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## A novel multi-target cancer therapy based on destabilization of short-lived mRNAs

Felicitas Rataj

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## THÈSE

Pour obtenir le grade de

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Présentée par

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préparée au sein du **Laboratoire Biologie du Cancer et l'Infection**  
dans l'**École Doctorale Chimie et Sciences du Vivant**

## **Nouvelle thérapie anti-tumorale multi-cibles basée sur la dégradation des ARNms à demi-vie courte**

**A novel multi-target cancer therapy based on  
destabilization of short-lived mRNAs**

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« Grimpez si vous le voulez, mais n'oubliez jamais que le courage et la force ne sont rien sans prudence [...]. N'agissez jamais à la hâte, prenez garde au moindre pas. Et dès le début, pensez que ce pourrait être la fin. »

≈

« Climb if you will, but remember that courage and strength are nought without prudence [...]. Do nothing in haste; look well to each step; and from the beginning think what may be the end. »

≈

« Klettere, wenn du willst, aber vergiss nicht, dass Mut und Kraft ohne Besonnenheit wertlos sind [...]. Übereile nichts, achte auf jeden Schritt und habe von Anfang an das Ende im Blick. Wir, die wir die Berge erklettern, wissen, dass jede Höhe durch geduldige und mühsame Anstrengung gewonnen werden muss. Wir wissen auch, dass ein entschlossener Wille sich den Weg bahnt, und wenn wir zu unseren täglichen Beschäftigungen zurückkehren, so sind wir für den Kampf des Lebens besser gerüstet und schöpfen aus der Erinnerung neue Kraft und Lebensfreudigkeit. »

*Edward Whymper (1840-1911),*

*Mountaineer*



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## List of content

INTRODUCTION.....	17
Chapter 1 Regulation of short-lived mRNA stability .....	19
1.1. The highway to degradation: Deadenylation-dependent mRNA decay .....	21
1.2. The highway code: Control of mRNA decay .....	23
1.2.1. <i>Cis</i> -acting elements: Focus on AU-rich elements .....	23
1.2.2. Trans-acting factors: Focus on ARE-binding proteins .....	26
1.2.2.1. Stabilizing ARE-binding proteins .....	27
1.2.2.2. Destabilizing ARE-binding proteins .....	28
1.3. Regulation of mRNA decay by signalling pathways.....	30
In conclusion .....	33
Chapter 2 Key player in mRNA decay: The TIS11 protein family .....	35
2.1. Members of the TIS11 protein family .....	37
2.1.1. Tristetraprolin (TTP) .....	37
2.1.2. TIS11b .....	39
2.1.3. TIS11d .....	42
2.1.4. Zfp3613 .....	43
2.2. Regulation of TIS11 protein expression.....	43
2.3. Protein domains and truncation of TIS11 proteins.....	46
2.4. Functions of TIS11 proteins .....	49
2.4.1. Key players in mRNA decay .....	50
2.4.2. Alternative functions of TIS11 proteins during mRNA lifecycle .....	51
2.5. Regulation of TIS11 protein activities .....	55
2.5.1. Post-translational modifications – phosphorylation .....	56
In conclusion .....	59
Chapter 3 The multi-step development of human cancer .....	61
3.1. The hallmarks of cancer .....	62
3.1.1. Enabling hallmark: Genome instability and mutation .....	62
3.1.2. Enabling hallmark: Tumor-promoting inflammation .....	63
3.1.2.1. Cytokines.....	65
3.1.2.2. Chemokines .....	68
3.1.3. Hallmark: Sustaining proliferative signalling.....	70
3.1.4. Hallmark: Evading growth suppressors.....	71
3.1.5. Hallmark: Resisting cell death.....	72
3.1.6. Hallmark: Enabling replicative immortality.....	73



3.1.7. Hallmark: Inducing angiogenesis .....	73
3.1.7.1. Physiological angiogenesis.....	73
3.1.7.2. Tumor angiogenesis .....	81
3.1.8. Hallmark: Activating invasion and metastasis .....	86
3.1.9. Emerging hallmark: Deregulating cellular energetics .....	87
3.1.10. Emerging hallmark: Evading immune destruction .....	89
3.2. The cancer niche or tumor microenvironment .....	90
3.2.1. Cell types of the tumor microenvironment.....	91
3.2.1.1. Cancer stem cells.....	91
3.2.1.2. Immune inflammatory cells.....	92
3.2.1.3. Cancer associated fibroblasts .....	92
3.2.2. Heterotypic signalling coordinates cells of the tumor stroma .....	93
3.2.3. Hypoxia .....	95
In conclusion .....	99
Chapter 4 Therapeutic targeting of cancer and its limits .....	101
4.1. Fight against cancer: Focus on anti-angiogenic and anti-inflammatory cancer .....	103
therapies.....	103
4.1.1. Targeting tumor vasculature: Anti-angiogenic therapies .....	103
4.1.1.1. Single-target angiogenesis inhibitors: the first generation .....	104
4.1.1.2. Second generation multi-targeted angiogenesis inhibitors: VEGF and related targets .....	106
4.1.1.3. Broad-spectrum of angiogenesis inhibitors: the next generation .....	106
4.1.1.4. Indirect pharmacological inhibitors of angiogenesis.....	107
4.1.2. Targeting the wound that never heals: Anti-inflammatory therapies .....	108
4.2. Limits of targeted cancer therapies.....	111
4.3. Emerging link between regulation of mRNA stability and cancer.....	113
4.3.1. Expression of TIS11 proteins in cancer cell lines .....	114
4.3.2. The impact of TIS11 proteins on cancer hallmarks.....	115
In conclusion .....	121
Chapter 5 Breaking through the other side: Cell penetrating peptides .....	123
5.1. Classes of CPPs .....	124
5.2. Different cellular uptake mechanisms of CPPs .....	125
5.3. The potential of CPPs in anti-cancer drug delivery.....	127
5.4. CTPs .....	129
In conclusion .....	131
Objectives.....	133

RESULTS.....	135
Article 1 .....	137
“A novel phosphorylation-dependent regulation of TIS11b stability and activity by cAMP-dependent protein kinase (PKA) reveals the important role of two conserved N- and C-terminal serines (S54 and S334)” .....	137
Introduction .....	139
Abstract .....	145
Introduction .....	147
Material and Methods.....	149
<i>Generation of stable HeLa Tet-off cells expressing wild type TIS11b and TIS11b mutants</i> .....	152
Results .....	154
<i>PKA signalling pathway regulates TIS11b expression and phosphorylation</i> .....	154
<i>Identification of PKA target sites within TIS11b sequence: conserved S54 and S334 are phosphorylated in vitro</i> .....	155
<i>TIS11b is phosphorylated at S54 and S334 in vivo</i> .....	156
<i>S54 and S334 regulate TIS11b mRNA-destabilizing activity</i> .....	158
<i>TIS11b protein stability is regulated by S54 and S334</i> .....	159
<i>S54 and S334 modulate the proteasome-dependent degradation of TIS11b</i> .....	159
<i>Mimicking a phosphorylation at S54 changes TIS11b subcellular localization</i> .....	160
<i>Mimicking a phosphorylation at S54 promotes the binding of TIS11b to endogenous 14-3-3 proteins</i> .....	161
<i>TIS11b interacts with endogenous CNOT1</i> .....	161
Discussion .....	162
Acknowledgements .....	166
References .....	167
Legends of the figures .....	171
Article 2.....	185
“Targeting AU-rich element-mediated mRNA decay using a mutant version of ZFP36L1/TIS11b zinc finger protein impairs major hallmarks of tumorigenesis” .....	185
Introduction .....	187
Abstract .....	191
Introduction .....	193
Material and Methods.....	195
Results .....	204
<i>The N- and C-terminal domains of TIS11b are active in mRNA decay</i> .....	204
<i>R9-ZnC and R9-ZnC<sup>S334D</sup> fusion proteins reduce luciferase reporter gene activity through VEGF-3'UTR</i> .....	205
<i>Deletion of the N-terminal domain of TIS11b doubles protein half-life in cellulo</i> .....	205

<i>R9-ZnC and R9-ZnC<sup>S334D</sup> protein overexpression and purification</i> .....	206
<i>Deletion of the N-terminal domain of TIS11b increases the stability of the purified protein under storage</i> .....	206
<i>R9-ZnC and R9-ZnC<sup>S334D</sup> are efficiently internalized into living cells</i> .....	207
<i>Purified R9-ZnC and R9-ZnC<sup>S334D</sup> proteins decrease VEGF mRNA and protein expression</i> ...	207
<i>R9-ZnC<sup>S334D</sup> inhibits proliferation and migration of breast cancer cells in vitro</i> .....	208
<i>R9-ZnC and R9-ZnC<sup>S334D</sup> reduce cancer cell invasion and anchorage-independent cell growth</i> .....	208
<i>R9-ZnC and R9-ZnC<sup>S334D</sup> downregulate the expression of EMT-markers</i> .....	209
<i>R9-ZnC and R9-ZnC<sup>S334D</sup> impair the formation of pseudo capillaries by endothelial cells</i> .....	209
Discussion .....	210
Acknowledgements .....	214
References .....	215
Legends of the figures .....	218
Supplementary data: <i>In vivo</i> characterization of R9-ZnC and R9-ZnC <sup>S334D</sup> as novel anti-tumoral agents.....	233
Results & Discussion.....	235
<i>Intratumoral injection of R9-ZnC or R9-ZnC<sup>S334D</sup> inhibits 4T1 luc tumor growth</i> .....	235
<i>R9-ZnC or R9-ZnC<sup>S334D</sup>-treatment of 4T1-luc tumors decreases VEGF expression and microvessel density as well as hypoxic areas</i> .....	237
<i>Decreased expression of several angiogenic and inflammatory cytokines upon R9-ZnC- or R9-ZnC<sup>S334D</sup> treatment in vivo</i> .....	239
<i>R9-ZnC and R9-ZnC<sup>S334D</sup> reduces expression of EMT-markers in vivo</i> .....	247
Conclusion.....	250
Material & Methods .....	251
DISCUSSION & CONCLUSIONS .....	255
BIBLIOGRAPHY .....	263
APPENDIX .....	293

