2001)
TNIII 3	$\alpha v\beta 3$, $\alpha 2\beta 1$	(Sriramarao et al., 1993)
	$\alpha v\beta 3$, $\alpha v\beta 6$	(Prieto et al., 1993)
	$\alpha v\beta 3$, $\alpha 9\beta 1$, $\alpha v\beta 6$	(Yokosaki et al., 1996)
	$\alpha v\beta 1$	(Probstmeier and Pesheva, 1999)
	$\alpha 8\beta 1$	(Denda et al., 1998; Schnapp et al., 1995)
TNIII 3-5	Perlecan, lecticans	(Chung and Erickson, 1997; Day et al., 2004)
TNIII 4-5	Neurocan	(Rauch et al., 2001)
TNIII 5-6	Heparin	(Fischer et al., 1995; Jang et al., 2004; Weber et al., 1995)
	Glypican	(Vaughan et al., 1994)
TNIII 5	Contactin	(Zisch et al., 1992)
TNIII A1-A4	RPTPβ	(Milev et al., 1997)
TNIII A1-B	NaN	(Srinivasan et al., 1998)
TNIII A1-D	Annexin II	(Chung and Erickson, 1994)
TNIII D	Integrin $\alpha 7\beta 1$	(Mercado et al., 2004)
TNIII 6	Contactin	(Zisch et al., 1992)
TNIII 6-8	NaN	(Srinivasan et al., 1998)
FBG	Heparin	(Fischer et al., 1995; Jang et al., 2004; Weber et al., 1995)
	Neurocan	(Rauch et al., 2001)
	CALEB	(Schumacher et al., 2001)
	Integrin $\alpha v\beta 3$	(Yokoyama et al., 2000)
	RPTPβ	(Milev et al., 1997)
	TLR4	(Midwood et al., 2009)

Table 3. ECM proteins and cell surface receptors that interact with or are activated by tenascin-C

2.1 Cell Adhesion, Proliferation and Survival

Tenascin-C has long been known to modulate cell adhesion, proliferation and survival. These effects, and the mechanisms by which they are mediated specifically in the context of tumor cell biology, are reviewed in detail in (Chiquet-Ehrismann and Tucker, 2011; Orend and Chiquet-Ehrismann, 2006). These data are also discussed in more detail in sections 2.3, 2.4 and 3 below.

2.2 Immune Modulation

Tenascin-C expression has also long been associated with sites of inflammation in a wide variety of pathologies, and in the last two decades a number of inflammatory functions for this molecule have been described. These have been recently reviewed (Midwood and Orend, 2009; Udalova et al., 2011) and are summarized in table 4.

Process	Function	Reference
Pathogen invasion	Supports streptococcal adhesion	(Vollmer et al., 2010)
	Stimulates sustained cytokine translation upon bacterial infection by promoting the expression of miR-155	(Piccinini and Midwood, 2012)
Thrombosis	Supports platelet adhesion and promotes platelet activation	(Schaff et al., 2010)
Innate immunity	Stimulates TLR4-mediated cytokine synthesis	(Liu et al., 2012; Midwood et al., 2009; Patel et al., 2011)
	Stimulates integrin mediated cytokine synthesis	(Kanayama et al., 2009)
	Inhibits myeloid cell migration	(Loike et al., 2001; Talts et al., 1999)
	Stimulates macrophage migration	(Sumioka et al., 2011)
	Stimulates hematopoesis	(Klein et al., 1993; Ohta et al., 1998; Seiffert et al., 1998)
	Promotes erythroid cell differentiation	(Tanaka et al., 2009)
Adaptive immunity	Stimulates Th2 and B cell activation	(Kuhn and Mason, 1995; Nakahara et al., 2006)
	Inhibits T cell activation	(Kuznetsova and Roberts, 2004)
	Promotes lymphocyte migration	(Clark et al., 1997; El-Karef et al., 2007; Sobocinski et al., 2010)
	Limits T cell actin polymerization and migration	(Huang et al., 2010)
	Drives Th17 cell polarization	(Kanayama et al., 2011; Ruhmann et al., 2012)

Table 4. Overview of the inflammatory properties of tenascin-C

Several inflammatory diseases, including inflammatory bowel disease, have been reported to increase the risk of cancer. Moreover, the tumor stroma is increasingly recognized as a pro-inflammatory environment complete with infiltration of tumor associated immune myeloid cells and lymphocytes that synthesize high levels of pro-inflammatory mediators. These mediators have been shown to directly impact tumor cell biology, for example TNF enhances the growth and metastasis of a number of tumor types including skin, ovarian,

pancreatic and bowel cancer, and IL-6 has tumor growth and survival promoting activities in a wide range of cancer models. Furthermore, chemokine networks elaborated by immune myeloid cells in the tumor have been shown to work together to facilitate tumor and immune cell migration, as well as angiogenesis, during primary disease, and to promote tumor cell egress during metastasis. Thus the inflammatory process is hijacked during the progression of cancer and used to the advantage of the tumor cell (reviewed in (Balkwill and Mantovani, 2012).

Tenascin-C has been shown to drive both innate and adaptive immune responses during tissue injury and infection (Midwood and Orend, 2009; Udalova et al., 2011). Whilst its recently uncovered roles in pathogen defence (Piccinini and Midwood, 2012; Vollmer et al., 2010) may not be relevant to the sterile inflammation that is the hallmark of the tumor environment, the ability of tenascin-C to induce the synthesis of several inflammatory cytokines, including TNF and IL-6, via activation of the cell surface receptors TLR4 (Liu et al., 2012; Midwood et al., 2009; Patel et al., 2011) and $\alpha 9$ integrins (Kanayama et al., 2009), may contribute to disease progression by raising the levels of these tumorigenic cytokines in the tumor stroma. Tenascin-C also drives the synthesis of chemokines such as IL-8 (Midwood et al., 2009), thus stimulating the formation of high gradients of chemoattractants within the tumor stroma and potentially inducing further cell infiltration into the tumor site. Tenascin-C may additionally promote immune cell migration at tumors by directly interacting with cells. One study has shown that it supports lymphoid cell tethering to and invasion through complex ECMs (Clark et al., 1997). *In vivo*, inflammatory infiltrates during immune mediated hepatitis were less intense in tenascin-C null mice (El-Karef et al., 2007) and tenascin-C null mice exhibit suppressed macrophage, but not neutrophil, invasion into cauterized corneas (Sumioka et al., 2011). It is possible here that tenascin-C may act at a structural level by altering the conformation of the matrix to make it less restrictive and more migration permissive. Ultrastructural analysis of lymph nodes revealed that tenascin-C was deposited along the reticular fiber network forming putative 'footholds' for T cell migration (Sobocinski et al., 2010). In addition, tenascin-C has been shown to modulate the synthesis and activity of ECM degrading proteases *in vitro* and so in this way it may enable increased degration of ECM barriers to migration. Tenascin-C also directly influences cell phenotype, for example modulating polarity, focal adhesion turnover or cytoskeletal architecture, all processess that contribute to sucessful migration. Indeed tenascin-C may drive all of these events to bring about coordinated and efficient cell movement into and out of tumor sites (reviewed in Midwood and Orend, 2009).

However, it is important to note that these data supporting a role for tenascin-C in promoting immune cell migration contrast with data showing that tenascin-C limits immune cell migration. One study examining myeloid cell migration in a model system with a direct relevance to tumor cells showed that significantly higher levels of monocyte and macrophage migration were observed in the stroma of mammary tumors in tenascin-C null mice compared to wild type mice (Talts et al., 1999). These data fit with *in vitro* studies showing that myeloid cells failed to effectively migrate through a matrix barrier containing tenascin-C, compared to matrices that did not contain tenascin-C (Loike et al., 2001). More recently, Huang *et al*. (2010) demonstrated that tenascin-C mediated cross-talk between glioma cells and neighboring T cells is key for limiting T-cell migration within the brain. Here, CD3-positive T cells accumulated within blood vessels stained strongly with tenascin-C in glioma tissue and did not migrate into the brain tissue (Huang et al., 2010). These data suggest an immune suppressive function of tenascin-C, whereby it prevents T cell mediated attack of the glioma.

Together these data exemplify the context specific nature of studies examining the role of tenascin-C, but do indicate that, in addition to direct modulation of tumor cell biology, tenascin-C may facilitate cancer progression by orchestrating the behaviour of tumor associated immune cells. Moreover, tenascin-C could comprise part of the molecular machinery that links inflammatory disease to susceptibility to developing cancer. Indeed, high circulating levels of tenascin-C are found in patients with inflammatory bowel disease (Riedl et al., 2001) and this aspect of tenascin-C biology warrants further examination.

2.3 Angiogenesis

The growth of a tumor strongly depends on its supply of nutrients and oxygen. In the course of tumor development the angiogenic switch is considered a crucial event in promoting malignancy (Hanahan and Weinberg, 2011). The angiogenic switch is characterized by sprouting, new vessel formation, vessel maturation, and the recruitment of perivascular cells (Bergers and Benjamin, 2003; Hanahan and Weinberg, 2011)). It can be triggered by pro-angiogenic growth factors and hypoxia as well as by the ECM of the tumor microenvironment. The vasculature of a tumor often differs from vasculature in healthy tissues and is characterized by chaotic vessels, a disorganized basement membrane, incomplete pericyte coverage and leakiness (Shang et al., 2012).

2.3.1 Tenascin-C as a Marker for Tumorigenic Blood Vessels

In several studies tenascin-C expression has been found to be preferentially localized around tumor blood vessels. In 86 gliomas, where tenascin-C expression increased with tumor malignancy, tenascin-C was strongly expressed around tumor blood vessels in glioblastoma grade IV (GBM). This was different in gliomas of WHO grade II and III with a reduced frequency of tenascin-C lined blood vessels. In addition perivascular tenascin-C staining in glioma grade II and III significantly correlated with a shorter disease free period (Herold-Mende et al., 2002). Also Behrem *et al.* (2005) observed that GBM with strong perivascular tenascin-C staining contained more newly formed blood vessels than tumors with moderate or weak tenascin-C expression, as assessed by staining of CD105 microvessels (Behrem et al., 2005). CD105 is defined as a proliferation associated endothelial cell marker (Tanaka et al., 2001). Furthermore in tissue samples from 63 patients with non-small cell lung cancer Ishiwata *et al*. (2005) found a correlation between serum tenascin-C and intratumoral vessel densitiy (Ishiwata et al., 2005). In juvenile nasopharyngeal angiofibroma tenascin-C expression was also found around blood vessels where expression correlated with vessel density, tumor stage and endothelial c-kit expression (Renkonen et al., 2012).

Berndt *et al*. (2010) showed that in CD31 positive blood vessels of clear cell renal cell carcinoma and atypical carcinoids of the lung tenascin-C is localized at the extraluminal side of the basement membrane (Berndt et al., 2010). By *in situ* hybridization tenascin-C mRNA was detected in astrocytoma tumor tissues in hyperplastic capillaries. Staining was observed lining the vascular lumen, indicating the presence of tenascin-C in endothelial cells (EC). But other cells could also be a source of tenascin-C, as additional staining was observed in the walls of the vascular structures (Zagzag et al., 1996). Indeed, data from Martina *et al.* (2010) showed that tenascin-C is expressed by pericytes in GBM but not by EC (Martina et al., 2010).

Recently three studies using proteomic approaches have identified tenascin-C as a marker preferentially expressed in the vasculature of tumors or at the metastatic site. Borgia *et al*. (2009) used *in vivo* perfusion of biotin for labelling of vascular proteins. Four different tenascin-C isoforms (containing TNIII domains A1, A2, A4, B) have been identified as molecules which were expressed in the vasculature of liver metastases in a syngeneic heterotopic model of colon cancer (Borgia et al., 2009). Hill *et al.* (2011) used laser capture microdissection of microvessels of invasive ductal carcinoma and identified tenascin-C as one of the proteins overexpressed in tumor vessels in comparison to vessels from adjacent healthy tissue (Hill et al., 2011). Similarly, by using laser capture microdissection and protein expression profiling tenascin-C was found to be exclusively expressed in tumor vessels of GBM but not in tissue with physiological angiogenesis (Mustafa et al., 2012).

In summary, these data show that in a number of tumors tenascin-C specifically marks tumor blood vessels. Tenascin-C expression often correlates with higher tumor stage and increased blood vessel density which argues for a role of tenascin-C in the tumor vasculature.

2.3.2 Tenascin-C Impacts on Endothelial Cell Behavior in Vitro

A number of studies showed that tenascin-C is able to modulate the behaviour of EC *in vitro*, including EC attachment and spreading, supporting migration and proliferation, as well as their sprouting. All these effects are known to be associated with angiogenesis and may contribute to the tumor angiogenesis promoting effect of tenascin-C.

Tenascin-C supported the switch from a non-angiogenic (resting) cobblestone phenotype to an angiogenic sprouting cord-forming phenotype in bovine aortic endothelial cells (BAEC) (Canfield and Schor, 1995). Schenk *et al*. (1999) showed that tenascin-C is exclusively expressed in the sprouts and cords of the sprouting but not in the resting BAEC. For the induction of sprouting by tenascin-C the growth factor basic fibroblast growth factor (bFGF) seems to be required. Furthermore the authors showed that the FBG domain of tenascin-C was responsible for the BAEC sprouting (Schenk et al., 1999). The authors argued that the sprout-supporting effect of tenascin-C might be explained with the anti-adhesive effect of tenascin-C. However, conflicting observations have been reported concerning this issue. Whereas an anti-adhesive effect of tenascin-C on EC of different origin was shown (Ballard et al., 2006; Sriramarao et al., 1993) significant stimulation of tenascin-C on EC adhesion was reported by others (Delaney et al., 2006; Zagzag et al., 2002).

The anti-adhesive tenascin-C effect reported has been linked to a reduction in focal adhesions in EC (Murphy-Ullrich et al., 1991). These authors showed that the TNIII A-D domain mediates the anti-adhesive effect, which can be reversed by blocking cell surface annexin II, a tenascin-C receptor (Chung et al., 1996).

Despite its anti-adhesive and anti-spreading effect early upon cell plating, most EC eventually do attach and spread on tenascin-C after culture for longer periods of time. This cell attachment can be blocked with an RGD peptide (Bourdon and Ruoslahti, 1989). EC attachment and spreading on tenascin-C was mediated by different tenascin-C cell surface receptors including annexin II (Chung and Erickson, 1994), $\alpha 2\beta 1$ and $\alpha v\beta 3$ integrins (Delaney et al., 2006; Joshi et al., 1993; Sriramarao and Bourdon, 1993). But in longer term assays EC secrete other ECM molecules such as fibronectin, and adhesion to fibronectin would be blocked with RGD peptides. Thus the described observation may be due to inhibiting adhesion to fibronectin with the RGD peptide rather than blocking the interaction with tenascin-C. Altogether these studies show that through different cell surface receptors adhesion and spreading of EC is modulated by tenascin-C, which could be crucial in tumor angiogenesis.

Tenascin-C was shown to enhance EC proliferation (Castellon et al., 2002; Chung et al., 1996; Delaney et al., 2006) and, to promote EC migration (Ballard et al., 2006; Castellon et al., 2002; Chung et al., 1996; Ishiwata et al., 2005; Martina et al., 2010; Zagzag et al., 2002). This was demonstrated in different assays and with EC of different origin. Moreover it has been demonstrated that in the presence of tenascin-C there is increased sprouting and tube formation of EC (Castellon et al., 2002; Martina et al., 2010). The different effects of tenascin-C on EC behavior are summarized in table 5.

Assay	Experimental details				Cell line	Reference
ADHESION						
⬇	10 min, 3hrs	Coating (10 µg/ml)	Compared to Collagen	Statistical difference, replicate experiments	CMEC	(Ballard et al., 2006)
=	45 min	Coating (0,1 µg/ml) – for other ECMs higher conc. used	Compared to plastic, FN, VN (but less then LN, Coll)	No statistical difference if compared to plastic, 3 experiments	HDMEC	(Delaney et al., 2006)
⬇	24h	Coating (no conc.	FN/TNC 1:100	Statistical difference,	HUVEC	(Alves et al., 2011)

		mentioned)	compared to FN/TNC 1:1	triplicate experiments		
MIGRATION						
⬆	Wound healing assay, 48h	TNC (30 µg/ml) added to medium	Compared to plastic	No statistics provided, 1.8 fold increase, 7 experiments	GM7373	(Chung et al., 1996)
⬆	Wound healing assay, 7d	TNC (40µg/ml) added to medium	Compared to IgG treatment	Statistical difference, 6 experiments	REC	(Castellon et al., 2002)
=	plating of EC aggregates on matrix, 22h	Coating of glass slides (100 µg/ml)	Compared to FN	Statistical difference, 3-10 observations	BREC	(Zagzag et al., 2002)
⬆	cell culture insert poly-ethylene tereph-thalate, underside of membrane coated, 5h	Coating (100 µg/ml)	Compared to FN	Statistical difference, 4-10 observations	BREC	(Zagzag et al., 2002)
⬆	Boyden Chamber, reverse side coated, 6h	Coating (100 µg/ml)	Compared to BSA	Statistical difference, number of experiments not indicated	HUVEC	(Ishiwata et al., 2005)
⬆	migration into collagen gel ontop, medium with VEGF-A or PDGF-AB	Coating (10 µg/ml)	Compared to collagen	Statistical difference, 4 experiments	CMEC	(Ballard et al., 2006)
⬆	Time lapse, 10 hr	Coating (200 ng FN or TNC in 72 well MicroWell Mini Trays + 20 µg/ml Collagen)	Coll/TNC compared to Coll/FN	Statistical differerence, 2 experiments	HUVEC	(Martina et al., 2010)
TUBE FORMATION						
⬆	Tube length, 48hrs	Coating (40 µg/ml) on BM matrix, or TNC added to the cells	Compared to no coating, LN, FN	Statistical difference, 3 experiments	REC	(Castellon et al., 2002)
⬆	Cumulative	TNC (20	Compared to	Statistical	HUVEC	(Martina et

	sprouts length, HUVEC spheroids in collagen gel, 48hr	µg/ml) incorporated in the gel/secreted by HEK 293 spheroids	BSA/control cells	difference, 3 experiments		al., 2010)
⬇	Number capillary like structures, HUVECs previously seeded for 24h on U373 cell derived matrix, seeded on matrigel for 16 hrs	α-TNC antibody incubation for 2h with U373 cell derived matrix	Compared to IgG treatment	Statistical difference, 3 experiments	HUVEC	(Alves et al., 2011)
⬇	Number capillary like structures, HUVECs previously seeded for 24h on U373 cell derived matrix, seeded on matrigel for 16 hrs	Coating with TNC (concen-tration not specified)	TNC or FN/TNC compared to FN	No statistics provided, decrease in number of capillary like structures by approx. 4.5 or 1.6 respectively, comparing TNC or FN/TNC compared to FN	HUVEC	(Alves et al., 2011)
PROLIFERATION						
⬆	BrdU incorporation , 12h	TNC (30; 70; 100 µg/ml) added to medium	Compard to plastic	Statistical difference, at least 3 experiments	GM7373, BAEC	(Chung et al., 1996)
⬆	[3H] thymidine incorpor-ation assay, 24h	TNC (40 - 100 µg/ml) added to medium	Compared to plastic	No statistics provided, approx. 1.6 fold increase, number of experiments not indicated	BAEC	(Chung et al., 1996)
⬆	Survival – secondary sprouting assay:	Coating (25 µg/ml) on BM matrix	Compared to no coating	Statistical difference, 2 experiments	REC	(Castellon et al., 2002)

	number of cells in sprouting colonies 14 days after tube collapse					
⬆	MTS Assay (0.5% and 10% serum), 6 days	Coating (10 – 50 µg/ml)	Compared to plastic	No statistics provided, 20-40% increase, 2 experiments	REC	(Castellon et al., 2002)

Table 5. Overview of tenascin-C effects on endothelial cells *in vivo*.

2.3.3 Tenascin-C Supports Angiogenesis – Potential Underlying Mechanism and Signaling

Several studies provide evidence linking tenascin-C to the expression of the proangiogenic factor VEGFA. The initial study by Tanaka *et al.* (2004) suggests that tenascin-C supports melanoma angiogenesis by regulating the expression of VEGFA. After injection of melanoma cells in immune compromised wild type (wt) and tenascin-C knockout (KO) mice tumors grown in KO mice were smaller and were less vascularized. Measuring the VEGFA content of the tumors by ELISA the authors observed a lower VEGFA content in tumors from the KO mice than those grown in wt mice. Also in co-cultures with melanoma cells and the mesenchyme derived from either wt or tenascin-C KO mice the authors measured a higher VEGFA level when the mice expressed tenascin-C (Tanaka et al., 2004). Other studies support the author's observation that tenascin-C expression correlates with VEGFA levels as e.g. in GBM (Behrem et al., 2005). In serum from non-small cell lung cancer patients tenascin-C levels correlated with the serum levels of VEGFA (Ishiwata et al., 2005). Moreover Sumioka *et al.* (2011) showed that occular fibroblasts derived from tenascin-C KO mice expressed less VEGFA than wt fibroblasts which was associated with less neovascularisation in tenascin-C KO mice in the corneal stroma (Sumioka et al., 2011). But how tenascin-C impacts on the expression of VEGFA is unknown.

Interestingly, tenascin-C has been demonstrated to regulate the expression or function of other proangiogenic growth factors such as PDGF-BB (Lange et al., 2008) and endothelin receptors EDNR-A and EDNR-B (Lange et al., 2007) in tumor cell lines derived from GBM, melanoma and urinary bladder carcinoma.

Wnt signaling is another pathway regulating physiological and pathological angiogenesis (Dejana, 2010). Tenascin-C has been shown to down regulate the Wnt inhibitor DKK1 in a GBM cell line (Ruiz et al., 2004). In tenascin-C-deficient MEFs reduced DKK2 levels were observed (Brellier et al., 2011a). DKK1 and DKK2 have been shown to play opposite roles in angiogenesis; while DKK2 is promoting angiogenesis through stimulating filopodial dynamics and EC sprouting which involves LRP6-mediated APC/Asef2/Cdc42 activation, DKK1 has been shown to antagonize the proangiogenic DKK2 effect (Min et al., 2011). Therefore it is intriguing to speculate that tenascin-C may promote tumor angiogenesis by shifting the balance between the two DKK molecules, leading to low expression of DKK1 in tumor cells and high expression of DKK2 in the stromal fibroblasts. Since DKK1 and DKK2 are soluble factors they could exert a paracrine proangiogenic effect on surrounding EC.

In summary these studies show that tenascin-C is strongly associated with tumor blood vessels. Moreover, tenascin-C appears to be able to promote the formation of tumor associated blood vessels. However, there remains a need to better understand the mechanisms and signaling by which tenascin-C is involved. Nevertheless, even without this knowledge targeting of tenascin-C, alone or in combination with other therapeutic approaches, in the tumor vasculature seems to be a promising strategy to counteract tumor neovascularization. This approach is discussed in more detail in chapter 4.

2.4 Stem Cell Biology

There is increasing evidence for the existence of tumor stem cell-like populations playing a role in tumor growth and metastasis (Reya et al., 2001). Several studies show that tenascin-C plays an important role in proliferation, migration, responsiveness to growth factors and differentiation of neural stem cells (NSC) or neural progenitors (Abaskharoun et al., 2010; Czopka et al., 2010; Garcion et al., 2001; Garcion et al., 2004; Moritz et al., 2008; von Holst et al., 2007; Yagi et al., 2010). A number of recently published papers also investigated the role of tenascin-C in stemness of tumor cells such as in neuroblastoma (Pezzolo et al., 2011), melanoma (Fukunaga-Kalabis et al., 2010) and breast cancer (Oskarsson et al., 2011).

2.4.1 Neural Stem Cells

Neural stem cells (NSC) are highly proliferative cells of neuronal origin which are capable of self-renewal and differentiation into astrocytes, neurons or oligodendrocytes. Two studies examined the impact of tenascin-C knockout on neural stem cells in the central nervous system (CNS). Garcion *et al.* (2001) investigated the behaviour of oligodendrocyte progenitors (OP), which give rise to myelin-forming oligodendrocytes, in tenascin-C-null transgenic mice. OP cells of tenascin-C KO mice exhibited an increased migration along the optic nerve at P0 and P2. Those results were confirmed by *in vitro* experiments with rat OP and astroglial matrices derived from wt or tenascin-C KO mice (Garcion et al., 2001).