## List of figures

Figure 1:	Complexity of RNA decay and enzymes involved in eukaryotic cells ( <i>Stoecklin &amp; Muhlemann, 2013</i> ). .....	18
Figure 2:	Mechanisms of deadenylation-dependent mRNA decay in eukaryotes ( <i>Planel et al., 2014</i> ).....	20
Figure 3:	Cis-elements located in 3'untranslated region (3'UTR) of mRNAs are recognized by specific trans-acting factors to modulate the rate of mRNA decay ( <i>Thapar &amp; Denmon, 2013</i> ).....	22
Figure 4:	Regulation of deadenylation-dependent mRNA degradation through AU-rich elements (AREs) recognized by ARE-binding proteins (ARE-BPs) in eukaryotes ( <i>Planel et al., 2014</i> ). .....	26
Figure 5:	Phenotype of TTP-knock out (KO-PBS) mice compared to wildtype (WT) and prevention of this phenotype by injecting anti-TNF $\alpha$ antibodies (KO-Ab) ( <i>Taylor et al., 1996</i> ). .....	36
Figure 6:	Optical projection tomography of vascular abnormalities in TIS11b <sup>-/-</sup> and TIS11b <sup>+/-</sup> embryos at E9.5 ( <i>Bell et al., 2006</i> ). .....	41
Figure 7:	Schematic presentation of TTP gene, mRNA and protein. TTP gene consists of one intron and two exons ( <i>Sanduja et al., 2012</i> ). .....	45
Figure 8:	Structure similarities between human TIS11 proteins ( <i>Ciais et al., 2013</i> ).....	47
Figure 9:	Predicted structure of human TTP tandem zinc finger (TZF) domain in a complex with 5'-UUAUUUAUU-3'-ARE (pink) based on original structure for TIS11d TZF domain ( <i>Carrick et al., 2004; Hudson et al., 2004</i> ).....	47
Figure 10:	Subcellular localization of TTP, TIS11b (CMG1) and TIS11d (11D) in leptomycin B-treated HEK 293 cells ( <i>Phillips et al., 2002</i> ). .....	49
Figure 11:	Multiple functions of TIS11 proteins during mRNA life cycle have been shown ( <i>Ciais et al., 2013</i> ). .....	52
Figure 12:	Subcellular localization of mRNAs is regulated by TIS11 proteins ( <i>Franks &amp; Lykke-Andersen, 2007</i> ).....	53
Figure 13:	Signalling pathways acting on TTP and TIS11b ( <i>Ciais et al., 2013</i> ). .....	56
Figure 14:	Central role of the p38-MAPK–MK2–PP2A–TTP axis in the regulation of TTP protein activity, stability and localization ( <i>Sandler &amp; Stoecklin, 2008</i> ). .....	57
Figure 15:	Different phases of tumor development ( <i>Thiery, 2002</i> ).....	60
Figure 16:	Hallmarks during tumor progression ( <i>Hanahan &amp; Weinberg, 2011</i> ). .....	60
Figure 17:	The intrinsic and extrinsic pathways that describe the link between cancer and inflammation ( <i>Mantovani et al., 2008</i> ).....	64
Figure 18:	The complex role of cytokines and chemokines during cancer initiation and progression ( <i>Landskron et al., 2014</i> ). .....	67
Figure 19:	Involvement of chemokines in angiogenesis of breast cancer ( <i>Palacios-Arreola et al., 2014</i> ). .....	69
Figure 20:	Interaction of CXCR4 with its ligand CXCL12 in primary tumor and metastasis of breast cancer ( <i>Mukherjee &amp; Zhao, 2013</i> ).....	70

Figure 21:	A balance between pro- and anti-angiogenic factors controls angiogenesis. ....	74
Figure 22:	Regulation of VEGF-A expression by alternative splicing ( <i>Eymin et al., 2014</i> ).....	75
Figure 23:	The binding specificities of VEGF proteins and VEGFR signalling complexes.....	78
Figure 24:	The multi-step process of angiogenesis including the activation phase, the sprouting and the maturation phase ( <i>David et al., 2009</i> ). ....	80
Figure 25:	The angiogenic switch during tumor development ( <i>Bergers &amp; Benjamin, 2003</i> )...	82
Figure 26:	Schematic representation of tumor angiogenesis including sprouting angiogenesis, vasculogenesis (bone marrow-derived endothelial progenitor cells are recruited to the tumor by different factors such as the stromal-derived factor 1 $\alpha$ (SDF-1 $\alpha$ ), the basic fibroblast growth factor (bFGF), IL-6 and G-CSF), intussusception and vessel co-option ( <i>Zhu et al., 2011</i> ). ....	84
Figure 27:	Tumor vasculature is abnormal in their structure and function ( <i>Carmeliet &amp; Jain, 2011</i> ). ....	85
Figure 28:	Abnormalities of tumor microvasculature ( <i>Jain, 2005; Goel et al., 2011</i> ).....	85
Figure 29:	The invasion-metastasis cascade ( <i>Thompson &amp; Haviv, 2011</i> ).....	87
Figure 30:	Complexity of the interactions between tumor cells and cells of the tumor microenvironment ( <i>Hanahan &amp; Weinberg, 2011</i> ).....	90
Figure 31:	Signalling network of cancer cells and the tumor microenvironment during tumorigenesis ( <i>Hanahan &amp; Weinberg, 2011</i> ). ....	94
Figure 32:	Oxygen-dependent regulation of HIF-1 $\alpha$ stability and HIF-1 signalling ( <i>Semenza, 2013</i> ). ....	96
Figure 33:	Therapeutic targeting of cancer hallmarks ( <i>Hanahan &amp; Weinberg, 2011</i> ).....	100
Figure 34:	The basic concept of anti-angiogenic cancer therapies ( <i>Zetter, 2008</i> ). ....	102
Figure 35:	The hypothesis of vessel normalization ( <i>Jain, 2005; Goel et al., 2011</i> ). ....	104
Figure 36:	Adaptive (evasive) resistance and intrinsic non-responsiveness are the two modes of tumor resistance to anti-angiogenic cancer therapies ( <i>Bergers &amp; Hanahan, 2008</i> ).....	110
Figure 37:	Mechanisms of adaptive resistance ( <i>Bergers &amp; Hanahan, 2008</i> ). ....	110
Figure 38:	Network of TTP-mediated post-transcriptional control of mRNAs encoding oncogenic and tumor-promoting factors ( <i>Ross et al., 2012</i> ).....	115
Figure 39:	Cellular uptake mechanisms and possible intracellular trafficking of CPPs ( <i>Chou et al., 2011</i> ). ....	126
Figure 40:	Activatable CPP, which is fused to an anionic inhibitory sequence via MMP-cleavable linker ( <i>Jiang et al., 2004</i> ).....	128
Figure 41:	Biodegradable pH-sensitive micelle delivery system ( <i>Sethuraman et al., 2008</i> )...	128
Figure 42:	Multi-target effect of truncated/mutated TIS11b fusion proteins on cancer hallmarks. ....	258

## List of tables

Table 1:	Classification of AU-rich elements after Chen et al. ( <i>Chen et al., 1995</i> ).....	25
Table 2:	Classification of AU-rich elements after Bakheet et al. ( <i>Bakheet et al., 2003</i> ).....	25
Table 3:	Overview of important ARE-binding proteins in eukaryotes ( <i>Planel et al., 2014</i> )....	27
Table 4:	Identified targets of TTP ( <i>Brooks &amp; Blackshear, 2013</i> ).....	38
Table 5:	Identified targets of TIS11b ( <i>Baou et al., 2009</i> ).....	40
Table 6:	TTP interaction partners ( <i>Brooks &amp; Blackshear, 2013</i> ). ....	50
Table 7:	Anti-angiogenic agents, their molecular targets and current indications for cancer therapy ( <i>Limaverde-Sousa et al., 2014</i> ).....	102
Table 8:	Examples of CPPs, their origin, structure and proposed mechanism of cellular uptake ( <i>Koren &amp; Torchilin, 2012</i> ).....	124



## List of abbreviations

5'/3'UTR	5'/3' untranslated region
ACTH	Adrenocorticotrophic hormone
AGO2	Argonaute 2
AMF	Autocrine motility factor
Ang1	Angiopoietin 1
Ang2	Angiopoietin 2
ARE	AU-rich elements
ARE-BP	ARE-binding proteins
ARED	AU-rich elements (AREs) containing mRNA database
Arg (R)	Arginin
Asp (D)	Aspartate
ASTRC	AUF1-and-Signal Transduction-Regulated Complex
BAC	Bovine adrenocortical cells
Berg36	B-cell early response gene encoding a 36-kDa protein
BM	Basement membrane
BMDC	Bone-marrow derived cells
BRF1/2	Butyrate Response Factor 1/2
BSA	Bovine serum albumin
C, CC(R)	C/CC-motif chemokine (receptor)
CA	Carbonic anhydrase
CAF	Cancer-associated fibroblast
CDKN2A	Cyclin dependent kinase inhibitor 2A
CHX	Cycloheximide
COX-2	Cyclooxygenase 2
CPP	Cell-penetrating peptide
CSC	Cancer stem cell
CSF-1	Colony-stimulating factor 1
CTD	C-terminal domain
CTP	Cell-targeting peptide
CXC, CX3C(R)	CXC/CXC3-motif chemokine (receptor)
Cul4B	Cullin 4B
Da	Dalton
Dll4	Delta-like 4
DRB	5,6-Dichloro-β-D-ribofuranosyl benzimidazol
EC	Endothelial cells
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	EGF receptor
ELAV	Embryonic-lethal abnormal visual in <i>Drosophila melanogaster</i>
EMT	Epithelial-mesenchymal transition
ENDO	Endonucleolytic mRNA decay
eNOS	Endothelial nitric oxide synthase
EPR	Enhanced Permeability and Retention effect
ERF-1/2	Epidermal Growth Factor-response factor 1/2
ERK	Extracellular-signal-regulated kinase
EXO	Exonucleolytic mRNA decay
FDA	Food and Drug Administration
FFF	Formalin-free fixation solution
FGF	Fibroblast growth factor (=bFGF)
Flk-1	Fetal liver kinase 1
Flt-1/4	Fms-like tyrosine kinase 1/4
GFP	Green fluorescent protein
G0S24	G0/G1 switch regulatory gene 24
GM-CSF	Granulocyte macrophage-colony stimulating factor
GLUT1-4	Glucose transporter 1 to 4
GSK3β	Glycogen synthase kinase-3 beta
GST	Glutathion-S-transferase
HEK	Human embryonic kidney
HER	Human epidermal growth factor receptor 2
HNF1α	Hepatocyte nuclear transcription factor 1α
HIF-1	Hypoxia-inducible factor-1
HNF1α	Hepatocyte nuclear transcription factor 1α
HPRT	Hypoxantin phosphoribosyl transferase
HRE	Hypoxia responsive element