Tenascin-C-null mice also exhibited a decreased number of proliferative cells in the subventricular zone (SVZ). In this region OP cells are generated from pre-progenitor cells during early postnatal development. In addition double immunostaining for BrdU and the OP marker NG2 showed a reduction in proliferating OP cells in the CNS of tenascin-C KO mice. *In vitro* the authors demonstrated that tenascin-C regulated the proliferation of OP by sensitizing OP cells to the growth factor PDGF. OP cells from KO mice grown on tenascin-C-deficient astroglia matrix did not show any response to PDGF while wt cells grown on a wt matrix showed an optimal response. An antibody blocking β3 integrin inhibited the mitogenic effect of PDGF on rat OP cells in the presence of tenascin-C sugessting that tenascin-C might have a positive impact on the formation of a complex comprised of integrin αvβ3 and PDGFR (Schneller et al., 1997).

Garcion *et al*. (2004) investigated in more detail the role of tenascin-C in regulating the responsiveness of neural stem cells to growth factors (Garcion et al., 2004). Early embryonic neural stem cells respond to bFGF signaling only while late embryonic and adult neural stem cells by expressing EGFR do also respond to the mitogenic EGF stimulus (Qian et al., 2000). By immunostaining the authors found delayed acquisition of EGFR in tenascin-C knockout brain tissue of embryonic mice. *In vitro* the authors confirmed that neural stem cells derived from tenascin-C KO mice do not respond to EGF stimulation. In the presence of EGF neurospheres were obtained from telencephalic cells from E10.5 wt mice while no neurospheres could be obtained from tenascin-C KO mice. In further support, tenascin-C-deficient cells isolated from E10.5 mice did not express EGFR in response to bFGF which stimulates the expression of EGFR while wt cells did and, this defect could be rescued by addition of exogenous tenascin-C (Garcion et al., 2004; Lillien and Raphael, 2000). Moreover the authors showed reduced proliferation in bFGF stimulated neural stem cells derived from E12.5 and P0 tenascin-C KO mice, indicating that tenascin-C enhances mitogenic bFGF signaling (Garcion et al., 2004). In contrast to an enhanced EGFR expression in NSC by bFGF, BMP was shown to inhibit EGFR expression (Lillien and Raphael, 2000). Interestingly the inhibition of EGFR expression by BMP4 was only shown in neural stem cells derived from E12.5 and P0 tenascin-C KO mice but not in wt mice, suggesting that tenascin-C also acts as an inhibitor of BMP4 signaling (Garcion et al., 2004). In summary the authors have shown that tenascin-C modulates the responsiveness of neural stem cells to mitogenic stimuli by promoting the expression of EGFR, this is mediated by enhanced bFGF signaling as well as by inhibition of BMP4 signaling (Garcion et al., 2004). Furthermore the authors showed that tenascin-C had an effect on the potential of NSC to differentiate into brain-forming cells (neurones, glial cells or oliogdendrocytes). Although there was no change in the number of glial cells between wt and KO mice, tenascin-C inhibited neurogenesis (Garcion et al., 2004).

As tenascin-C has been described to undergo alternative splicing (see section 1.3) it is not surprising that also in NSC different tenascin-C isoforms were detected (von Holst et al., 2007). By using primers flanking the alternatively spliced region of tenascin-C in RT-PCR analysis followed by dot blot hybridization analysis the expression of 20 tenascin-C isoforms in E13 neurospheres were shown. Tenascin-C isoforms contained up to 6 alternatively spliced TNIII domains. Addressing the underlying mechanism of alternative splicing of tenascin-C in NSC, the authors showed that the paired box transcription factor Pax6, a regulator of tenascin-C expression (Gotz et al., 1998), enhances the expression of tenascin-C isoforms with more than three additional TNIII domains while the expression of isoforms with one or without any alternatively spliced TNIII domains was even decreased. Although Pax6 involvement could not explain the appearance of the seemingly NSC specific A1A4BD isoform, it was clearly shown that Pax6 differentially regulates alternative splicing of tenascin-C mRNA. Alternative splicing of tenascin-C by Pax6 seems to be restricted to NSC, as overexpression of Pax6 in MEF or in an astrocytic cell line did not alter tenascin-C isoform identity or abundance. Moritz *et al*. (2008) showed that tenascin-C reduced the expression of Sam68 in NSC (Moritz et al., 2008). Sam68 is an RNA binding protein of the STAR family involved in mRNA splicing (Itoh et al., 2002; Tremblay and Richard, 2006). Conversely, overexpression of Sam68 in NSC grown as neurospheres was shown to promote the expression of large tenascin-C isoforms, suggesting that Sam68 regulates alternative splicing of tenascin-C (Moritz et al., 2008).

Czopka *et al,* (2010) analysed in more detail the processes underlying tenascin-C induced retardation of OP differentiation into myelin basic protein (MBP)-positive oligodendrocytes. The authors demonstrated that tenascin-C bound to the cell adhesion molecule contactin and the Src family kinase Fyn, located in the lipid rafts of the OP membrane, diminished Akt phosphorylation and downstream inhibited expression of MBP and Sam68 (Czopka et al., 2010).

Yagi *et al*. (2010) showed that tenascin-C expressed by NSC exhibits the human natural killer-1 (HNK-1) epitope on the cell surface. Similar to tenascin-C, HNK-1 is downregulated during the process of differentiation and facilitates the formation of neurospheres and enhances NSC proliferation. Furthermore the authors show that both tenascin-C and HNK-1 regulate the expression of EGFR in NSC which given the demonstrated impact of tenascin-C on EGFR expression suggests a potential interdependence of tenascin-C and HNK-1 in regulating EGFR expression (Yagi et al., 2010). It remains to be seen whether HNK-1 is mediating this effect in a tenascin-C dependent manner.

Altogether, these studies demonstrated that tenascin-C modulates the responsiveness of neural stem cells to mitogenic stimuli and therefore enhances their proliferation. Furthermore tenascin-C impacts on progenitor migration and differentiation of neural stem cells and progenitors.

2.4.2 Tumor Derived EC

Ricci-Vitiani *et al.* (2010) and Wang *et al.* (2010) first described that CD133+ glioblastoma stem cells are able to transdifferentiate into tumor-derived endothelial cells (TEC) and contribute to the formation of a tumor vasculature (Ricci-Vitiani et al., 2010; Wang et al., 2010b). Pezzolo et al (2011) showed that tenascin-C contributes to the process of tumor stem cell transdifferentiation into TEC, which was demonstrated in neuroblastoma. Tenascin-C was mostly co-expressed with the neuroblastoma stem cell marker Oct4 in perivascular regions. This coexpression of tenascin-C and Oct4 was also seen in human neuroblastomas and in their orthotopic murine xenografts. Upon injection of cells not expressing tenascin-C orthotopic tumors lacked the lining of endothelial microvessels with TEC. In addition orthotopic tumors with expression of tenascin-C exhibited a higher microvessel density. *In vitro* the authors demonstrated the plasticity of tenascin-C expressing neuroblastoma cells where neuroblastoma cells were only able to grow as neurospheres if they expressed tenascin-C. Furthermore it was demonstrated that tenascin-C contributed to the differentiation into TEC by showing that tenascin-C expressing cells displayed an increased tube formation in matrigel. Upon culturing in VEGF-containing medium tenascin-C expressing cells acquired expression of endothelial-specific markers as PSMA, VE-Cadherin and CD31 while selected cells not expressing tenascin-C did not. Since in the applied

FACS sorting approach it cannot be excluded that other molecules than tenascin-C are contributing to the described effect the published results await a confirmation by a tenascin-C knockdown approach (Pezzolo et al., 2011)). Together these results suggest that tenascin-C is a characteristic marker of a stem cell microenvironment and could trigger stem cell proliferation. Moreover tenascin-C also plays a role in the plasticity of these cells contributing to their differentiation into TEC.

2.4.3 Melanoma Cancer Stem Cells

Studies by Fukunaga-Kalabis *et al.* (2010) suggest that tenascin-C is a marker for stem cells in melanoma. By using oncosphere growth as read out for stemness the authors showed that tenascin-C is expressed in WM 3734 melanoma cells when they were grown as spheres but not when cultured in adherent conditions. It was demonstrated that tenascin-C is crucial for sphere growth of the melanoma cells since a tenascin-C knock down decreased sphere formation (Fukunaga-Kalabis et al., 2010). In addition, tenascin-C knock down led to a decrease of the stem cell like side population expressing the ATP binding cassette transporter ABCB5. Expression of this transporter mediates the efflux capacity for the chemotherapeutic drug doxorubicin. Upon tenascin-C knock down the melanomaspheres were significantly sensitized to doxorubicin. These results suggest that tenascin-C expression in melanoma cells does not only contribute to the stemness phenotype but also promotes their drug resistance.

2.4.4 Breast Cancer Stem Cells of the Metastatic Niche

The study of Oskarsson *et al.* (2011) demonstrated that in metastatic breast cancer cells tenascin-C expression was important for the formation and fitness of oncospheres. Tenascin-C also had an impact on the expression of the adult stem cell markers Mushashi and Lgr5, which appear to be crucial for inititation of metastasis. However tenascin-C was dispensable for the expression of the pluripotency markers Nanog, Oct4 and Sox2. Furthermore tenascin-C did not affect the CD44+CD24- antigen profile, which has been shown to be a characteristic marker for breast cancer stem cells (Oskarsson et al., 2011). Altogether these studies suggest that tenascin-C plays an important role in the stemness phenotype by supporting recruitment, proliferation and plasticity of stem cells, progenitors and cancer stem cells. Therefore it is crucial to elucidate the downstream signaling pathways regulated by tenascin-C, especially with the knowledge that cancer stem cells seem to be crucial for tumor growth and metastasis with the need for therapeutic approaches specifically targeting these cells.

3 The Role of Tenascin-C in Metastasis Formation

Metastasis occurs when tumor cells spread from the primary site and form a new tumor at a different site within the same or another organ. This process involves tumor cell migration, vessel invasion and extravasation into the distant organ tissue. While tumor cells travel in the blood or lymphatic circulation they need to survive. Only a few selected tumor cells that have gained the ability to leave the circulation and penetrate into the tissue of the secondary organ will grow into a new tumor (Chambers et al., 2002).

3.1 Tenascin-C Promotes Metastasis Formation

Several studies showed that tenascin-C is not only a predictor for poor prognosis (see section 1.2) but in several cancers high tenascin-C expression also correlates with metastasis to distant organs such as lymph nodes, liver and lung (for an overview see table 6). In contrast to these studies supporting a role of tenascin-C in metastasis there are also studies showing that tenascin-C expression does not correlate with metastasis or that a high tenascin-C expression exhibits an inverse correlation with metastasis (see table 6). This discrepancy of the described studies might be due to the analysed patient material or differences in the protocol used for the disease assessment and there is clearly a need for further investigations.

Organ:	Tenascin-C correlation with metastasis and/or invasion	References
Breast		
Small node-negative carcinoma	Staining at the invasion border but general stromal staining is no predictor for metastasis	(Jahkola et al., 1996)
Intraductal carcinoma	Stromal staining correlates with early invasion	(Jahkola et al., 1998a)
Axillary node-negative carcinoma	Expression at invasion boarder serves as prognostic factor for local recurrence	(Jahkola et al., 1998b)
Breast carcinoma	Positivity in cancer cells and stroma correlates with lymph node metastasis	(Ishihara et al., 1995)
Invasive breast carcinoma	No correlation with lymph node metastasis	(Shoji et al., 1993)
Benign tumors and ductal and lobular carcinoma	Increased expression and stromal staining in infiltrating carcinomas	(Gould et al., 1990)
Carcinoma	Changes in expression during menstrual cycle; increased stromal staining of infiltrating carcinomas	(Ferguson et al., 1990)
Primary invasive breast carcinoma	Tenascin-C expression at the invasive front was positively correlated with lymph node status	(Ioachim et al., 2002)
Infiltrating ductal carcinoma	Expression of tenascin-C in tumors with lymph node metastasis is higher than in those without lymph node metastasis	(Wang et al., 2010a)
Primary breast cancer	Tenascin-C mRNA expression no correlation with nodal status, tenascin-C was significantly associated with metastasis free survival of adjuvant tamoxifen-treated patients	(Helleman et al., 2008)
Breast cancer	Tenascin-C expression in primary tumor and lung metastatic foci is associated with lung metastatic relapse.	(Oskarsson et al., 2011)
Kidney		
Bladder neoplasia	Strong staining in stroma of invasive tumors	(Deen and Ball, 1994)
Inflammation and neoplasm of urinary bladder	Increased staining with inflammation and more intense in transitional cell carcinomas with strong stromal staining in infiltrating carcinoma cells	(Tiitta et al., 1993)
Clear cell renal cell carcinoma	Tenascin-C expression was an independent predictor of metastasis in patients with stage 1-3 disease.	(Ohno et al., 2008)
Lung		
Cancers with variety of clinicopathological features	Large tenascin-C isoforms found in cancer tissue and tenascin-C degradation is a marker for the metastatic potential	(Kusagawa et al., 1998)
Mesothelioma	Staining of malignant tumors at invasive front	(Procopio et al., 1998)
Non-small cell lung cancer	Tenascin-C expression was frequently observed in tumors with LN metastasis (P = 0.06)	(Han et al., 2003)
Female genital tract and ovaries		

Endometrial carcinoma	Tenascin-C correlates with metastasis, muscle and vascular invasion	(Doi et al., 1996)
Salivary glands		
Hyalinizing clear cell carcinoma of the salivary gland	Stromal marker for invasive front	(Felix et al., 2002)
Salivary gland tumors	Is higher in carcinomas ex-pleomorphic adenomas than in pleomorphic adenomas and correlates with disease progression (metastasis)	(Felix et al., 2004)
Other glands		
Pancreatic carcinoma	Identification of invasion promoting stroma	(Linder et al., 2001)
Pancreatic carcinoma	Is increased in carcinomas but does not correlate with poor differentiation, decreased survival, clinical stage or metastasis	(Juuti et al., 2004)
Pancreatic adenocarcinoma	Fibrilliar tenascin-C expression is associated with liver metastasis	(Chen et al., 2009)
Gastro-intestinal tract		
Gastric carcinoma	Strong stromal staining correlates with low stage but not with nodal status or metastasis	(Wiksten et al., 2003)
Colitis, colon adenoma and colorectal carcinoma	No BM staining at bottom of crypts in normal tissue, loss of this polarity in colitis, increased stromal staining in adenomas and carcinomas; correlation with lymph node metastasis	(Riedl et al., 1992)
Adenoma and carcinoma	Correlation between transcript levels and depth of invasion and frequency of metastasis to lymph nodes	(Hanamura et al., 1997)
Gastric carcinoma	No correlation with invasion, metastasis, survival	(Ilunga and Iriyama, 1995)
Gastric carcinoma and lymph node metastasis	No correlation with invasion, metastasis or prognosis	(Ikeda et al., 1995)
Colonic carcinoma with and without lymphogeneous metastasis	Very strong expression in every non-metastatic case; good correlation with prognosis	(Sugawara et al., 1991)
Colorectal cancer	Tenascin-C expression in tumor invasive area was significantly correlated with tumor progression, lymphatic invasion, lymph node metastasis and advanced pTNM stage	(Ide et al., 2007)
Gastric adenocarcinoma, colorectal adenocarcinoma, gastric adjacent non-cancerous mucosa and colorectal adjacent non-cancerous mucosa	Tenascin-C expression was negatively correlated with liver metastasis, but not with depth of invasion, venous invasion or lymph node metastasis.	(Zheng et al., 2007)
Diffuse and intestinal type gastric carcinoma	Enhanced staining in the stroma of invasive tumors	(Tiitta et al., 1994b)
Head and neck		
Oral squamous cell carcinoma	High transcript levels correlate with lymph	(Nagata et al., 2003)

	node metastasis	
Oral tongue squamous cell carcinoma	Tenascin-C mRNA expression showed no statistical significance both in negative and in positive lymph node metastasis patients although a trend was observed. Tenascin-C is a prognostic factor for survival.	(Wang et al., 2010b)
Squamous carcinoma of the floor of the mouth	Marker for *in situ* and invasive squamous carcinoma	(Regezi et al., 2002)
Laryngeal squamous cell carcinoma, dysplasias, papilloma	Stromal staining correlates with malignancy but not the histological grade of invasive carcinomas	(Goussia et al., 2000)
Laryngeal squamous carcinoma	Strong staining in invasive carcinomas	(Hagedorn et al., 1999)
Laryngeal carcinoma	stromal marker around cancer nests, cytoplasmic staining of cancer cells in majority of invasive carcinomas	(Yoshida et al., 1999)
Laryngeal squamous carcinoma	Stromal marker of carcinoma *in situ* and invasive carcinomas	(Uhlman and Niehans, 1999)
Oral squamous carcinoma	Intracellular tenascin-C staining in cancer cells of the invasive front	(Mori et al., 1996)
Oral squamous cell carcinoma	Enhanced stromal expression in invasive tumors with stongest expression at advancing edges of tumors	(Tiitta et al., 1994a)
Leukoplakia and oral squamous cell carcinoma	Increase in submucosa correlating with degree of hyperplasis/dysplasia and more intense and extending into stroma in SCC at the infiltrating tumor margin	(Shrestha et al., 1994)
Skin		
Melanoma	Higher expression in lesions of greater dermal invasiveness	(Natali et al., 1990)
Merkel cell carcinoma	Tenascin-C expression increased with tumor size and malignancy at sites of invasive growth, no correlation with metastasis	(Koljonen et al., 2005)
Primary melanoma	Absence of tenascin-C in stroma at invasion front correlates with lower risk for metastasis	(Ilmonen et al., 2004)
Extramammary Paget's disease	No correlation with level of invasion	(Kuivanen et al., 2004)
benign, dysplastic and malignant melanocytic tumors	Correlation with malignancy and metastasis	(Tuominen and Kallioinen, 1994)
Primary melanomas	Intensity of tenascin-C staining correlated with incidence of sentinel node micrometastases.	(Kaariainen et al., 2006)
Skeleton and teeth		
Odontogenic tumors	Staining at epithelial-mesenchymal interfaces in ameloblastomas and adenomatoid tumors and widespread stromal staining in fibromas and odontomas	(Mori et al., 1995)
Osteosarcoma	Correlation with metastasis and poor survival	(Tanaka et al., 2000)
Primary giant cell tumors	Tenascin-C expression (microarray) correlates with metastasis	(Pazzaglia et al., 2010)
Soft tissues, lymphomas		
Pedriatic rhabdomyosarcoma	All tumors stain positive without correlation to	(Saxon et al., 1997)

	tumor differentiation or metastasis	
Prostate		
Prostatic adenocarcinoma	Expression of large splice variants by carcinoma cells at tumor invasion front	(Katenkamp et al., 2004)

Table 6. Correlation of tenascin-C expression in cancer with invasion and metastasis. These data are adapted from supplementary table 1 in (Orend and Chiquet-Ehrismann, 2006). Recent publications arising since this review are highlighted in grey.

Three studies from the same laboratory on a large cohort of human breast cancer specimen support the possibility that tenascin-C may be a predictor for lung metastasis (Minn et al., 2005; Oskarsson et al., 2011; Tavazoie et al., 2008). A metastasis promoting impact of tenascin-C in breast cancer was demonstrated in murine xenograft experiments. By tail vein injection of human MDAMB-231 cells into immune compromised nude mice tumor cells were selected that homed to the lung. Upon transcriptomic microarray analysis on the highly lung metastatic versus the low lung metastatic cell line tenascin-C was identified in a gene signature that correlated with lung metastasis (Minn et al., 2005; Tavazoie et al., 2008). Similarly, Calvo et al. (2008) observed tenascin-C to be increased in MMTV-Myc induced breast tumors overexpressing VEGF that gave rise to micro- and macrometastasis which was in contrast to mice not overexpressing VEGFA where tenascin-C expression and the mestastasis rate were low. By comparison of the genes upregulated in the MMTV-Myc/VEGF tumors and upon comparison to genes upregulated in human breast cancer with metastasis, tenascin-C again turned out as a candidate of a lung specific metastasis signature (Calvo et al., 2008).

In contrast to these studies with a positive link of tenascin-C to lung metastasis in breast cancer, in two other studies no correlation of tenascin-C expression and metastasis was observed. Ramaswamy *et al.* (2003) compared the gene expression profile of adenocarcinoma derived metastasis of multiple tumors including breast cancer to unmatched primary adenocarcinomas (Ramaswamy et al., 2003). Landemaine *et al.* (2008) compared the gene expression signature from lung breast cancer derived metastasis with that of other non-pulmonary sites (Landemaine et al., 2008). This surprising discrepancy warrants further investigation.

There is evidence that tenascin-C plays also a role in bone metastasis. The authors showed that upon knock down of tenascin-C in lung, bone and brain metastatic breast cancer cell lines there is less metastasis in the lung and bones (Oskarsson et al., 2011). However, it needs to be determined why and how tenascin-C specifically influences the seeding, survival or proliferation of metastasis-initiating cells in certain organs while at other sites tenascin-C does not seem to play a role. It is possible that other ECM molecules or microenvironmental factors contribute to this site specific seeding of cancer cells, which may occur in conjunction with or independently of tenascin-C.

By using different murine cancer models the authors tried to elucidate the role of tenascin-C in lung metastasis. Tavazoie *et al*. (2008) used a xenograft model with immunodeficient NOD/SCID mice, where LM2 cells, which are MDAMB231 cells selected for lung metastageneicity, were intravenously injected. Upon knock down of tenascin-C the injected cells exhibited a reduced ability to colonize the lung (Tavazoie et al., 2008). Oskarsson *et al.* (2011) confirmed these results by using MDAMB231-LM2 and CM34-LM1 cell lines that also displayed a knock down for tenascin-C. Upon injection into the mammary fat pad or the tail vain of NOD/SCID mice less metastatic lesions in the lung were noticed (Oskarsson et al., 2011). Calvo *et al*. (2008) used another xenograft model, where tenascin-C null MDAMB 435 cells were injected into the mammary fat pad of nude mice. Also in this model a lowered tenascin-C expression correlated with a reduced lung metastasis (Calvo et al., 2008). Since tenascin-C has been shown to have immuno-modulatory functions (see section 2.2) the presented data might be hampered by the fact that the studies have been done in immunodeficient mice. Also the stromal

compartment in a murine host is different from that of the human tumor (Fantozzi and Christofori, 2006) and, human cells are not fully adapted to grow in a murine environment (Kuperwasser et al., 2005). In this context the study by O'Connell *et al.* (2011) is relevant since the authors have used the 4T1 immunocompetent Balb/c grafting model. Upon intravenous injection of the 4T1 cells into a tenascin-C KO host less metastatic lesions were observed in the lung (O'Connell et al., 2011). Altogether these studies suggest that tenascin-C plays a role in promoting breast cancer lung metastasis and that tenascin-C expressed by both the tumor cells and the host is relevant.

In contrast to the presented grafting studies Talts *et al.* (1999) had used a genetic model with stochastic breast tumor development and lung metastasis. Tenascin-C KO mice were crossed with MMTV/PyMT (polyomavirus (PyV) middle T oncogene under the transcriptional control of the mouse mammary tumor virus (MMTV) long-terminal repeat) transgenic mice, which spontaneously develop adenocarcinomas in the mammary gland and metastasize to the lung. In this model the authors observed neither a difference in tumor growth and size nor in the number of metastases in the lung (Talts et al., 1999). But organization of the tumor nests and ECM was different and the tumors were more infiltrated by macrophages in the absence of tenascin-C. These data suggest that other mechanisms may have promoted lung metastasis in the absence of tenascin-C. What these mechanisms are and whether they are a direct consequence of the absence of tenascin-C remains to be seen.

In summary there is increasing evidence that tenascin-C is an important component of the tumor microenvironment which promotes metastasis in several cancers. The correlation of high tenascin-C expression in the primary tumor to the formation of lung metastasis as demonstrated in several immunohistochemistry and gene expression analysis based studies on human cancer tissues has been recapitulated in a couple of murine tumor models, even if there is the deficit of a proper transgenic mouse model.

Not only does the overall expression of tenascin-C in the primary tumor seem to be important for a higher risk of metastasis but also its organization, the source and the place of expression. Chen et al (2009) showed that only the fibrillar organization of tenascin-C in pancreatic cancer correlated with metastasis. As MMP2 expression correlated with fibrilliar tenascin-C, the authors suggested that for the fibrilliar organization of tenascin-C the presence of MMP2 is required. Indeed in *in vitro* experiments co-culture of stromal fibroblasts with metastatic pancreatic cancer cells triggered fibrilliar tenascin-C organization, and most importantly this was suppressed by a MMP2 inhibitor (Chen et al., 2009). Moreover, non-metastatic pancreatic cancer cells deposited fibrilliar tenascin-C only upon addition of exogenous MMP2. A concomitant high expression of MMP2 and tenascin-C in the identified gene signature for breast cancer lung metastasis (Calvo et al., 2008) seems to support a potential link of tenascin-C and MMP2 to metastasis.