HUVEC	Human umbilical vein endothelial cells
ICAM	Intercellular adhesion molecule
IFP	Interstitial fluid pressure
IFN $\gamma$	Interferon $\gamma$
IL	Interleukin
IP	Immunoprecipitation
IP-10	Interferon $\gamma$ -induced protein 10
IPTG	Isopropyl-beta-D-thiogalacto-pyranoside
IRE	Iron response element
IREBP	IRE-binding protein
IRES	Internal ribosome entry sites
JAK	Janus-activated kinase
JNK	C-jun-N-terminal kinase
$\alpha$ KG	$\alpha$ -ketoglutarate
KA	<i>in vitro</i> kinase assay
KH-domains	K homology-domains
KO	Knock out
KSRP	K homology splicing regulatory protein
LDHA	Lactate dehydrogenase
LH	Luteinizing hormone
LHR	LH receptor
LLC	Lewis lung carcinoma
LOX	Lysyl-oxydase
LPS	Lipopolysaccharide
MCP-1	Monocyte-chemoattractant protein 1
MCT4	H <sup>+</sup> /monocarboxylate transporter 4
MEK1/2	Mitogen-activated protein kinase kinase1/2
MET	Mesenchymal-epithelial transition
MIP-1	Macrophage inflammatory protein 1
miRNA	micro RNA
MK2	p38MAPK-activated protein kinase 2
MMP	Matrix-metalloprotease
MR	Mineralocorticoid receptor
MSC	Mesenchymal stem cell
mTOR	
NES	Nuclear export sequence
NF- $\kappa$ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGD	No-go mRNA decay
NLS	Nuclear localization sequence
NMD	Nonsense-mediated mRNA decay
NOV	Nephroblastoma overexpressed gene
NRP1/2	Neuropilin 1/2
NTD	N-Terminal domain
NSAIDs	Non-steroidal anti-inflammatory drugs
OE	Overexpression
P-body (PB)	Processing body
p38 MAPK	p38 mitogen activated protein kinase
PABP	Poly(A)-binding protein
PARN	Poly(A)-specific ribonuclease
PBS	Phosphate buffer saline
PDGF	Platelet-derived growth factor
PFA	Paraformaldehyde
PHD	Prolyl-hydroxylases
PI3K	Phosphatidylinositol 3'-kinase
PIMO	Pimonidazol
PKA	cAMP-protein kinase A
PKB	Protein kinase B (=AKT)
PKC	Protein kinase C
PLGF	Placental growth factor
PMA	Phorbol myristyl acetate
PP2A	Protein phosphatase 2A
PTD	Protein transduction domain
PTEN	Phosphatase and tensin homologue deleted on chromosome 10
PTX3	Pentraxin 3
PVDF	Polyvinylidene fluoride
RB	Retinoblastoma proteins
RBP	RNA-binding protein
RGD	Arginine-glycine-aspartate

RISC	RNA-induced silencing complex
RONS	Reactive oxygen/nitrogen species
RPL13A	Ribosomal protein 13a
RRM	RNA Recognition Motifs
SDF-1	Stromal-derived factor 1
SDS	Sodium dodecyl sulfate
Ser (S)	Serine
SG	Stress granule
siRNA	Small interfering RNA
SLBP	Stem-loop-binding protein
SSC	Saline sodium citrate
TAM	Tumor-associated macrophage
TAN	Tumor-associated neutrophils
TAT	Transcription-transactivating protein of HIV-1
TBS	Tris buffer saline
TCA	Tricarboxylic acid cycle (Krebs cycle)
TGFβ	Transforming growth factor beta
Th	T-helper cell
Thr	threonine
TIL	Tumor-infiltrating leukocyte
TIMP	Tissue Inhibitor of metalloprotease
TIS11	TPA-inducible sequence 11
TKI	Tyrosine kinase inhibitor
TNFα	Tumor necrosis factor alpha
TPA	12-O-tetradecanoylphorbol-13-acetate
TSP1/2	Thrombospondin 1/2
TTP	Tristetraprolin
TTR-BPs	Turnover and translation regulatory RNA-binding proteins
TZF	Tandem zinc finger
Ub	Ubiquitin
uPA(R)	Urokinase-type plasminogen activator (receptor)
Y2H	Yeast two hybrid
VECAM	Vascular cell adhesion protein
VEGF	Vascular endothelial growth factor
VEGFR	VEGF receptor
VHL	Von Hippel Lindau factor
VPF	Vascular permeability factor
WST-1	4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene disulfonate
WT	Wildtype
ZF	Zinc finger
ZFP36 (L1/L2/L3)	Zinc finger protein 36 (like 1/2/3)



# INTRODUCTION

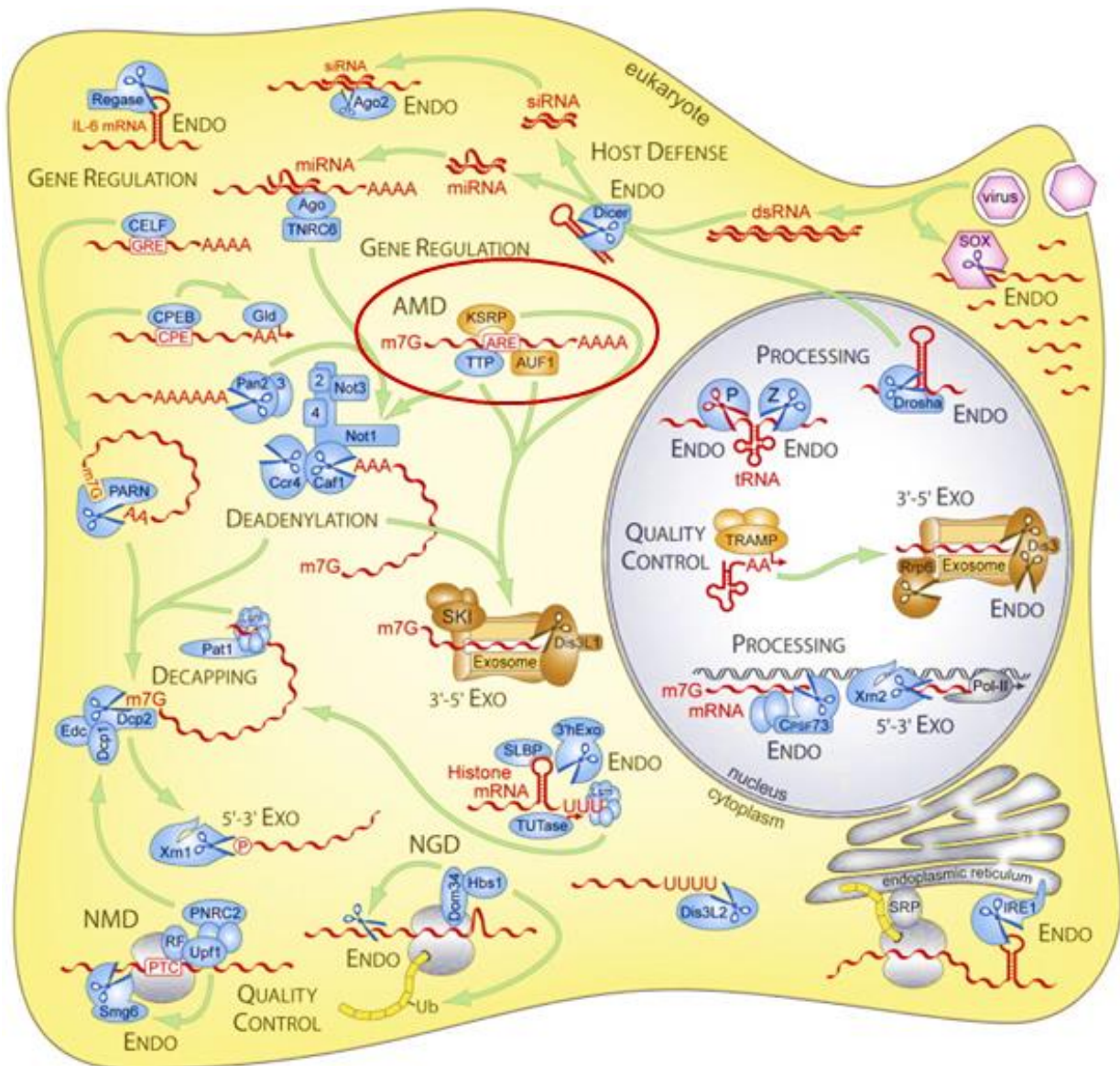


Figure 1: Complexity of RNA decay and enzymes involved in eukaryotic cells (Stoecklin & Muhlemann, 2013). Among other mechanisms, ARE-mediated mRNA decay (AMD) is an important regulatory process of mRNA stability and is further described in this chapter. ENDO, endonucleolytic mRNA decay; 5'-3' EXO, 5'-3' exonucleolytic mRNA decay; 3'-5' EXO, 3'-5' exonucleolytic mRNA decay; NGD, no-go mRNA decay; NMD, nonsense-mediated mRNA decay.

## Chapter 1 Regulation of short-lived mRNA stability

Gene expression is a tightly controlled mechanism. Beside transcription, post-transcriptional mechanisms are also important for controlling gene expression. Multiple regulatory systems ensure the balance between gene products (RNA or protein) and their need for cellular growth, function and fate. Especially during development, the presence of certain transcripts during specific time windows is crucial. Protein concentration is determined by the cytoplasmic concentration of the corresponding mRNA, which depends on mRNA synthesis and decay rates, and also on post-translational modifications regulating protein stability. Post-transcriptional regulatory mechanisms, sub-divisible into processes determining translatability of mRNAs and processes defining equilibrium between transcription rate and degradation of the transcript, gained a lot of interest during the last years. Pre-mature mRNA processing, nuclear mRNA export, RNA interference, mRNA sequestration, codon usage, translational repression by microRNAs (miRNAs) or proteins, and the control of mRNA turnover are limiting mechanisms for mRNA synthetic rate. Figure 1 illustrates the fascinating biology of eukaryotic ribonucleases and how they are involved in the mRNA life cycle (*Stoecklin & Muhlemann, 2013*). Although most mRNAs are degraded through the deadenylation-dependent decay pathway triggered either by RNA-binding proteins or microRNAs, deadenylation-independent mechanisms exist (*Fabian et al., 2010; Garneau et al., 2007*). For certain mRNAs, degradation is initiated by endonuclease cleavage within the nucleotide sequence, followed by further degradation via either the exosome (3'→5' decay) or the exoribonuclease Xrn1 (5'→3' decay). Mammalian cells have developed several mRNA surveillance mechanisms to clear defective transcripts and to avoid the synthesis of abnormal proteins. Nonsense-mediated mRNA decay (NMD) seems to be restricted to newly synthesized mRNAs that contain premature termination codons. The translation of these transcripts would lead to truncated proteins with aberrant function. No-go mRNA decay (NGD) induces the degradation of faulty transcripts associated with stalled ribosomes, to release sequestered components of the translation machinery. Transcripts lacking a stop codon are degraded by the non-stop mRNA decay pathway, thus preventing translation to proceed along the poly(A)tail of the transcript and to result in aberrant proteins.

However, this chapter will focus on the post-transcriptional regulation of mRNA stability, in particular on AU-rich element-mediated control of mRNA half-life, which enables cells to quickly adjust transcript levels and their translational potential in response to various stimuli (*Guhaniyogi & Brewer, 2001*).