Several studies showed that not the overall expression of tenascin-C in the tumor is important but that the expression of tenascin-C at the tumor invasion front correlates with metastasis. This has been shown in colorectal carcinoma (Ide et al., 2007), primary invasive breast carcinoma (Ioachim et al., 2002; Oskarsson et al., 2011), early breast cancer and axillary node-negative breast carcinoma (Jahkola et al., 1998a; Jahkola et al., 1998b; Jahkola et al., 1996). These results suggest a role for tenascin-C in EMT, cancer cell migration and invasion, processes which have been demonstrated to be fundamental for metastasis formation.

3.2 Tenascin-C Promotes Cancer Cell Migration, Invasion and EMT

In several *in vitro* models with a variety of tumor cells (breast, colon cancer, glioma, chondrosarcoma, squamous cell carcinoma) tenascin-C has been demonstrated to increase migration and invasion (for summary see table 7).
A particular role of tenascin-C was demonstrated by a tenascin-C knockdown approach. Tumors derived from GBM cells knocked down for tenascin-C and engrafted into nude mice consisted of less infiltrating tumor cells and less tumor cell clusters in the surrounding brain tissue, despite the fact that no difference in tumor growth and proliferation was observed (Hirata et al., 2009).

Cell line	TNC	Assay		Reference
Breast cancer				
mouse mammalian cancer cell line, GHOM5E, was established from a spontaneously developing tumor in a congenic Tn-C-null mouse of the GRS/A strain	TNC (3 µg/ml) added to the medium	Transwell migration assay	increased migration compared to no treatment	(Tsunoda et al., 2003)
MDAMB 231	Added to the medium (10 µg/ml)	Matrigel invasion assay - Cells were plated on the cell-culture inserts pre-coated or untreated with Matrigel, 6h	Stimulates invasion together with TGFβ1	(Ilunga et al., 2004)
LM2 (derived from MDAMB 231, selected to metastasize to the lung, for details see (Minn et al., 2005)	TNC kd	Transwell invasion	decreased invasion compared to CTR SH	(Tavazoie et al., 2008)
M630 (Cell line from a Myc/VEGF tumor)	TNC blocking Ab	Wound closure	decreased migration compared to control, IgG treatment did not effect migration	(Calvo et al., 2008)
MDAMB 435	TNC kd	Wound closure	decreased migration compared to CTR SH	(Calvo et al., 2008)
MDAMB 231, MCF-7, T47D, MDDAMB 468	Overexpression of TNC isoforms with TNIII D or TNIII BD domains	Tumor cell invasion in real time with modified Boyden Chamber Assay	increased invasion compared to empty vector control	(Hancox et al., 2009)
MCF-7, T-47	Overexpression of the TNC isoform containing TNIII BAD1D domains	Tumor cell invasion in real time with modified Boyden Chamber Assay, up to 48 hrs	increased invasion compared to emtpy vector control	(Guttery et al., 2010b)
MCF-7	adding 10 µg/ml TNC to the medium	Wound healing	increased migration	(Nagaharu et al., 2011)
MDAMB231-LM2	TNC kd	Matrigel	decreased invasion	(Oskarsson et al.,

(Minn et al., 2005)		invasion/Transwell membrane	compared to SH CTR	2011)
Brain tumor				
U251.3	TNC coating (20 µg/ml)	Coating of undersurface of transwell membranes, 4 hrs	Increased migration compared to FN or BSA	(Deryugina and Bourdon, 1996)
U251.3	TNC coating (10 µg/ml)	Spheroid outgrowth assay, 1 or 2 days	Increased migration compared to FN	(Deryugina and Bourdon, 1996)
U178, U251	10 µg/ml embedded in the matrix	3D collagen I matrix, transwell invasion chamber	increased invasion	(Sarkar et al., 2006)
LN229	TNC knock down	Wound healing assay, 14h	decreased migration	(Hirata et al., 2009)
LN229	TNC knock down	Single cell locomotion assay, 12h	decreased migration, reversed by coating with 10 µg/ml TNC	(Hirata et al., 2009)
WM35, WM983, WM983B	TNC coating (2µg/cm²) together with collagen	Single cell tracking	Increased speed but no difference for displacement/track length) compared to collagen coating alone	(Grahovac et al., 2012)
Colon cancer				
HCT-8/E11, PC/AA/C1	Blocking TNC with BC24 Antibody	Invasion of cells seeded on top of collagend I, 24h	decreased invasion compared to IgG treatment	(De Wever et al., 2004)
HCT-8/E11	TNC (soluble or matrix-embedded – 1 or 2 µg/ml)	Invasion of cells seeded on top of collagen I, 24h	Increased invasiveness together with SF/HGF	(De Wever et al., 2004)
Squamous cell carcinoma				
SCC12	siRNA mediated knock down in CAF	Cells seeded on organotypic matrix enriched with CAF	No effect	(Gaggioli et al., 2007)
Bone cancer				
JJ012 chondrosarcoma	TNC coating (10 µg/ml)	Collagen based cell invasion assay	Increased invasion compared to FN or BSA	(Galoian et al., 2007)

Table 7. Tenascin-C modulates tumor cell migration and invasion *in vitro.*

Tenascin-C also appears to play a role in the intricate cross talk of myofibroblasts with tumor cells. Myofibroblasts isolated from colon cancer tissue stimulated the invasive behaviour of colon cancer cells in a tenascin-C dependent manner (De Wever et al., 2004). Colon cancer cells co-cultured with myofibroblasts rapidly invaded a collagen gel which could be blocked by treatment with an antibody against the tenascin-C

EGFR repeats, suggesting that deposition of tenascin-C by the myofibroblasts in the gel drives the invasion of the cancer cells. The authors linked this tenascin-C stimulated pro-invasive behaviour of carcinoma cells to down-regulation of RhoA signaling (De Wever et al., 2004). Similarly Gaggioli and colleagues (2007) observed that squamous carcinoma cells invade an organotypic matrix upon co-culture with stromal fibroblasts that were derived from oral or vulval squamous cell carcinoma. Stromal fibroblasts invaded and strongly modified the matrix by promoting degradation and deposition of matrix including tenascin-C and fibronectin. The collagen matrix within these tracks was organized into thick bundles (Gaggioli et al., 2007). The authors demonstrated that those tracks promoted carcinoma cell invasion. However, tenascin-C and fibronectin seemed not to be crucial for the invasion, as a knockdown of these molecules in fibroblasts did not change the number of invading carcinoma cells. What the role of tenascin-C in this context is remained elusive.

It is conceivable that the fibrillar tenascin-C matrix observed in pancreatic cancer (Chen et al., 2009) and in melanoma (Kaariainen et al., 2006) has similarities with the tenascin-C matrix tracks observed in cell culture (Gaggioli et al., 2007). Kaariainen et al., (2006) showed that in melanoma where tenascin-C mRNA expression correlated with invasiveness and metastatic lesions, tenascin-C is assembled together with several other ECM such as fibronectin, laminins and pro-collagen I within channels or matrix tracks. It was shown that melanoma cells are located within these matrix channels which supports a potential contribution of these channels to melanoma dissemination and metastasis (Kaariainen et al., 2006). Also other studies revealed a high expression of several matrix molecules (laminin-5 and collagens) together with tenascin-C in metastatic conditions, as shown in MMTV-VEGF/c-myc transgenic tumors compared to non-metastatic c-myc tumors (Calvo et al., 2008). It is conceivable that a combined high expression of several ECM molecules and their assembly into matrix tracks or channels contributes to metastasis by e.g. providing dissemination cues and/or local niches that promote survival of disseminated tumor cells. The role of tenascin-C expression in matrix tracks has been furthermore extensively addressed in reviews by Midwood et al., 2011 and Van Obberghen-Schilling et al., 2011.

However it is unknown what the exact role of tenascin-C within these matrix channels would be, as e,g, promotion of survival, proliferation and/or migration. A potential supportive role in these events is not unlikely given the promoting effect of tenascin-C on survival (Oskarsson et al., 2011), proliferation (Huang et al., 2001; Orend and Chiquet-Ehrismann, 2006) and migration of tumor cells (see table 7). Another question is whether and how these tenascin-C matrix channels play a role in tumor cell dissemination throughout the entire body. These matrix channels were not lined by blood nor lymphatic endothelial cells (excluding that they are a physical component of functional vessels) but exhibited erythrocytes which supports a connection of the tumor mass to the circulation through the tenascin-C matrix channels. Although highly intriguing proof is missing that would demonstrate an active role of these matrix channels in tumor cell dissemination and metastasis. Moreover, it is unknown whether tenascin-C plays a role in the formation and/or function of these matrix channels.

Grahovac *et al.* (2012) showed that the EGFL repeats of tenascin-C in particular might regulate migration and invasion of melanoma cells. Overexpression of the tenascin-C EGF-like domain in melanoma cells increased their invasion into matrigel (Grahovac et al., 2012). This could be blocked by a rho-associated protein kinase (ROCK)-inhibitor, suggesting the involvement of a tenascin-C EGF-like induced cytoskeletal alteration in the process of matrigel invasion. However in 2D assays overexpression of tenascin-C EGF-like rather decreased cell migration suggesting that the 3D organization of tenascin-C EGF-like region is instrumental. These observations also raise the possibility that specific domains in tenascin-C might induce different cell responses. An overrepresentation of specific tenascin-C domains mostly encompassing the alternativley spliced TNIII repeats but not the EGF-like repeats had been demonstrated in several cancers. In particular in breast cancer the expression of tenascin-C isoforms, containing TNIII D, TNIII BD or TNIII BAD1D domains, were associated with increased tumor cell invasion (Adams et al., 2002; Guttery et al., 2010b; Hancox et al., 2009). The exposition of certain domains within tenascin-C could also arise from cleavage of the molecule by MMPs and other proteases. Cleavage of tenascin-C by memprin-beta1 was shown to abrogate the syndecan-4 inhibitory activity of tenascin-C on a mixed fibronectin/tenascin-C substratum (Ambort et al., 2010).

Tenascin-C itself might also increase the expression and activity of MMPs stimulating the invasive behaviour of cancer cells. Ilunga *et al.* (2004) showed that MDAMB231 invasion into matrigel was stimulated by the addition of both tenascin-C and TGFβ1, which could be blocked by a MMP-inhibitor (Ilunga et al., 2004). Similarly, tenascin-C also stimulated the invasion of glioblastoma cells by increasing protein kinase C δ activity and MMP12 expression and migration could be inhibited by MMP-inhibitors or an antibody specific for MMP12 (Sarkar et al., 2006; Sarkar and Yong, 2010).

Epithelial mesenchymal transition (EMT), characterized by the loss of epithelial cell-cell contacts and a gain of a fibroblast-like motile phenotype that enables tumor cell migration and invasion (Huber et al., 2005) is considered as a key feature toward metastasis. There is evidence that EMT is promoting metastasis and that EMT often occurs at the invasive front of a tumor, where tenascin-C has been shown to be highly expressed (see table 6). This finding supports a potential role of tenascin-C in EMT.

Indeed, in breast carcinoma tenascin-C expression correlated with the expression of the mesenchymal marker vimentin and, in several cancer cell lines tenascin-C and vimentin were found to be coexpressed (Dandachi et al., 2001). Moreover, tenascin-C is highly expressed at the invasive front of colorectal tumors at sites with nuclear β-catenin (Beiter et al., 2005). Nuclear β-catenin has been demonstrated to be an EMT marker (Kim et al., 2002). Knock down of β-catenin in colon cancer cell lines indeed resulted in reduced tenascin-C expression and the authors provided evidence that β-catenin is directly regulating tenascin-C promoter activity (Beiter et al., 2005) which suggests a positive feedback regulation. Nagaharu and colleagues (2011) demonstrated that upon a combined treatment with tenascin-C and TGFβ-1 MCF7 and T47-D breast cancer cells acquire an EMT-like phenotype, characterized by loss of membranous E-cadherin and β-catenin, which was linked to increased cell migration (Nagaharu et al., 2011). Furthermore tenascin-C seemed to be involved in regulating the EMT process in the mouse lense epithelium upon injury (Tanaka et al., 2010). In another study tenascin-C promoted a partial EMT in MCF7 cells that changed their cobblestone epithelial morphology into a fibroblastoid phenotype upon growth on a tenascin-C substratum. This was linked to an altered expression of the adaptor protein 14.3.3tau (Martin et al., 2003). These observations suggest that tenascin-C promotes EMT but may also be regulated by EMT, as it was shown to be a β-catenin target gene. Thus highly expressed tenascin-C at the tumor invasion front will enhance cancer cell migration and invasion.

In summary, tenascin-C might increase cancer cell invasion and migration by promoting EMT and/or by enhancing the activity or expression of MMPs. Organisation of tenascin-C into fibrillar tenascin-C channels presumably plays another important role in metastasis.

3.3 Tenascin-C in the Metastatic Niche

Disseminated tumor cells which form metastasis in the tissue of a secondary organ need a perceptive microenvironment or niche that promotes survival and expansion at the secondary site. The seed and soil hypothesis argues that this niche can either be prepared by the arriving tumor cells (the seed) or by the organ microenvironment (the soil) that is already modulated by soluble factors secreted from cells of the primary tumor (Paget, 1989). The metastatic niche therefore is described as a specialized microenvironment which supports maintenance and growth of metastasis-initiating cells (Psaila and Lyden, 2009). Fibronectin and MMPs in the (pre-)metastatic niche seem to enable the seeding and survival of arriving metastatic cells (Psaila and Lyden, 2009). Given the involvement of tenascin-C in the primary tumor and in metastasis it is possible that tenascin-C plays a role both in the pre-metastatic and metastatic niche.

Two recent studies addressed whether tenascin-C plays a role in preparing the metastatic niche. O'Connell *et al.* (2011) demonstrated that S100A4 (Mts1/FSP) positive stromal cells, which are mesenchymal cells including fibroblasts are a source for tenascin-C. S100A4+ cells significantly contributed to metastasis in the lung upon injection of 4T1 breast cancer cells into a syngeneic host, since ablation of S100A4+ cells decreased the number of metastatic lesions in the lung. Moreover tenascin-C expression in the metastatic lung tissue was strongly reduced upon ablation of S100A4+ cells (O'Connell et al., 2011). This argued for an

important role of stromally expressed tenascin-C in the colonization and outgrowth of tumor cells in the lung. By means of 4T1 cells injected into tenascin-C KO mice the authors confirmed that indeed stromally provided tenascin-C is important for the colonization of tumor cells at the metastatic site. However a lack of tenascin-C expression by stromal cells did not induce a difference in angiogenesis. The authors further showed that VEGFA secreted by the S100A4+ cells is important for the seeding of the tumor cells in the lung (O'Connell et al., 2011). Whether VEGFA plays a role in the preparation of a pro-angiogenic microenvironment as claimed by the authors or whether VEGFA supports survival (Lichtenberger et al., 2010) is an open question. Together these results suggested that tenascin-C secreted by S100A4+ stromal cells promotes lung metastasis by preparation of a permissive microenvironment.

Oskarsson *et al* (2011) demonstrated that tenascin-C provided by disseminated breast cancer cells plays an important role for the survival and growth of metastasis-initiating cells in the lung(Oskarsson et al., 2011). However, expression of tenascin-C by the cancer cells itself seemed only to be crucial at early time points of the metastatic outgrowth while later knock down of tenascin-C did not affect growth of the metastatic lesions. This might be due to the fact that metastatic cells have already activated the surrounding stroma to express tenascin-C, most likely involving the S100A4+ cells identified by O'Connell et al (2011). The authors further elucidated by which mechanism tenascin-C regulated the lung metastasis potential of breast cancer cells (Oskarsson et al., 2011). Tenascin-C decreased apoptosis of cells in the metastasis site while it did not change their proliferation. Tenascin-C knock down in breast cancer oncospheres showed that tenascin-C indeed specifically promoted the survival of metastasis-initiating cells but not their self-renewal capacity. In this model tenascin-C expression was significantly enhanced in comparison to monolayer cultures and correlated with the expression of the pluripotency markers Sox2, Oct4 and Nanog and the adult stem cell markers Musahsi (MSI1) and LGR5. However, tenascin-C knock down revealed that tenascin-C is not regulating the expression of these putative pluripotency markers, while the activity of Wnt and notch signaling was shown to be reduced in a tenascin-C dependent manner (Oskarsson et al., 2011). Using knock down and blocking strategies the authors concluded that tenascin-C regulates lung metastasis by stimulating the expression of the Wnt target Lgr5 and suppressing JAK2-STAT5 signaling which would result in MSl1 induction and Notch pathway activation.

Other important ECM components playing a role in tumor progression and formation of metastasis have been shown to be co-expressed with tenascin-C and even bind to tenascin-C. In particular fibronectin and periostin have been shown to interact with tenascin-C. Interactions between fibronectin and tenascin-C were extensively reviewed by Van Obberghen-Schilling et al., 2011. Kii et al., 2010 demonstrated that periostin is important for the incorporation of tenascin-C into the matrix meshwork probably due to bridging binding of tenascin-C to other ECM components such as fibronectin (Kii et al., 2010) although tenascin-C can also directly bind to fibronectin (Huang et al., 2001). Recently, Malanchi et al, (2011) demonstrated an important role for periostin in lung metastasis derived from breast cancer. The authors demonstrated Wnt ligand binding to periostin which seems to stimulate the colonization of metastasis-initiating cells (Malanchi et al., 2011). It is highly interesting to speculate that tenascin-C and periostin act together to promote the survival of metastasis initiating cells (Oskarsson and Massague, 2011); while periostin presents Wnt ligands to the cancer cells (Malanchi et al., 2011), tenascin-C downregulates the Wnt inhibitor DKK1, stabilizes β-catenin (Ruiz et al., 2004) and promotes expression of the Wnt target gene Lgr5 (Oskarsson et al., 2011).

In summary, tenascin-C seems to promote metastasis by different poorly understood mechanisms. Tenascin-C expression by either tumor or stromal cells in the primary tumor promotes cancer cell dissemination and survival in the circulation. A promoting role of tenascin-C on proliferation, migration, invasion and EMT and the formation of particular tenascin-C rich matrix channels is in agreement with this concept. Moreover, in addition to the expression of tenascin-C in the primary tumor early expression of tenascin-C by tumor cells and later expression by stromal cells also plays a crucial role in metastasis. Recent publications suggest a role for tenascin-C in cooperating with other ECM molecules in inducing notch and Wnt signaling that would promote the survival and colonization of cancer cells at the metastatic site. Despite these important insights it is still an enigma how tenascin-C in the primary tumor can impact on the pre-metastatic niche. Which domains of

tenascin-C and which tenascin-C receptors are involved? Which effects are triggered by a direct contact of tenascin-C with the cells, which effects are indirect and mediated by alterations in tissue organization and stiffness? How are the identified tenascin-C linked signaling pathways regulated by tenascin-C? For addressing these questions more appropriate 3D co-culture models with defined tenascin-C subdomain molecule expression and immune competent tumor models with a defined tenascin-C expression and a stochastic development of tumors and metastasis are needed.

4 Clinical Impact

Cancer is a leading cause of death in humans with a high complexity and often patient-specific etiopathology. It therefore requires the development of new and advanced strategies for anti-cancer treatment. The most commonly used ways to treat cancer are resection, chemotherapy and radiation. Whereas in some cancer types these treatments are highly effective, other tumor types are resistant to cytotoxic agents or ionizing radiation and severe side-effects to intact organs frequently occur. In the meantime, new concepts have been developed such as vaccination, the use of antibodies, peptides, nucleic acids or protein inhibitors to target cancer-specific signaling pathways or proteins, including tenascin-C.

4.1 Targeting Tenascin-C in Cancer

In adult tissues, the expression of tenascin-C is largely absent but it is induced in a large number of cancerous tissues (see section 1). Extensive research, the most recent of which has been summarized in the preceeding sections of this review, has shown that tenascin-C plays an important role in different phases of tumor progression and is therefore a promising target to improve current diagnostic and therapeutic approaches in anti-cancer treatment. This is evidenced by the high number of filed patents which protect the use of specific tenascin-C domains, the production and use of monoclonal antibodies against these domains or the development of novel therapeutic concepts targeting tenascin-C expression based on nucleic acids. In the following section, we will first summarize some of the most advanced strategies of anti-cancer treatments that target tenascin-C that are currently in development or have advanced into clinical testing (see also table 8). Subsequently, some of these strategies will be discussed in more detail.

4.2 Anti-tenascin-C Antibodies in Anti-cancer Therapy

The generation of monoclonal antibodies with strong affinities to a specific molecule is the most applied strategy in the development of novel anti-cancer drugs. Such antibodies can be modified and coupled with cytotoxic agents such as cytokines. Several antibodies, mostly recognizing isoforms of tenascin-C containing the alternatively spliced domains A1 to D, have been developed and a few have been examined in preclinical or clinical trials (table 8, figure 3). A detailed summary of the use of anti-tenascin-C antibodies in radiotherapy of cancer can be found in the book “Monoclonal Antibody and Peptide-Targeted Radiotherapy of Cancer” (Reilly, 2010).

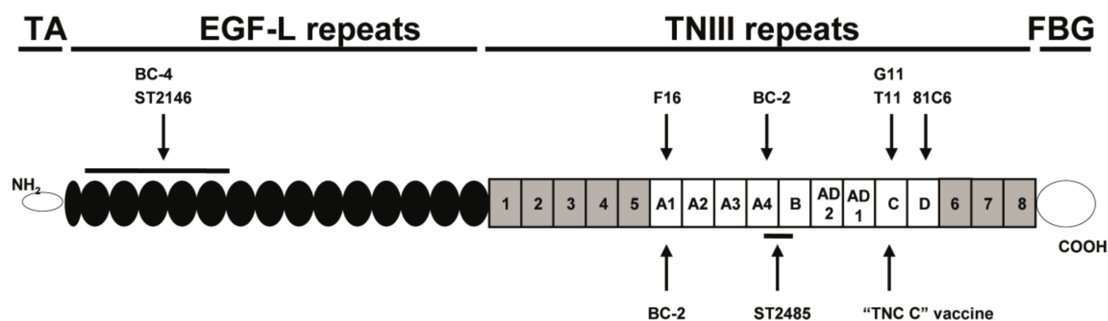

Figure 3. Overview of monoclonal antibodies and vaccine generated for targeting tenascin-C in anti-cancer treatments. Black arrows indicate the specific tenascin-C targeting domain of the different antibodies. "TNC C" vaccine is based on a fusion protein of thioredoxin and a peptide comprising TNIII C repeat. A detailed summary including references can be found in table 8.

The monoclonal antibody **81C6** was generated from mice which were immunized with the glioma cell line U-251 MG. It exhibits specific binding to tenascin-C of different cancer types including glioma (Bourdon et al., 1983) and specifically targets TNIII-domain C/D (Bourdon et al., 1985). The potential of this antibody in cancer therapeutics for delivery of radioisotopes was tested in the 1980s in subcutaneous and intracranial xenograft mouse models of glioma. Intravenous injection of iodine labeled 81C6-^{131}I inhibited tumor growth compared to non-specific isotype control antibody (Lee et al., 1988a; Lee et al., 1988b). After improvement of this antibody leading to higher tumor accumulation and enhanced stability (Sampson et al., 2006) several phase I and II clinical trials were performed to determine dose, efficacy and toxicity on glioma patients with the purpose to target remaining tumor cells after resection. **81C6-^{131}I** was delivered by injection into the surgically created resection cavity followed by either fixed radiotherapy dosing (Reardon et al., 2006) or patients-specific 44-Gy boost (Reardon et al., 2008). Another phase I study used α-particle-emitting radionuclide ^{211}At with shorter range and more potent cytotoxicity. 81C6 coupled to ^{211}At (**81C6-^{211}At**) was injected into the resection cavity of glioma patients (Zalutsky et al., 2008). So far, these three studies showed safe administration of radionuclide coupled 81C6 with low toxicity and survival benefit in some patients but a significant therapeutic improvement could not be observed in the low numbers of patients included in the studies. A follow up phase III clinical trial for combination therapy of 81C6-^{131}I with Temozolomide and radiotherapy after tumor resection was planned but terminated due to funding issues. Another phase II trial for combination therapy with the anti-angiogenic drug Bevacizumab was requested by Bradmer Pharmaceuticals but the status is currently unknown.