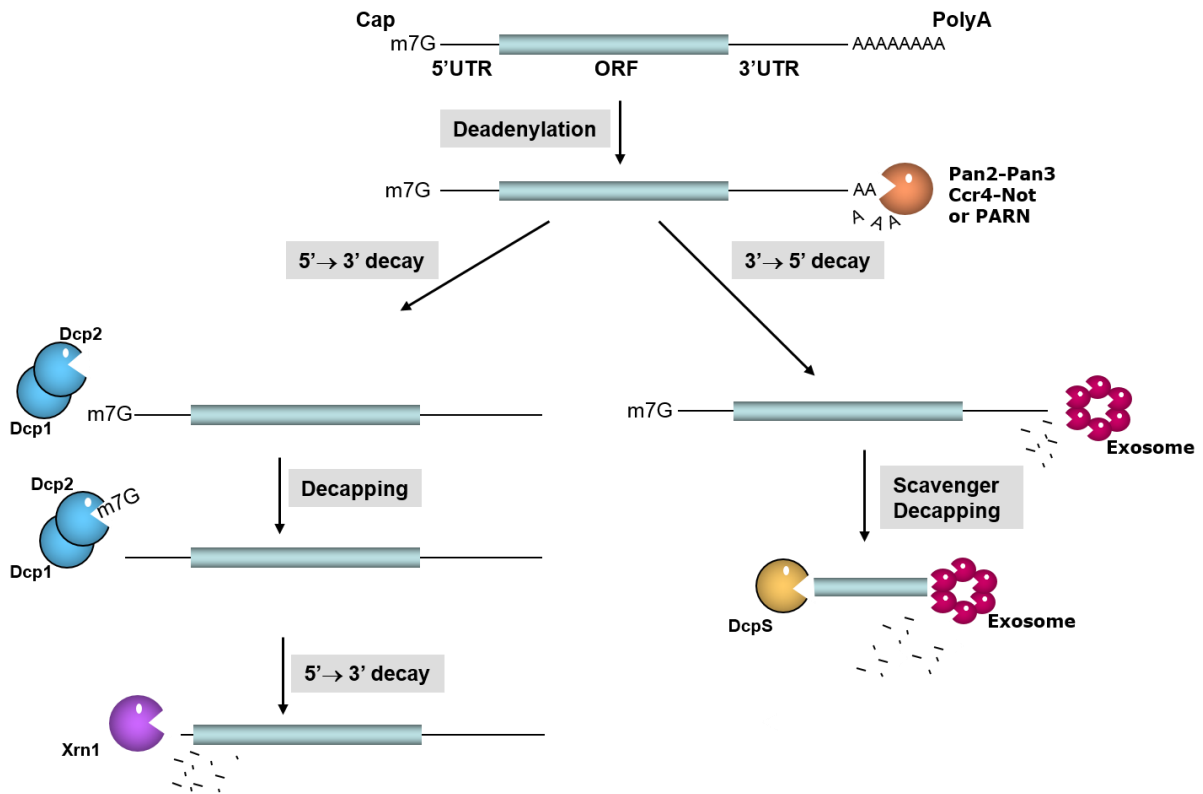


Figure 2: Mechanisms of deadenylation-dependent mRNA decay in eukaryotes (Planel *et al.*, 2014).

### 1.1. The highway to degradation: Deadenylation-dependent mRNA decay

Eukaryotic mRNAs contain two stabilizing structures, the 5'-end 7-methylguanosine cap (m<sup>7</sup>G-cap) and the poly(A) tail located at the 3'-end, which are added to the pre-mature mRNA after transcription. These two structure elements are recognized by the cytoplasmic proteins eIF-4F and the poly(A)-binding protein (PABP), to enhance translation initiation and to protect mRNAs from unspecific exonuclease activity. Translocation of the mature mRNA occurs at the level of cytoplasmic polysomes. In response to a still unknown signal, mRNAs are deadenylated and transported to processing bodies (P-bodies). In P-bodies, mRNAs can be either stored for return to translation or recruited to the mRNA decay machinery for degradation. P-bodies are dynamic, cytoplasmic foci, which harbour non-translatable mRNAs. In addition, translation repressors and mRNA decay factors are present in these cellular structures (*Kulkarni et al., 2010*).

The decay of the majority of eukaryotic mRNAs is initiated by a rate-limiting process called deadenylation, which leads to the shortening of the poly(A) tail (Figure 2). Several deadenylases, located in the cytoplasm of eukaryotic cells as components of P-bodies, are well characterized.

Pan2-Pan3 is a PABP-dependent deadenylase unit, which trims the nascent poly(A) tail (*Brown et al., 1996*). Ccr4-Not is a large protein complex, which consist of nine subunits including the conserved canonical subunits Ccr4, three Caf proteins (Caf1/Pop2, Caf40/Not9, Caf130/Not10) and five Not proteins (Not1, Not2, Not3, Not4 and Not5) (*Wahle & Winkler, 2013; Inada & Makino, 2014*). In this complex, Ccr4 and Caf1 are the catalytic subunits, whereas Not1 functions as a central scaffold. The role of the other subunits remains unknown. Deadenylation in eukaryotic cells seems to be a two-phase process, where Pan2-Pan3 is performing a first, incomplete shortening of the poly(A) tail, followed by the entire poly(A) tail degradation through Ccr4-Not1 deadenylase activity (*Zheng et al., 2008*). A third mammalian deadenylase is the poly(A)-specific ribonuclease (PARN), which plays an important role during embryogenesis (*Godwin et al., 2013*). PARN activity is cap-dependent and is increased by interacting with the 5'-end m<sup>7</sup>G-cap. The naked 3'-end is further attacked by the exosome, a macromolecular complex of 10-11 subunits. Nine subunits form the central core of the exosome. Two other subunits of multi-domain polypeptides, which carry the catalytical activity of the eukaryotic exosome, associate with the central core depending on cellular localization, leading to mRNA degradation (Figure 2) (*Chlebowski et al., 2013*). The 5'-cap on the remaining nucleotide sequence is metabolized by the scavenger decapping enzyme DcpS.



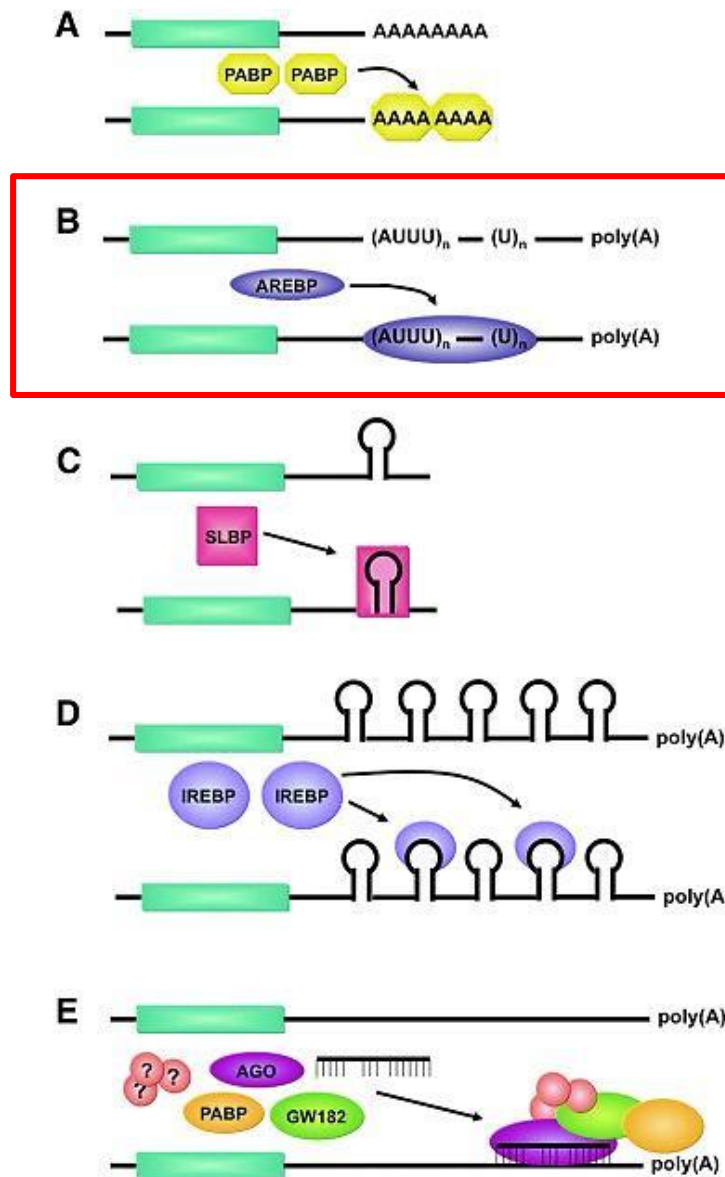


Figure 3: *Cis*-elements located in 3'untranslated region (3'UTR) of mRNAs are recognized by specific *trans*-acting factors to modulate the rate of mRNA decay (Thapar & Denmon, 2013). (A) Poly(A) tail and PABP. (B) AU-rich elements (AREs) and ARE-binding proteins (ARE-BP). (C) Histone stem-loop and the stem-loop-binding protein (SLBP). (D) Iron response elements (IREs) and the IRE-binding protein (IRE-BP). (E) MiRNAs targeting mRNA sequences and recruiting the RNA-induced silencing complex (RISC) complex.

Alternatively to the exosome-dependent pathway, deadenylation can be followed by removing the 5'-cap through the major activity of decapping enzymes Dcp1 and Dcp2 (Figure 2) (Arribas-Layton *et al.*, 2013). Within this complex, Dcp2 has the intrinsic decapping activity. Xrn1, a 5'→3'exoribonuclease, recognizes the unprotected 5'-end leading to 5'→3'decay of the mRNA (Nagarajan *et al.*, 2013).

## 1.2. The highway code: Control of mRNA decay

Traditionally, transcriptional regulation has been the major focus of gene expression studies. However, recently the important contribution of mRNA decay mechanisms has emerged and numerous pathways linked to this aspect were reported. Most regulatory sequences (*cis*-acting elements) involved in these control mechanisms are located in the 5' and 3'untranslated regions (UTR) of mRNAs, which function as platforms for association of multi-subunit complexes initiated by RNA-binding proteins (RBPs) and other *trans*-acting factors (miRNAs, long non-coding RNAs). Whereas the 5'UTR is more important in translational control including cap-dependent and internal ribosomal entry site (IRES)-mediated processes, determinants of mRNA stability, cellular location and translatability are predominately found in the 3'UTR (Moore, 2005; Pickering & Willis, 2005). Interestingly, the length of 3'UTR could act as determining factor of mRNA stability. Experiments have shown a positive correlation between length of 3'UTR and number of *cis*-acting elements, respectively, and mRNA half-life (Akashi *et al.*, 1994). Importantly, these mechanisms are often altered during cancer development and other life-threatening diseases, suggesting that they might contribute to human pathologies (Vislovukh *et al.*, 2014).

The following paragraphs will describe how mRNA fate is controlled by a balance between stabilizing and destabilizing RNA-binding factors which recognize regulatory *cis*-acting sequences located in the 3'UTR.