The company Philogen developed a fully humanized antibody called "**F16**" which specifically recognizes the TNIII-domain A1 of tenascin-C. Injected in the small immunoprotein (SIP) format, which comprises a protein derived from the variable region fragment of an antibody to tenscin-C, in a brain cancer xenograft model, the antibody accumulated selectively in the tumor but not in other organs (Brack et al., 2006). Using this antibody for tissue staining showed tenascin-C expression in many different cancer types such as in lymphoma (Schliemann et al., 2009), renal cell carcinoma (Berndt et al., 2010), lung cancer (Pedretti et al., 2009), head and neck cancer (Schwager et al., 2011), glioblastoma multiforme (GBM) (Pedretti et al., 2010) and melanoma (Frey et al., 2011) suggesting a possible application in many cancer types. The antibody was coupled to interleukin-2 (IL2) with the aim to improve classical treatment such as chemotherapy. IL2 is approved for treatment of cancer (Proleukin®, Novartis) and shows activity against renal cell carcinoma, melanoma, lymphoma or leukemia. Applied in the conjugated scFv format (**F16-IL2**, Teleukin) in a breast cancer xenograft model in combination with the chemotherapeutic agents paclitaxel or doxorubicin showed a significant reduced tumor volume as compared to single treatment with IL2 or chemotherapy alone (Marlind et al., 2008). The antibody was also tested in preclinical models of brain cancer together with standard chemotherapy of

Temozolomide. The combined treatment led to complete tumor remission in animals with subcutaneous tumors. In an orthotopic setting, tumors were smaller and the survival rate of the animals was prolonged (Pedretti et al., 2010). Currently, a phase I/II clinical trial is in progress in patients with solid tumors (including breast and lung cancer) where F16-IL2 is applied together with paclitaxel or doxorubicine. First results from phase Ib trials showed that the combined treatment can safely be administered with disease stabilization in a few cases (De Braud et al., 2011). Another phase I/II clinical trial using F16 is currently in progress. Here, F16 is coupled to iodine (**^{131}I-F16SIP**, Tenarad) for use in radiotherapy in several cancer types. A preliminary evaluation of a low number of patients suffering from Hodgkin's disease and refractory to conventional treatment showed acceptable toxicity. Some of the patients showed disease stabilization with reduced number and/or size of lesions (Aloj et al., 2011).

A few other monoclonal antibodies have been raised against tenascin-C for a cancer-specific radionuclide delivery. Some of them were modified to reach higher purity levels for pharmaceutical use or techniques have been developed to enhance sensitivity of current applications. Some of these antibody-based methods are listed in table 8, but are not discussed in more detail. None of them reached clinical trials beyond phase II. For the moment, antibody-based cancer-therapeutics targeting tenascin-C have only been tested in phase I/II clinical trials for safety, dose and toxicity issues using small patient cohorts, mainly suffering from brain tumors. Most of these studies show that the agents can be safely administered and some indicate that the developed strategy might be beneficial in severe cases of cancer which are refractory to conventional therapies. Studies on larger patient cohorts need to be performed to evaluate a beneficial therapeutic effect justifying an application in the clinic.

4.3 Nucleic Acid-based Strategies to target Tenascin-C Expression in Cancer

The development of nucleic acid-based strategies is an alternative to the use of antibodies in cancer therapeutics. Such novel drugs include ribozymes, small interfering RNAs (siRNA) or aptamers which are already in clinical trials for different pathologies, including cancer (Burnett and Rossi, 2012).

Interesting results for a potential use of **RNA interference** (RNAi) in cancer therapy was demonstrated for the first time by Barciszewski and colleagues in human glioma (Rolle et al., 2010; Wyszko et al., 2008; Zukiel et al., 2006). 46 patients (grade II, III and IV) were injected with **ATN-RNA**, a double stranded RNA homologous to tenascin-C mRNA, into areas of neoplastic infiltration after resection of the primary or recurrent tumor. Tumor growth was followed by MRI or CT imaging and median survival rates were compared to 48 patients which had obtained brachytherapy (radiation). This comparison showed that ATN-RNA application prolonged survival by 4.8, 13.2 and 13.9 weeks in glioma patients with grade II, III and IV disease, respectively. It further improved quality of life as determined by the Karnofsky Performance Scale. A beneficial effect was even better for patients with recurrent tumors (Rolle et al., 2010). Although the authors claim that the use of dsRNA ATN-RNA has many advantages in terms of natural target for RISC, no interferon induction capacity, non-requirement of further stabilization and no off-target effects (Rolle et al., 2010), knock down of tenascin-C will only occur transiently at the site of injection and therefore needs repetitive administration with unknown side effects and high costs. Beside these first promising results it is conceivable that the development of a long-lasting knock down for tenascin-C with systematic administration could be successful in targeting TNC in glioblastoma.

Aptamers are short oligonucleotides with specific binding to a target molecule with very high affinity and selectivity, similar to monoclonal antibodies (Tuerk et al., 1992). The characteristics (e.g. specificity, stability, size) can easily be modified. Aptamers can be linked to other molecules such as cytokines. Using the SELEX technology applied to U251 glioblastoma cells or purified tenascin-C protein, the aptamer "**TTA1**" was generated with a high specificity and selectivity for human tenascin-C (Hicke et al., 2001). Further modifications improved biodistribution and injection of radionuclide-labeled **^{99}mTc-TTA1** showed a tumor specific uptake in mouse models of the xenografted human glioma (Schmidt et al., 2004), colon, breast and rhabdomyosarcoma

(Hicke *et al.*, 2006). This suggests a possible application in various cancer types. In the same group another aptamer was generated (**GBI-10**). Although *in vivo* studies are not published the authors present data where GBI-10 was used for affinity purification of target proteins. Liquid chromatography tandem mass spectroscopy revealed the interaction of GBI-10 with several tenascin-C peptides. These were located along the whole protein sequence demonstrating target-specific binding of the aptamer to several sites within tenascin-C (Daniels et al., 2003). Recently, Ko and colleagues further extended the aptamer approach and generated a multimodal nanoparticle-based Simultaneously Multiple Aptamers and RGD Targeting (**SMART**) probe targeting nucleolin, integrin $\alpha v\beta 3$ and tenascin-C at once. Compared to single-target probes TTA1, RGD or AS1411 the multi-target probe SMART provided a strongly enhanced specificity and binding intensity when applied to different human cancer types *in vitro*. These included C6 brain, DU145 prostate, HeLa cervical, NPA thyroid papillary and A549 non-small lung cancer cell lines (Ko et al., 2011). However, no proof-of-concept has yet been performed in pre-clinical *in vivo* models.

Compared to the similar approach of using radionuclide-labeled monoclonal antibodies, aptamers might be advantageous as they are smaller, faster to produce by chemical synthesis and thus cheaper than antibodies. As has been shown for example with GBI-10, aptamers can also have several binding sites within the target protein whereas monoclonal antibodies only bind to one specific region. This might become important to specifically target one or several splice variants of tenascin-C which are unique for specific cancer types or which are associated with enhanced malignancy. Other advantages of aptamers are that they can easily be modified to improve their pharmacological properties, their resistance to degradation or to delay their renal elimination (Burnett and Rossi, 2012).

4.4 Vaccination

The development of therapeutic vaccines for treating existing tumors is another novel approach in cancer-therapeutics. To date, only one vaccine is approved by the FDA for use in patients with metastatic prostate cancer (Gardner et al., 2012) and many more are currently tested in clinical trials for different cancer types. A vaccination approach targeting several tumor specific antigens, including TNIII C-domain, is currently under development by Olsson and colleagues which was recently protected by a patent (Olsson and Hellman, 2011). This strategy is based on six different antigens which are preferentially expressed in the tumor vasculature but not in healthy tissue or normal blood vessels: fibronectin extra-domains A and B (FN-EDA, FN-EDB), extra-domain C of tenascin-C (**TNC C**), annexin A1, endosialin (CD248) and magic roundabout (MR). The invention contains the development of a vaccine using one or a combination of these six proteins which are modified in a way that the immune system recognizes the modified self-protein as a non-self protein. To date, the *in vivo* proof of concept was only shown for FN-EDB. A fusion protein between the 91 amino acid long FN-EDB and *E.coli* thioredoxin was generated (TRX-EDB) and injected into mice which then showed high titers of anti-EDB antibodies. Then, T241 fibrosarcoma cells were grafted subcutaneously into these mice. Mice treated with TRX-EDB developed much smaller and highly necrotic tumors which lacked a structured organization compared to a control group. Consistently, tumor vessels showed an altered morphology and the presence of macrophages within the endothelium indicating destruction of the blood vessels by the immune system (Huijbers et al., 2010). Furthermore, vaccination of mice and rabbits against **TNC C** showed the presence of TNC C-specific antibodies in serum of these animals. A combination therapy against EDA, EDB and TNC C together is currently under investigation and will be tested in xenograft experiments or models of spontaneous tumor formation.

The strategy was further applied in a second independent model of spontaneous tumor formation. Upon immunization against EDB, Rip1-Tag2 mice showed a reduced number of pancreatic tumors compared to control animals (Huijbers, 2012). This tumor model better mimics tumor formation in humans than xenograft experiments and therefore suggests that this therapeutic approach could be applied in patients with already established tumors. Compared to the use of monoclonal antibodies, the vaccination approach would overcome cost-intensive repetitive injections as the organism produces an immune response and the tumor antigen specific antibodies by itself. This approach may even allow vaccination against several tumor antigens simultaneously.

Compound	Strategy	Cancer type	Latest clinical stage	Observation	Reference
Antibody-based					
81C6-^{131}I (Neuradiab)	Regional targeted radiotherapy; Injection into cavity after resection; Co-treatment with chemotherapy	Glioma patients	Phase I/II	Feasible, No/low toxicity, Non-significant but encouraging overall outcome	(Reardon et al., 2006; Reardon et al., 2008)
81C6-^{211}At					(Zalutsky et al., 2008)
81C6-^{131}I (Neuradiab)	Co-treatment with Bevacizumab	Glioma patients	Phase II (unknown status) NCT00906516	-	http://clinicaltrials.gov/ct2/show/NCT00906516.
F16-IL2 (Teleukin)	Co-treatment with Temozolomide	U87 (GBM)	Preclinical	Growth inhibition; Survival prolongation	(Pedretti et al., 2010)
	Co-treatment with Doxorubicine	MDA-MB231; Advanced solid tumors, Breast cancer, Lung cancer	Phase Ib/II (2008-2013) NCT01131364	Safe administra-tion; disease stabilization	(De Braud et al., 2011; Marlind et al., 2008)
	Co-treatment with Paclitaxel		Phase Ib/II (2008-2013) NCT01134250		
F16-^{131}I (Tenarad)	Radionuclide therapy	Hodgkin's disease, Solid tumors	Phase Ib/II NCT01240720	Good partial response, stabilization of Hodgkin's disease	(Aloj et al., 2011)
BC-2-^{131}I, BC-4-^{131}I	Regional targeted radiotherapy	Glioma patients	Phase II	Stabilization, partial or complete remission	(Riva et al., 1994)
BC-4-biotin + avidin + ^{99}Y-biotin	Pre-targeted Antibody-Guided RadioImmunoTherapy (PAGRIT)	Glioma patients	Phase I	Stabilization or partial remission	(Paganelli et al., 2001)
ST2146, ST2185	PAGRIT; combination of anti-tenascin-C antibodies	HT-29 (colon), U-118 (brain)	Preclinical	Tumor-specific localization	(De Santis et al., 2006; Petronzelli et al., 2005)
G11, G11-IL2	mAb generation targeting TNIII C-domain	Lung, brain	Preclinical	Tumor-specific localization	(Silacci et al., 2006)
TN11	mAb generation targeting TNIII C-	-	-	Suitable for histological	(Carnemolla et al., 1999)

	domain			staining (breast, brain cancer)	
Nucleic-acid based					
anti-TNC dsRNA (ATN-RNA)	interference RNA intervention	Brain cancer	46 patients after tumor resection	Prolonged survival; Improved quality of life	(Rolle et al., 2010)
Aptamer ^{99}mTc-TTA1	Targeted radiotherapy	Various xenograft models	Preclinical	Tumor-specific uptake	(Hicke et al., 2001; Hicke et al., 2006; Schmidt et al., 2004)
Aptamer GBI-10	Aptamer generation by SELEX to target tenascin-C	-	-	Interaction with several tenascin-C peptides	(Daniels et al., 2003)
SMART Simultaneous Multiple Aptamers and RGD Targeting	Nanoparticles coated with aptamers and peptides targeting nucleolin, tenascin-C and RGD at once	Several human cancer cell lines	-	Enhanced sensitivity compared to mono-targeting strategy	(Ko et al., 2011)
Vaccination					
TRX-TNC C	Fusion proteins of thioredoxin with tumor vessel specific antigens (TNC C domain, FN-EDA, FN-EDB, ANXA1, CD248, MR)	Subcutaneous mouse xenograft; spontaneous Rip1-Tag2 model	Preclinical	High anti-TNC antibody titers in immunized mice or rabbits; Reduced tumor volume (FN-EDB)	(Huijbers, 2012; Huijbers et al., 2010; Olsson and Hellman, 2011)

Table 8. Overview of strategies for tenascin-C targeted cancer therapeutics. For each approach, the compound name, its envisaged therapeutic strategy and targeting cancer type is listed. The last known clinical stage and short summary of main observations which can be found in the indicated references are specified. If known, NCT identifiers are indicated for clinical studies listed on the ClinicalTrials.gov webpage.