### 1.2.1. Cis-acting elements: Focus on AU-rich elements

Several *cis*-acting sequences, such as the poly(A) tail, iron-responsive elements (IRE), Jun-kinase response elements, histone stem-loop and AU-rich elements (AREs) are located in the 3'UTR of the mRNA to determine its stability (Figure 3) (Knapinska *et al.*, 2005; Matoulkova *et al.*, 2012). Stem-loop destabilizing elements are present in mRNAs encoding for Interleukin-2/6 or the granulocyte-stimulating factor (G-CSF). This sequence motif requires at least one stem-loop structure to be functional. Furthermore, CU-rich regulatory elements confer instability to mRNAs. The poly(A)-tail promotes longer stability of transcripts and efficient translation. AREs

are the landmark *cis*-acting elements of short-lived mRNAs (Figure 3) (Matoulkova *et al.*, 2012). AREs were originally identified as instability determinants (Shaw & Kamen, 1986). Shaw & Kamen gave the initial evidence of the destabilizing function of AREs as they demonstrated that the half-life of the otherwise stable  $\beta$ -globin mRNA was decreased when fused to the 3'UTR of the granulocyte macrophage-colony stimulating factor (GM-CSF) which is known to harbour AREs. AREs are generally conserved between species (Halees *et al.*, 2008). Initially, it was estimated that 8-10 % of total mammalian mRNAs contain AREs (<http://brp.kfshrc.edu.sa/ARED/>) (Halees *et al.*, 2008). More recently, a database elaborated for a comprehensive investigation of AREs reported 3 275 protein encoding genes which harbour at least one ARE in their 3'UTR, underscoring the importance of these sequences (Halees *et al.*, 2008; Gruber *et al.*, 2011). By searching AREs also in the introns of human genes, 9 114 additional genes were found, meaning that around 50 % of human genes are part of the ARE-regulome (Halees *et al.*, 2008). ARE appearance correlates with rapid patterns of mRNA decay for genes encoding for factors involved in proliferation, inflammation, transcription, immune response, development and signalling or proto-oncogenes (Shaw & Kamen, 1986; Shyu & Wilkinson, 2000). ARE-bearing mRNAs are intrinsically labile short-lived mRNAs with half-lives of a few hours.

AREs are 40- to 150-nt long adenylate uridylate-rich sequences with various copies of an AUUUA motif. UUAUUUAWW (W=A/U) is considered as the minimally functional ARE. Historically, AREs were categorized into three groups based on the number of the AUUUA pentamer (ARE length) and functional characteristics (Table 1) (Chen & Shyu, 1994). Class I presents several isolated AUUUA motifs flanked by U-rich sequences found in early-response-gene mRNAs like transcription factors. Genes categorized in class II harbour clustered or tandem, overlapping pentamers. Representatives of this group are cytokines like the GM-CSF, the tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), the Vascular endothelial growth factor (VEGF) or Interleukin-3 (IL-3). Class III represents U-rich sequences in absence of the AUUUA pentanucleotide. All three ARE-categories induce mRNA decay, but via different mechanisms and kinetics. Class I, such as *c-fos* ARE, and class III, like *c-jun* ARE, mediate mRNA degradation through a synchronous shortening of the poly(A) tail, followed by the rapid digest of the mRNA core molecule. In contrast, mRNAs containing class II AREs, such as GM-CSF or IL-3 are eliminated asynchronously – poly(A) tail is removed simultaneously to endo-/exonuclease activity (Chen *et al.*, 1995). Recently, an alternative classification, which takes also the context of the AUUUA pentamer into account, was proposed and is the basis of the ARE-mRNA database “ARED” (Table 2) (Bakheet *et al.*, 2003; Bakheet *et al.*, 2006). The

computationally predicted 13 bp sequence WWUUAUUUAUUWW is the most consensus motif in labile mRNAs. Sequence variations around this core motif are the basis of ARED classification into five groups. Cluster I-IV contains two to five AUUUA pentanucleotides. Cluster V harbours only one 13 bp consensus motif. Interestingly, AREScore, a recent study using an algorithm to identify mRNAs containing AUUUA motifs described further potential ARE-regulated mRNAs, which are not listed in ARED (*Spasic et al., 2012*).

	Motif	Example	Deadenylation
Class I	several AUUUA motifs flanked by U-rich sequences	early-response-genes, e.g. c-fos	synchron
Class II	clustered or tandem, overlapping AUUUA motifs flanked by U-rich sequences	cytokines, e.g. GM-CSF, TNF $\alpha$ , VEGF, IL-3	asynchron
Class III	U-rich sequences in the absence of AUUUA pentamers	c-jun	synchron

Table 1: Classification of AU-rich elements according to *Chen et al. (Chen et al., 1995)*. GM-CSF, Granulocyte macrophage-colony stimulating factor ; TNF $\alpha$ , Tumor necrosis factor  $\alpha$ ; VEGF, Vascular endothelial growth factor; IL-3, Interleukin-3.

	Motif	Example
Cluster I	AUUUAUUUAUUUAUUUAUUUA	GM-CSF, TNF $\alpha$ , IL-1 $\beta$
Cluster II	AUUUAUUUAUUUAUUUA	INF- $\alpha$
Cluster III	WAUUUAUUUAUUUAW	COX-2, IL-2
Cluster IV	WWAUUUAUUUAWW	FGF2, VEGF
Cluster V	WWWUUAUUUAWWW	u-PAR

Table 2: Classification of AU-rich elements according to *Bakheet et al. (Bakheet et al., 2003)*. INF $\alpha$ , Interferon  $\alpha$ ; COX-2, Cyclooxygenase 2; FGF2, Fibroblast growth factor 2; uPAR, Urokinase-type plasminogen activator receptor

### 1.2.2. Trans-acting factors: Focus on ARE-binding proteins

AU-rich elements are specifically recognized by *trans*-acting factors, called ARE-binding proteins (ARE-BPs), which belong to a group of proteins, called turnover and translation regulatory RNA-binding proteins (TTR-BPs) (Pullmann *et al.*, 2007). Therefore, ARE-BPs are involved in processing, nuclear export, cellular localization, degradation and translation of mRNAs. The final effect on mRNA strongly depends on the primary mRNA regulatory function of the bound ARE-BP. mRNA stability is determined by a balance between counteracting factors which compete for their binding to AREs or co-activate each other due to direct interactions (Cherradi *et al.*, 2006; Hinman & Lou, 2008; Kedar *et al.*, 2012). mRNAs are either stabilized and their translation is enhanced or they are tagged for degradation and translationally repressed. A common structure characteristic of ARE-BPs is the RNA-binding domain, which could be of different nature, including RNA Recognition Motifs (RRMs), K homology (KH)-domains and CCCH tandem zinc fingers. The N- and C-terminal part of these factors is less conserved and contains numerous interaction sites with other proteins. Therefore ARE-BPs are considered as linkers between mRNAs and the mRNA decay machinery by recruiting components of these protein complexes implicated in every step of mRNA degradation (Figure 4).

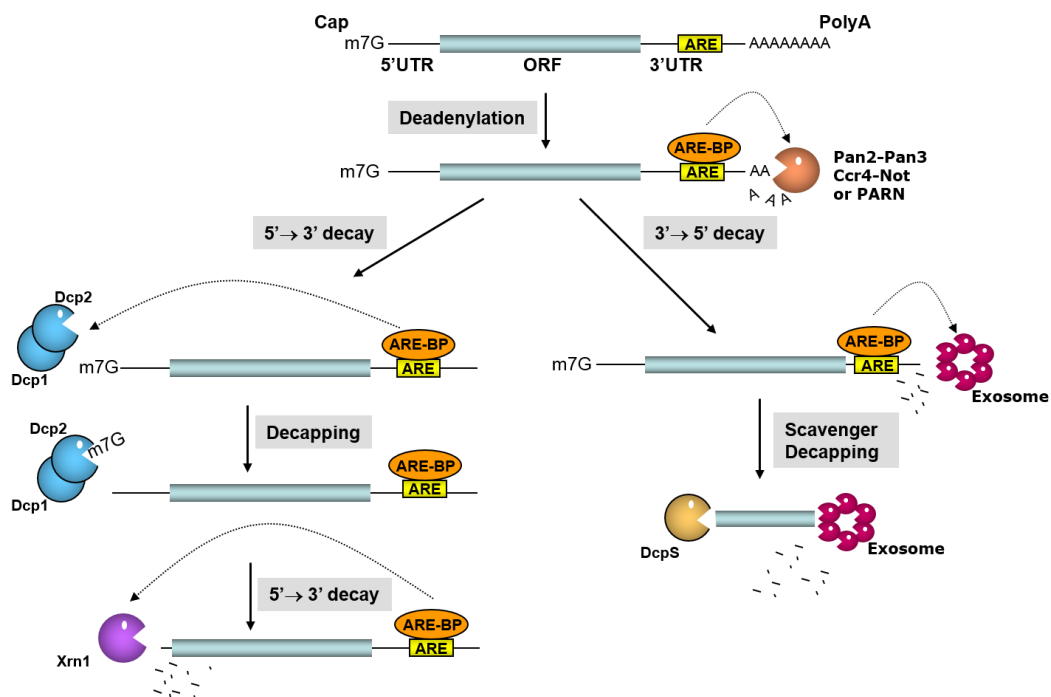


Figure 4: Regulation of deadenylation-dependent mRNA degradation through AU-rich elements (AREs) recognized by ARE-binding proteins (ARE-BPs) in eukaryotes (Planel *et al.*, 2014).

To date, around twenty ARE-BPs are known, the most important are listed in Table 3.

ARE-binding protein	Function	RNA-binding domain	Mode of action	Modifications
HuR (ELAV proteins)	Stabilizing	RRM	Competes with destabilizing proteins for ARE-binding; might relocalize mRNAs away from the decay machinery	CARM-1 mediated methylation reduces stabilizing function; phosphorylation by Chk2, PKC $\alpha$ and PKC $\delta$ regulates HuR shuttling and RNA-binding affinity
PAIP2 (PABP)	Stabilizing	RRM	Binds to the poly(A)tail and protects the transcript from deadenylase attacks; Interacts physically with HuR	Post-translational modifications such as methylation/acetylation which may alter interaction of PABP with mRNA stability-controlling factors
AUF1 (hnRNP D) four splice isoforms (p37, p40, p42, p45)	Usually destabilizing, sometimes stabilizing	RRM	Recruits the exosome, RNA remodeling	Phosphorylation leads to modifications in RNA conformation and/or in AUF1 RNA-binding activity
KSRP	Destabilizing	KH domain	Recruits decay enzymes: PARN and exosome	Phosphorylation by p38-MAPK pathway leads to reduced RNA-binding affinity
CUG-BP	Destabilizing	RRM	Recruits PARN	Phosphorylated by myotonic dystrophy protein kinase
RHAU	Destabilizing	RNA helicase	Recruits decay enzymes: PARN and exosome	unknown
TIA-1, TIAR	Translational silencing	RRM	Induces aggregation into stress granules	Phosphorylated by FAST
Tristetraprolin (TTP, TIS11, ZFP36), TIS11b (BRF1, ZFP36L1), TIS11d (BRF2, ZFP36L2)	Destabilizing	CCCH-type zinc finger	Recruit decay enzymes: Ccr4, Dcp1, Exosome (PM/Scf-75, Rrp4)	Phosphorylation by p38-MAPK pathway leads to association with 14-3-3 proteins

Table 3: Overview of important ARE-binding proteins in eukaryotes (*Planel et al., 2014*).

#### 1.2.2.1. Stabilizing ARE-binding proteins

The best studied stabilizing ARE-BP is HuR, a ubiquitously expressed protein (Table 3). This protein is a member of the ELAV (embryonic-lethal abnormal visual in *Drosophila*

*melanogaster*) protein family, comprising the nervous-system specific members HuB, HuC and Hu-D (Ma et al., 1996). HuR harbours three RNA recognition motifs through which it interacts with ARE-containing mRNAs. HuR targets a variety of mRNAs including cytokines, proto-oncogenes, growth factors and cell cycle regulators, leading to transcript stabilization and/or enhanced translation (Hinman & Lou, 2008; Lebedeva et al., 2011). Upon cell stimulation, nuclear HuR translocates reversibly into the cytoplasm (Fan & Steitz, 1998). The mechanism of mRNA stabilization remains unknown. Based on experimental observations, one could assume a competition between HuR and destabilizing ARE-BPs for the ARE (Cherradi et al., 2006; Hinman & Lou, 2008). Alternatively, HuR could interact directly or indirectly with components of the mRNA decay machinery, avoiding the assembly of these protein complexes, or strengthening the interaction of PABP to the poly(A) tail. In addition to its role in regulation of mRNA stability, HuR can also affect the expression of target proteins at the level of translation. HuR may serve as enhancer or repressor of translation. Furthermore, Hu proteins have been shown to influence alternative splicing and polyadenylation of the pre-mature transcript (Hinman & Lou, 2008).

It is worth mentioning that HuR-triggered mRNA functions are regulated by post-translational modifications of HuR involving several signalling cascades (Doller et al., 2008). HuR is target of various kinases such as MAPKs, MAPK-activated protein kinase-2 (MK2), AMP-activated kinase (AMPK) or the cell-cycle checkpoint kinase 2 (Chk2) leading to the phosphorylation of the protein. Furthermore, methylation and acetylation as well as ubiquitination are described for HuR. Altogether these post-translational modification regulate HuR subcellular localization, impact the sequestration of HuR and HuR binding to ligand proteins (e.g. 14-3-3). However, detailed mechanisms are poorly understood.

#### **1.2.2.2. Destabilizing ARE-binding proteins**

The antagonists of stabilizing ARE-BPs are factors promoting mRNA decay by binding to AREs. Overexpression of such factors is associated with rapid degradation of ARE-containing mRNAs. Once bound to the ARE, destabilizing ARE-BPs function as platforms for assembly of protein complexes implicated in mRNA decay by interacting directly or indirectly with mRNA-degrading proteins. In addition, ARE-BPs have been found in cellular micro-organelles, such as P-bodies, where mRNA decay takes place (Garneau et al., 2007). It needs to be emphasized that these *trans*-acting factors do not harbour an intrinsic nuclease activity.

The most important and best-studied destabilizing ARE-BPs, including AUF1, K homology splicing regulatory protein (KSRP), RHAU and TIS11 proteins are listed in Table 3.

Due to alternative splicing, four isoforms (p37<sup>AUF1</sup>, p40<sup>AUF1</sup>, p42<sup>AUF1</sup>, p45<sup>AUF1</sup>) of the *trans*-acting ARE-BP AUF1 with unique functions are present in mammals (*White et al., 2013*). AUF1 binds with high affinity via its RRM domain to ARE-containing mRNAs of Myc, Fos and GM-CSF. A current model suggests that p37<sup>AUF1</sup> recognizes the ARE-containing mRNA and orchestrates the formation of a large multi-subunit complex, the AUF1-and-Signal Transduction-Regulated Complex (ASTRC), composed of translation initiation factors and molecular chaperones. Furthermore, the ASTRC seems to recruit specific deadenylases and exosome components accelerating deadenylation and consequent degradation of targeted mRNAs. The interaction of AUF1 with either translation initiation factors or components of the mRNA decay machinery of the ASTRC supports the double-edged function of AUF1 as either stabilizing or destabilizing ARE-BP.

The ubiquitously expressed destabilizing protein KSRP is a more general post-transcriptional regulator of gene expression (*Briata et al., 2013*). Despite other functions of KSRP during mRNA life-cycle, this protein can bind AREs via its KH-domains to form complexes with the deadenylase PARN, the exosome components and the decapping enzyme Dcp2, favouring rapid mRNA decay. mRNAs of c-fos, TNF $\alpha$ , IL-8 as well as other cytokine and growth factor mRNAs are part of the KSRP target-repertoire. In addition to its role in mRNA decay, KSRP serves as a component of the complexes Dicer and Drosha thus promoting the maturation of miRNAs (*Trabucchi et al., 2009*).

Like KSRP, RHAU interacts with the deadenylase PARN and exosome components, thus inducing deadenylation and destabilization of ARE-bearing mRNAs (*Tran et al., 2004*).

Tristetraprolin (TTP), TIS11b and TIS11d, classified as TIS11 protein family, are well known as potent mediators of ARE-mediated mRNA decay. All three proteins will be introduced in more detail in Chapter 2 as they are the main subject of the present work. TIS11 proteins bind through a tandem CCCH type zinc finger mainly to AUUUA motifs flanked by additional uridylylate residues (*Brewer et al., 2004*). Mounting evidence indicates that the main function of TIS11 proteins is to recruit the deadenylation machinery on their target mRNA thus enhancing the shortening of the poly(A) tail and promoting mRNA destabilization. Co-Immunoprecipitation experiments have demonstrated the interaction between TTP and different deadenylases (*Lai et al., 2003; Marchese et al., 2010*). Very interestingly, a recent study demonstrated the direct interaction of TTP and Not1, the scaffold protein of the Ccr4-Not1 deadenylation complex (*Sandler et al., 2011; Fabian et al., 2013*). *In vitro*, TIS11 proteins could also induce PARN activity, while having no impact on ARE-lacking transcripts (*Lai et al., 2003*). In addition, TIS11 proteins seem to be also implicated in later steps of mRNA decay, as co-immunoprecipitation



experiments have identified the decapping enzyme Dcp2, the cytoplasmic 5'→3' exoribonuclease Xrn1 and components of the exosome as TIS11 protein interaction partners (*Fenger-Gron et al., 2005; Lykke-Andersen & Wagner, 2005; Hau et al., 2007*). Based on the phenotype of TTP knock out mice, which exhibit a syndrome of systemic inflammation due to TNF $\alpha$  overexpression, TNF $\alpha$  was identified as one of the first TTP target mRNAs in macrophages (*Taylor et al., 1996; Carballo et al., 1998*). Other important factors destabilized by TIS11 proteins are GM-CSF, VEGF, cyclooxygenase 2 (COX-2), IL-1, IL-8, IL-3 and hypoxia-inducible factor-1 (HIF-1) (*Stoeklin et al., 2002; Boutaud et al., 2003; Ciais et al., 2004; Chen et al., 2006; Marderosian et al., 2006; Essafi-Benkhadir et al., 2007; Suswam et al., 2008; Kim et al., 2010; Bourcier et al., 2011; Chamboredon et al., 2011*). Even though mRNA destabilization remains their best-characterized role, TIS11 proteins seem to have alternative functions throughout the life of an mRNA, which will be discussed in Chapter 2.

### **1.3. Regulation of mRNA decay by signalling pathways**

Even though mRNA decay is currently intensively investigated, the question of how extracellular stimuli or cell-cycle checkpoint signals are interacting with cytoplasmic mRNA turnover and how key players within this game are regulated remains unclear. During the last years, an additional level of complexity regarding the sophisticated regulation of mRNA turnover has appeared. Almost all ARE-BPs are targets of post-translational modifications, which alter their RNA-binding affinity or protein-protein interactions. Regulatory post-translational modifications, including phosphorylation, ubiquitination and methylation have been described for ARE-BPs. However, the impact of these chemical modifications on ARE-BP functions is not well understood. Phosphorylation is one of the best studied post-translational modifications of ARE-BPs. Several signal transduction pathways are implicated and the most important ones will be briefly introduced (*Thapar & Denmon, 2013*). Most data derived from disease conditions, like cancer and inflammatory syndromes, where altered mRNA turnover due to unbalanced activity of ARE-BPs was observed. In most cases, phosphorylation was shown to repress the mRNA destabilizing activity while enhancing ARE-BP protein stability. These reversible post-translational modifications ensure the immediate availability of ARE-BP to allow cells rapid adaptation to environmental changes.

The p38 mitogen activated protein kinase (p38 MAPK) pathway is one of the most important signalling cascades regulating ARE-mediated mRNA decay. Activation of p38 MAPK by environmental stress leads to the stabilization of class II ARE-containing mRNAs, such as TNF $\alpha$ , VEGF, Macrophage inflammatory protein 1 $\alpha$  (MIP-1 $\alpha$ ), GM-CSF, COX-2, matrix-

metalloprotease 1 (MMP-1) and MMP-3 (Wang *et al.*, 1999; Dean *et al.*, 2001; Reunanen *et al.*, 2002; Tebo *et al.*, 2003; Wilson *et al.*, 2003; Cha *et al.*, 2011). Therefore, it is not surprising, that stimulation of this pathway is associated with inflammatory diseases and cancer. Among other ARE-BPs, TTP is the best studied target of the p38 pathway. Upon activation, the protein kinase 2 (MK2), a downstream target of p38, phosphorylates TTP at two Serines (Ser). TTP thus loses its mRNA binding affinity and interacts with 14-3-3 proteins. This leads to an altered localization of TTP within the cell and prevents its destabilizing activity (Chrestensen *et al.*, 2004). The inhibition of KSRP-induced myogenin mRNA decay via its p38 phosphorylation has been described (Briata *et al.*, 2012).

The Extracellular-signal-regulated kinase (ERK or MAPK) pathway plays a central role in cell growth and cell cycle progression under healthy conditions as well as in uncontrolled cell proliferation in cancer and in inflammatory response of macrophages and eosinophils. For example, the ARE-BP AUF-1 induces exosome-mediated decay of GM-CSF mRNA in unstimulated eosinophils (Shen *et al.*, 2005). Stimulation of the ERK signalling cascade in these cells ends in phosphorylated AUF-1, which dissociates from the ARE, leading to GM-CSF mRNA stabilization. The constitutive activity of ERK in Ras-transformed fibroblasts inhibited TTP-mediated destabilization of VEGF mRNA (Essafi-Benkhadir *et al.*, 2007). Interestingly, the RNA-binding capacity of TTP to AREs in the VEGF 3'UTR was not changed by ERK activity. Furthermore, inducible TTP overexpression in melanoma cell lines with constitutive ERK activity revealed that ERK triggers the proteasome-dependent degradation of TTP (Bourcier *et al.*, 2011).