4.5 Tenascin-C and its Value in Cancer Prediction, Treatment and Diagnosis

As described in detail in section 1 tenascin-C is highly expressed in most malignant solid tumors (Orend and Chiquet-Ehrismann, 2006) as evidenced by strong tissue staining and high mRNA expression levels in cancer tissue. As a potential prognostic marker its presence was also analyzed in body liquids and it could be detected in serum of patients with several cancers such as colorectal carcinoma (Riedl et al., 1995; Takeda et al., 2007), malignant melanoma (Burchardt et al., 2003), pancreatic cancer (Esposito et al., 2006) or non-small cell lung

cancer (Ishiwata et al., 2005). Unfortunately, tenascin-C in serum is a questionable general tumor marker as many patients display normal tenascin-C levels which do not correlate with tumor progression. Moreover, in prostate cancer tenascin-C serum levels rather correlate with signs of inflammation and infection than with cancer (Schenk et al., 1995).

However, tenascin-C still may become a valid predictive marker in some cancer types as its high expression in the tumor tissue often correlates with poor prognosis or metastasis (Orend and Chiquet-Ehrismann, 2006) (table 1, table 6). Here we discuss GBM as the cancer type for which specific tenascin-C expression has been most been exploited as unique tool for imaging or treatment.

GBM is one of the most aggressive tumors with poor prognosis. In glioma patients, high stromal tenascin-C expression levels were associated with tumor invasion and shorter disease-free survival (Leins et al., 2003). Surgery and radiotherapy have only a short benefit on overall survival of GBM patients and tumors often re-grow after resection. Due to strong vascularization of these tumors anti-angiogenic therapies are currently under clinical testing (Chi et al., 2009). Phase I/II clinical trials using Bevacizumab (Avastin, Genentech), a monoclonal antibody targeting VEGFA, demonstrated improved progression-free survival in recurrent GBM with or without combination therapies (Friedman et al., 2009; Kreisl et al., 2009). Yet, a significant number of patients did not respond to the treatment (Vredenburgh et al., 2007) or even showed tumor regrowth and accelerated clinical decline after drug withdrawal (Zuniga et al., 2010). It seems that the use of anti-angiogenic treatment is questionable in some cancer patients such as GBM and may even worsen prognosis.

This was further investigated in different *in vivo* tumor models. Anti-angiogenic tumor therapies targeting VEGF receptor signaling showed beneficial anti-tumor effects but at the same time it accelerated metastasis formation and decreased survival (Ebos et al., 2009) or it increased tumor invasiveness and supported the formation of local and distant metastasis (Paez-Ribes et al., 2009) after drug withdrawal. It was observed that after anti-angiogenic treatment endothelial cells were eliminated but that empty sleeves of extracellular matrix comprising collagen IV remained intact. It was further shown that these matrix sleeves provided a scaffold for rapid revascularization upon end of treatment (Mancuso et al., 2006). Lumen forming tube-like structures rich of extracellular matrix have already been described elsewhere and it is speculated that they could serve as scaffold for tumor cell dissemination. Tube-like extracellular matrix structures contain tenascin-C in melanoma (Kaariainen et al., 2006; Maniotis et al., 1999) and in Rip1-Tag2 tumors (our unpublished data) and were seen in conduits of the thymus and lymph nodes (Drumea-Mirancea et al., 2006). We had previously speculated that a genetic program for the formation of tenascin-C-rich conduits in secondary lymphoid organs potentially is turned on in cancer (Midwood and Orend, 2009). Thus, tenascin-C could be a therapeutic target to counteract the rebound effect, re-vascularization and worse tumor progression in GBM after discontinuation of the anti-angiogenic treatment.

The different strategies presented above might also become useful for non-invasive *in situ* tumor detection by MRI, PET or ultrasound imaging, techniques by which tumors only bigger than 1 cm can be detected (Weissleder, 2006). Molecular imaging is becoming increasingly important in cancer diagnosis by using cancer-specific target-based imaging probes. Using probes coupled to fluorescent dyes or radioisotopes *in vivo* had been shown in preclinical models (Banerjee et al., 2010; Shah et al., 2009). The monoclonal antibodies 81C6 and F16 (Frey et al., 2011; Schwager et al., 2011), aptamer TTA1 (Hicke et al., 2006) and the muli-target strategy SMART (Ko et al., 2011) may provide perspectives for live imaging of tumors for an improved early detection of metastasis.

Most of the above mentioned monoclonal antibodies are specific for the large tenascin-C isoform. However, monospecificity to a single domain, antibody size or not fully humanized molecules might be disadvantageous for some cancer types. Here, other approaches such as the use of aptamers, RNAi or vaccination might be more useful since the production is easier and cheaper and multi-targeting approaches are possible. Unfortunately, advanced clinical trials are still missing. An increased knowledge about the tumor-, patient- and stage-specific expression of tenascin-C and its splice variants is required to develop more specific and flexible therapies for

targeting tenascin-C. These approaches may even be useful for overcome resistance against cytotoxic or anti-angiogenic drugs.

5 Conclusions and Future Perspectives

Since the discovery of tenascin-C 30 years ago, and the subsequent early murine studies which provided the first indication that targeting tenascin-C in tumors may have significant therapeutic benefit, what we know about how this molecule acts within the tumor ECM, and at the metastatic site, has advanced considerably. This is reflected in the sheer volume of peer reviewed journal articles and patents that detail the promise of this approach. However, what is also clear is that none of these approaches have yet yielded a proven mode of treatment effective in each of the tumor types characterized by elevated tenascin-C expression. The recent data that we highlight in this review exemplifies the vast complexity of tenascin-C expression, splicing and cell type specific effects of this ECM molecule. A greater understanding of precisely how tenascin-C works within a complex, 3D ECM to control tumor behavior is needed. The contribution of tenascin-C to metastasis must also be deciphered in more detail, since it becomes apparent that targeting the primary tumor alone is not optimally effective. Moreover, the tumor specific nature of the role of tenascin-C must be elucidated to enable the tailoring of appropriate treatments to the correct patient subsets. Over the next 30 years we hope to be able to report on the answers to some of these questions.

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Abbreviations:

ANXA1, annexin 1
BAEC, bovine aortic endothelial cells
bFGF, basic fibroblast growth factor
BM, basement membrane
BMP, bone morphogenetic protein
BrdU, 5-bromo-2'-deoxyuridine
BREC, bovine retinal endothelial cells
BSA, bovine serum albumin
CAF, cancer associated fibroblasts
CALEB, chicken acidic leucine-rich EGF like domain containing brain protein
CMEC, cardiac microvascular endothelial cells
CNS, central nervous system
Coll, collagen
CT, computer tomography
CTR, control
DKK1/2, dickkopf 1/2
DOC2, disabled homolog 2
EC, endothelial cells
ECM, extracellular matrix
EDNRA/B, endothelin receptor type A/B
EGF, epidermal growth factor
EGF-L, epidermal growth factor-like repeats
EGFR, epidermal growth factor receptor
EMT, epithelial mesenchymal transition
FBG, fibrinogen-like globe
FN, fibronectin
FN-EDA, fibronectin extra domain A
FN-EDB, fibronectin extra domain B
GBM, glioblastoma multiforme
GFAP, glial fibrillary acidic protein
GM7373, bovine endothelial cell line (fetal aortic endothelial cell line)
HDMEC, human microvascular endothelial cells
HGF, hepatocyte growth factor
HEK, human embryonic kidney cells
HNK-1, human natural killer-1
HUVEC, human umbilical vein endothelial cells
IgG immunoglobulin G
IL2, interleukin-2
Kd, knock down
KO, knockout
LN, laminin
LyN, lymph node
mAb, monoclonal antibody
MBP, myelin basic protein
MEF, mouse embryonic fibroblasts
miR-155, micro-RNA 155

MMP, matrix metalloprotease
MMTV, mouse mammary tumor virus
MR, magic roundabout
MRI, magnetic resonance imaging
Msl1, Musashi 1
NaN, sodium channel subunit β2
NSC, neural stem cells
OP, oligodendrocyte progenitors
PDGF, platelet-derived growth factor
PDGFR, platelet-derived growth factor receptor
PET, positron emission tomography
PyV, polyomavirus
REC, retinal endothelial cells
ROCK, rho-associated protein kinase
RPTPβ, receptor protein tyrosine phosphatase β/ζ
RNAi, ribonucleic acid interference
SCC, squamous cell carcinoma
scFv, single chain variable fragment
SELEX, systematic evolution of ligands by exponential enrichment
SF, scatter factor
siRNA, small inducing ribonucleic acid
SIP, small immunoprotein
SMART, simultaneous multiple aptamers and RGD targeting
SMOC1, SPARC-related modular calcium-binding protein 1
SOCS, suppressor of cytokine signaling
SPARC, secreted protein acidic cysteine-rich
SVZ, subventricular zone
TA, tenascin-C assembly domain
TEC, tumor-derived endothelial cells
TGFβ1, transforming growth factor beta 1
Th cell, T helper cell
TIMP, tissue inhibitor of metalloproteinase
TLR4, toll-like receptor 4
TNIII, fibronectin type III-like repeats in tenascin-C
TNC, tenascin-C
VEGF, vascular endothelial growth factor
VN, vitronectin
Wt, wildtype

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Anja SCHWENZER

Mécanisme et conséquences de la répression de DKK1 par la ténascine-C, une molécule du microenvironnement tumoral

La Ténascine-C (TNC) est un composé majeur de la matrice extracellulaire tumorale et sa forte expression est directement corrélée à l'angiogenèse tumorale et au processus métastatique. Lors de ma thèse j'ai pu démontrer que la TNC dérégulait DKK1, un inhibiteur de la voie de signalisation Wnt et par ce biais augmentait l'activité de cette voie impliquée dans la cancérogenèse. La diminution de la formation des fibres de stress en présence de TNC est l'un des mécanismes majeurs qui contribue à la diminution de DKK1. L'activité de MKL1, facteur co-transcriptionnel de SRF et régulable par l'actine, s'avère diminuée en présence de TNC. Mes données indiquent que la fonction de MKL1 n'est peut-être pas le mécanisme majeur de la régulation de DKK1 par la statu de l'actine. D'autres facteurs, probablement liés aux fibres de stress d'actine pourraient être impliqués. L'augmentation de l'activité de la voie de signalisation Wnt, dépendante de DKK1, est probablement le mécanisme majeur par lequel la TNC active la progression tumorale. Cette étude a permis de mettre en évidence un nouveau mécanisme de régulation de DKK1 faisant intervenir l'intégrité du cytosquelette d'actine.

Mots-clés : microenvironnement tumoral, matrice extracellulaire, ténascine-C, voie de signalisation Wnt, DKK 1, cytosquelette

Tenascin-C (TNC) is a major component of the tumor specific extracellular matrix and its expression has been linked to tumor angiogenesis and metastasis. I demonstrated that TNC downregulates the expression of the Wnt signalling inhibitor DKK1 and by that enhances Wnt/β-catenin signalling. Reduced stress fibre formation in the presence of TNC was identified as a major mechanism contributing to DKK1 downregulation. The activity of the actin-regulated SRF co-transcription factor MKL1 was found to be reduced in the presence of TNC. My results indicate that TNC-regulated MKL1 function maybe one, but not the major mechanism of DKK1 regulation by the actin status and that other factors, presumably regulated by actin stress fibres, are involved. Enhanced Wnt signalling activity downstream of TNC-induced DKK1 downregulation might be a major mechanism by which TNC promotes tumor progression. Furthermore, this study discovered a novel mechanism of regulating the Wnt inhibitor DKK1 by the integrity of the actin cytoskeleton.

Keywords: tumor microenvironment, extracellular matrix, tenascin-C, Wnt signalling, DKK1, cytoskeleton