As another important signalling pathway, the phosphatidylinositol 3-kinase (PI3K) and its most important target the Ser/Thr kinase AKT (or protein kinase B) need to be mentioned. Downstream effectors of AKT are implicated in cell survival, metabolism, cell cycle and protein synthesis. Transcriptome studies of human glioblastoma cells upon PI3K inhibition revealed an increased mRNA degradation of around 20 genes (Graham *et al.*, 2010). SiRNA experiments identified TIS11b and KSRP as ARE-BPs responsible for this effect. In addition, TIS11b has been described as target of AKT (Schmidlin *et al.*, 2004). Phosphorylation by AKT facilitates the interaction between TIS11b and 14-3-3 proteins in the cytoplasm leading to a decrease of its ARE binding-affinity and a consequent mRNA stabilization. Similar observations were reported for KSRP (Briata *et al.*, 2012). Phosphorylation of KSRP and consequent inhibition of this ARE-BP increased the half-life of  $\beta$ -catenin and myogenin mRNAs in myoblasts.



**In conclusion**, gene expression, from transcriptional initiation to translation of a mature protein, is a highly controlled mechanism. In between this start- and end-point, a mRNA, as a part of a messenger ribonucleoprotein complex, needs to pass several key steps during its life-cycle, including processing of the pre-mature mRNA, nuclear export, quality assessment as well as translational repression and de-repression. Among these processes, the regulation of mRNA stability is an additional key control point. This post-transcriptional regulation allows for rapid changes in mRNA levels during cell adaptation to extracellular stimuli.

The majority of eukaryotic mRNAs is degraded via the deadenylation-dependent mRNA decay. The steady-state level of an mRNA is determined by *cis*-acting sequences, mainly located in the 3'UTR, which are recognized by *trans*-acting factors, which are directly or indirectly bound to components of the translational or mRNA decay machinery. mRNA stability is determined by the balance between stabilizing factors, like HuR, and destabilizing factors, such as TTP, AUF1 or KSRP, which bind to *cis*-acting AU-rich elements pre-dominantly located in the 3'UTR of mRNAs. ARE-BPs are implicated in each step of the deadenylation-dependent mRNA decay.

ARE-BPs themselves undergo post-translational modifications, mainly phosphorylation, upon activation of signalling cascades, including p38 MAPK, ERK and PI3K/AKT pathway. Phosphorylation of ARE-BPs impairs their interaction with the mRNA decay machinery and increases their protein stability.

Importantly, AREs and ARE-BPs are associated with a rapid decay of growth factor, inflammatory cytokine and proto-oncogene mRNAs. In addition to their phosphorylation by overactive signalling pathways, the altered abundance of ARE-BPs could lead to impaired half-lives of short-lived mRNAs and has pathological consequences. It is therefore not surprising that several diseases, such as cancer and inflammation, are correlated with deregulated mRNA stability. Before discussing this emerging link, TIS11 proteins as major regulators of ARE-mediated mRNA decay are introduced in the next chapter.



## Chapter 2      Key player in mRNA decay: The TIS11 protein family

During a genetic screening of murine fibroblasts (3T3 cells) treated with the tumor promoting phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), *Varnum et al.* identified in the late 1980s an early response gene that they called “TPA-Inducible Sequence 11” (TIS11). At the same time, several other groups confirmed *Varnum et al.*'s observations, which led to the definition of the TIS11 family (*Varnum et al., 1989; DuBois et al., 1990; Gomperts et al., 1990; Lai et al., 1990; Varnum et al., 1991; Nie et al., 1995*). These proteins are nearly undetectable under quiescent conditions, but show a rapid, transient induction of their mRNA in response to external stimuli.

TIS11 proteins are tandem CCCH zinc finger-containing RNA-binding proteins which are ubiquitously expressed and play a crucial role in embryonic development and mRNA decay of short-lived mRNAs. By targeting specific response elements (AU-rich elements) located in the 3'UTR of mRNAs, TIS11 proteins promote mRNA degradation. In addition to this key function, TIS11 proteins seem to be also implicated in mRNA transcription, splicing, polyadenylation as well as translation of mRNAs. Even if the different members of this protein family share common structural characteristics and functions *in vitro*, each TIS11 protein holds a unique role *in vivo*. TIS11 proteins are post-translationally modified, mainly due to kinase-mediated phosphorylations. These modifications regulate their localization, activity and stability. Depletion of TIS11 proteins causes the abnormal stabilization of target short-lived mRNAs and is associated with cancer and systemic inflammatory diseases.

This chapter aims at introducing the TIS11 protein family by highlighting the different members, their function and regulation.

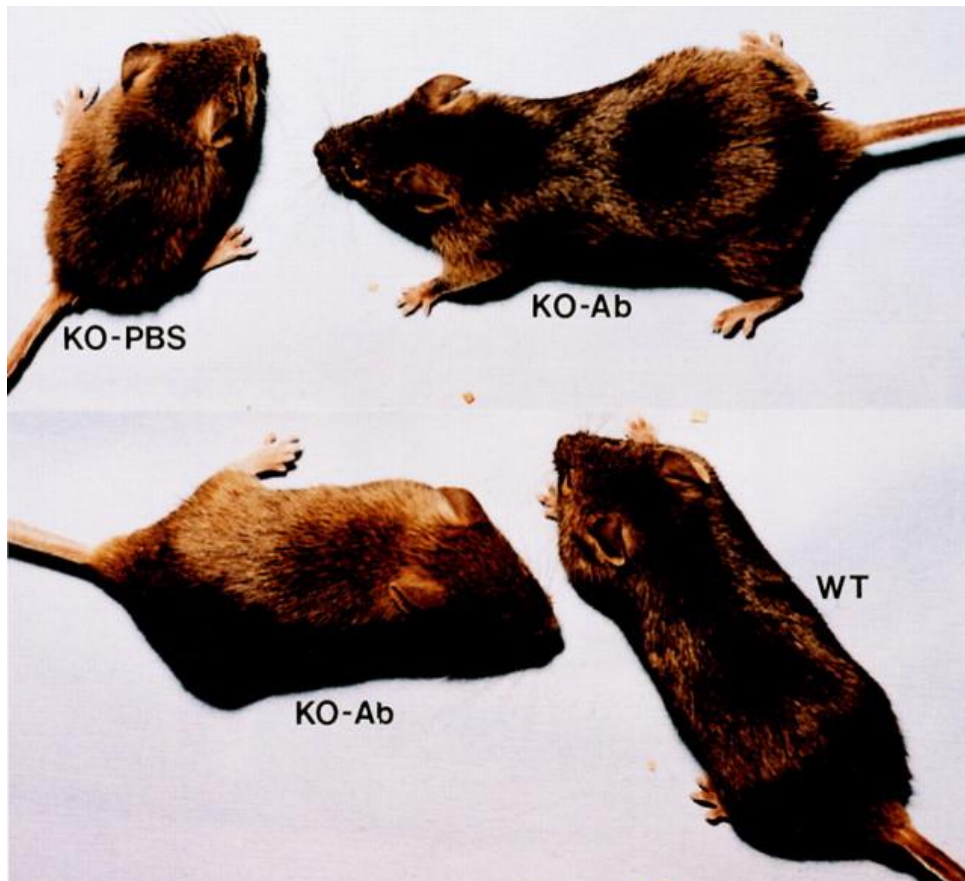


Figure 5: Phenotype of TTP-knock out (KO-PBS) mice compared to wildtype (WT) and prevention of this phenotype by injecting anti-TNF $\alpha$  antibodies (KO-Ab) (Taylor *et al.*, 1996).

## 2.1. Members of the TIS11 protein family

Three members of the TIS11 protein family are known in mammals: TTP, TIS11b and TIS11d (*Lai et al., 1990*). In 2005, *Blackshear et al.* discovered a fourth member, ZFP36L3, which is exclusively expressed in placenta of rodents (*Blackshear et al., 2005*). TIS11 proteins are encoded by different chromosomes and are therefore products of distinct genes. Nevertheless, TIS11 proteins have several common structural characteristics. All members are able to bind to ARE-containing mRNAs and to induce their deadenylation and degradation *in vitro* (*Baou et al., 2009*). By contrast, each member of the TIS11 family possesses a unique role *in vivo* as demonstrated by gene knock out-studies in mice which will be deciphered in the following paragraphs.

### 2.1.1. Tristetraprolin (TTP)

Tristetraprolin (TTP), the prototype of the tandem CCCH zinc finger TIS11 protein family, is also known as 12-O-tetradecanoylphorbol-13-acetate-inducible sequence (TIS11) or Zinc finger protein 36 (ZFP36) as well as Nup475 or G0/G1 switch regulatory gene 24 (G0S24). Full-length TTP was initially cloned from either insulin-, phorbol- or serum-induced murine fibroblasts and further described for humans (*DuBois et al., 1990; Lai et al., 1990*). In addition, TTP expression was shown to be induced by lipopolysaccharide (LPS), cinnamon polyphenols, and green tea extract (*Cao et al., 2004; Cao et al., 2007a; Cao et al., 2007b*). TTP is localized in the nucleus of quiescent fibroblasts, then is phosphorylated rapidly after induction due to external stimuli and translocates into the cytoplasm (*Taylor et al., 1995; Taylor et al., 1996*). By contrast, TTP is exclusively localized in the cytoplasm in macrophages (*Carballo et al., 1998*). To characterize the *in vivo* function of TTP, a murine knock out model was generated (*Taylor et al., 1996*). These mice are viable, but lose weight and body fat several weeks after birth (Figure 5). The TTP-KO mice exhibit furthermore growth retardation, cachexia, arthritis, inflammation and autoimmunity. The high similarity between the phenotype of TTP-KO mice and TNF $\alpha$  overexpressing mice led the authors to hypothesize that TNF $\alpha$  could be the major cause of the systemic inflammatory syndrome seen in TTP-deficient mice (*Keffer et al., 1991*). Indeed, this phenotype could be prevented by frequently treating TTP-KO mice with anti-TNF $\alpha$  antibodies early after birth (*Taylor et al., 1996*). Thus, this KO model described for the first time, a link between TTP and the pro-inflammatory cytokine TNF $\alpha$  and revealed the function of TTP in mRNA decay. To identify the origin of TNF $\alpha$  overproduction in TTP-KO mice *Carballo et al.* transplanted bone marrow of TTP-KO or TTP-WT mice in irradiated immunodeficient mice (*Carballo et al., 1997*).



Gene symbol	Common or protein name	Cell type
<i>Tnf</i>	Tumor necrosis factor	Mouse macrophages
<i>Csf2</i>	Granulocyte-macrophage colony-stimulating factor	Mouse bone-marrow derived stromal cells
<i>Il3</i>	Interleukin-3	Misc. Cells
<i>Ptgs2</i>	Cyclooxygenase 2	Misc. Cells
<i>Pitx2</i>	Pituitary homeobox 2	Mouse pituitary alpha T3-1 cells
<i>SERPINB2</i>	Plasminogen activator inhibitor type 2	Human HEK293 cells
<i>Zfp36</i>	Tristetraprolin	Mouse RAW 264.7 cells
<i>Nos2</i>	Inducible nitric oxide synthase	Human colon carcinoma DLD1 cells
<i>Il2</i>	Interleukin-2	Mouse splenocytes
<i>Il18</i>	Interleukin-1 beta	Mouse RAW 264.7 cells
<i>Il12</i>	Interleukin-12	Mouse J774 macrophages
<i>Cxcl2</i>	C-X-C motif chemokine ligand 2	Mouse J774 macrophages
<i>Ccl20</i>	C-C motif chemokine ligand 20	Mouse J774 macrophages
<i>Il6</i>	Interleukin-6	Misc. Cells
<i>Ier3</i>	Immediate early response 3	Mouse fibroblasts
<i>Lif</i>	Leukemia inhibitory factor	Mouse fibroblasts
<i>Plk3</i>	Polo-like kinase 3	Mouse fibroblasts
<i>HIV-1</i>	HIV-1	Genomic RNA
<i>Ccnd1</i>	Cyclin D1	Mouse fibroblasts
<i>Myc</i>	c-Myc protooncogene	Mouse fibroblasts, human liver cancer cells
<i>Ccl2</i>	C-C motif chemokine ligand 2	Mouse macrophages, fibroblasts
<i>Ccl3</i>	C-C motif chemokine ligand 3	Mouse macrophages, fibroblasts
<i>Vegfa</i>	Vascular endothelial factor A	Misc. Cells
<i>Tcf3</i>	E47	Mouse B cells
<i>Il8</i>	Interleukin-8	Misc. Cells
<i>Cxcl1</i>	C-X-C motif chemokine ligand 1	Mouse macrophages
<i>Il10</i>	Interleukin-10	Mouse macrophages
<i>Clmp</i>	CXADR-like membrane protein precursor	Sertoli TM4 cells
<i>Ifng</i>	Interferon gamma precursor	Mouse T cells
<i>UBE3A</i>	Ubiquitin-protein ligase E3A isoform 1	Human cervical cancer cells
<i>Il1α</i>	Interleukin-1 alpha	Mouse macrophages
<i>TLR4</i>	Toll-like receptor 4	Human THP-1 cells
<i>PLAU</i>	Urokinase-type plasminogen activator	Human breast cancer cells
<i>PLAUR</i>	Urokinase-type plasminogen activator surface receptor	Human breast cancer cells
<i>MMP1</i>	Interstitial collagenase isoform 1 preproprotein	Human breast cancer cells
<i>ECSCR</i>	Endothelial cell surface expressed hemotaxis and apoptosis regulator	HeLa cells
<i>Hif1α</i>	Hypoxia-inducible factor 1-alpha	Endothelial cells?
<i>LATS2</i>	Serine/Threonine-protein kinase LATS2	Human A549 lung cancer cells
<i>Dusp6</i>	Dual specificity protein phosphatase 6	HEK293, A375
<i>SLC10A2</i>	Ileal sodium/bile acid cotransporter	Human Caco-2 cells
<i>Thbd</i>	Thrombomodulin	Rabbit monocytes
<i>IL23α</i>	Interleukin-23 subunit alpha precursor	Mouse dendritic cells, macrophages
<i>Cdkn1α</i>	Cyclin-dependent kinase inhibitor 1	Mouse fibroblasts
<i>IL17</i>	Interleukin-17	Human T cell lines
<i>Pim1</i>	Serine/Threonine-protein kinase pim-1	Mouse fibroblasts
<i>SERPINH1</i>	Serpin H1 precursor	Human hepatoma cells

Table 4: Identified targets of TTP (Brooks & Blackshear, 2013).

After several months, mice which received TTP-KO bone marrow developed TTP-KO phenotype, suggesting that hematopoietic progenitors are responsible for the described syndrome. Additional *in vitro* studies showed an increase in TNF $\alpha$  secretion by LPS-stimulated TTP-KO macrophages that was accompanied by an increase in TNF $\alpha$  mRNA levels. Finally, *Carballo et al.* demonstrated the physical interaction between TTP and the ARE located in the 3'UTR of TNF $\alpha$  (*Carballo et al., 1998*). They concluded that TTP-KO phenotype is caused by an accumulation of TNF $\alpha$  mRNA in macrophages due to increased TNF $\alpha$  mRNA stability in the absence of TTP. These results could be further strengthened by two additional *in vivo* models. Interbreeding of TTP-KO mice with TNF $\alpha$  receptor-deficient mice protected the animals from the systemic inflammatory syndrome (*Carballo & Blakeshear, 2001*). The same effect was observed in the murine TNF $\alpha$ - $\Delta$ ARE model (*Kontoyiannis et al., 1999*).

Another well-known target of TTP is the Granulocyte-macrophage colony-stimulating factor (GM-CSF), a growth factor for myeloid cells. GM-CSF 3'UTR contains AU-rich elements for TTP binding (*Shaw & Kamen, 1986*). *Carballo et al.* observed increased GM-CSF secretion by LPS-induced TTP-KO-bone marrow-derived stromal cells compared to the wildtype due to increased GM-CSF mRNA half-life (*Carballo et al., 2000*). Our laboratory identified HIF-1 $\alpha$  mRNA as target of a TTP in endothelial cells submitted to hypoxia (*Chamboredon et al., 2011*). TTP directly interacts with AREs located in the 3'UTR of HIF-1 $\alpha$  mRNA and induces destabilization of the transcript. Interleukin 2 (IL-2) (*Ogilvie et al., 2005*), IL-3 (*Carballo et al., 1998*), IL-6 (*Stoecklin et al., 2001*), IL-8 (*Winzen et al., 2007; Bourcier et al., 2011*), IL-23 (*Lee et al., 2013*), c-fos (*Raghavan et al., 2001*), cyclooxygenase 2 (COX-2) (*Sawaoka et al., 2003*), c-myc (*Marderosian et al., 2006*), Vascular endothelial growth factor (VEGF) (*Essafi-Benkhadir et al., 2007*) and many other transcripts were also identified as TTP targets (Table 4). It is worth mentioning that no correlation between these potential targets and the TTP-KO model has been reported.

### **2.1.2. TIS11b**

The second member of the TIS11 protein family, TIS11b or Zinc finger protein 36-like 1 (ZFP36L1), cMG1, Butyrate Response Factor 1 (BRF1), Epidermal Growth Factor-response factor 1 (ERF-1) or B-cell early response gene encoding a 36-kDa protein (Berg36) was described by *Gomperts et al.* in 1990 (*Gomperts et al., 1990*). This study reported that growth factor-stimulated epithelial cells of rat intestine displayed an induced expression of an early response gene showing high sequence homology with TIS11. ZFP36L1 mRNA expression was induced by several mitogens and growth factors like EGF, Insulin, LPS, TPA, Phorbol myristyl

Gene symbol	common or protein name
<i>Tnf</i>	Tumor necrosis factor
<i>Csf2</i>	Granulocyte-macrophage colony-stimulating factor
<i>Il3</i>	Interleukin-3
<i>Vegf</i>	Vascular endothelial growth factor
<i>c-IAP2</i>	Human inhibitor of apoptosis protein-2
<i>Star</i>	Steroidogenic acute regulatory protein
<i>Notch1</i>	Notch homolog 1, translocation-associated
<i>BCL2</i>	B-cell lymphoma 2
<i>Dll4</i>	Delta-like protein 4
<i>NR3C2</i>	Mineralocorticoid receptor

Table 5: Identified targets of TIS11b (*Baou et al., 2009*).

acetate (PMA) or Angiotensin II, as already shown for TTP (*Gomperts et al., 1990; Corps et al., 1995; Cao et al., 2004; Cao & Lin, 2008*). Interestingly, the kinetic of gene expression induction differs between TIS11b and TTP. By contrast, redundancy of TIS11b and TTP was observed in cell-free deadenylation assays and cell-free RNA-binding as well as transfection experiments (*Lai et al., 2000; Lai et al., 2003*).

Less is known about the physiological role of TIS11b compared to TTP, so far. *Storch et al.* described a regulation of cardiac and hepatic TIS11b expression by the circadian rhythm proposing a potential role of ZFP36L1 in the circadian variability of cytokine levels in the blood stream (*Storch et al., 2002*). Stabilization of IL-3 in cells expressing a TIS11b mutant was observed by *Stoecklin et al.* (*Stoecklin et al., 2002*). In this study, an elegant functional approach based on the idea of translating mRNA stability changes into a fluorescent signal to identify regulators of mRNA turnover in mammalian cells was used. A reporter-gene (Green fluorescent protein (GFP) or  $\beta$ -globin) was fused to the ARE-containing 3'UTR of IL-3 and transfected *in vitro*. Cells underwent several rounds of mutagenesis, followed by subsequent analysis of the reporter mRNA stability. Clones showing increased GFP half-life were supposed to have a loss of function mutation of an ARE-binding protein and were re-transfected with a retroviral cDNA library. By using this strategy, TIS11b was identified as regulator of GM-CSF, TNF $\alpha$ , IL-2, IL-3 and IL-6 mRNA stability (Table 5) (*Stoecklin et al., 2001*). Ectopic expression of TIS11b in this context could restore the rapid decay of these mRNAs.

Our lab was first to demonstrate that TIS11b induction was concomitant with the decrease of the mRNA of the angiogenic cytokine VEGF in adrenocorticotrophic hormone (ACTH)-stimulated adrenocortical cells (*Chinn et al., 2002*). A functional interaction between TIS11b and the 3'UTR of VEGF mRNA was observed in NIH-3T3 cells. Indeed, co-transfection of TIS11b and

luciferase reporter-gene fused to the 3'UTR of VEGF mRNA led to a significant decrease of luciferase activity and mRNA. Later on, using ribonucleoprotein complex immunoprecipitation, our laboratory was first to show a direct binding of TIS11b to a 75 bp-long sequence located in the 3'UTR of VEGF mRNA (Ciais *et al.*, 2004). In addition, TIS11b was found to be a negative regulator of basal and ACTH-stimulated VEGF mRNA expression in adrenocortical cells. The key role of TIS11b in angiogenesis was even more strengthened by our team who discovered that Delta-like 4 (Dll4), another important factor in blood vessel formation, is a target of TIS11b in endothelial cells (Desroches-Castan *et al.*, 2011). Surprisingly, direct interaction between TIS11b and AREs located near the poly(A)-site in the 3'UTR of Dll4 mRNA did not influence Dll4 mRNA stability. However, the binding of TIS11b modulated the 3'-end maturation of Dll4 mRNA. These results described for the first time an alternative function of TIS11b in posttranscriptional regulation. Germ line deletion of TIS11b caused an intra-uterine death of TIS11b<sup>-/-</sup> embryos at E10.5, demonstrating a crucial role of this zinc finger protein in mouse early development (Stumpo *et al.*, 2004). Stumpo *et al.* claimed frequent chorioallantoic fusion defects leading to placental insufficiency in TIS11b<sup>-/-</sup> embryos. Bell *et al.* confirmed these results and provided a more detailed analysis of the murine TIS11b KO model (Bell *et al.*, 2006). Homozygous deletion of TIS11b led to extra- and intraembryonic vascular defects already visible at E9.5, when heart beat was still detectable in TIS11b<sup>-/-</sup> embryos (Figure 6).

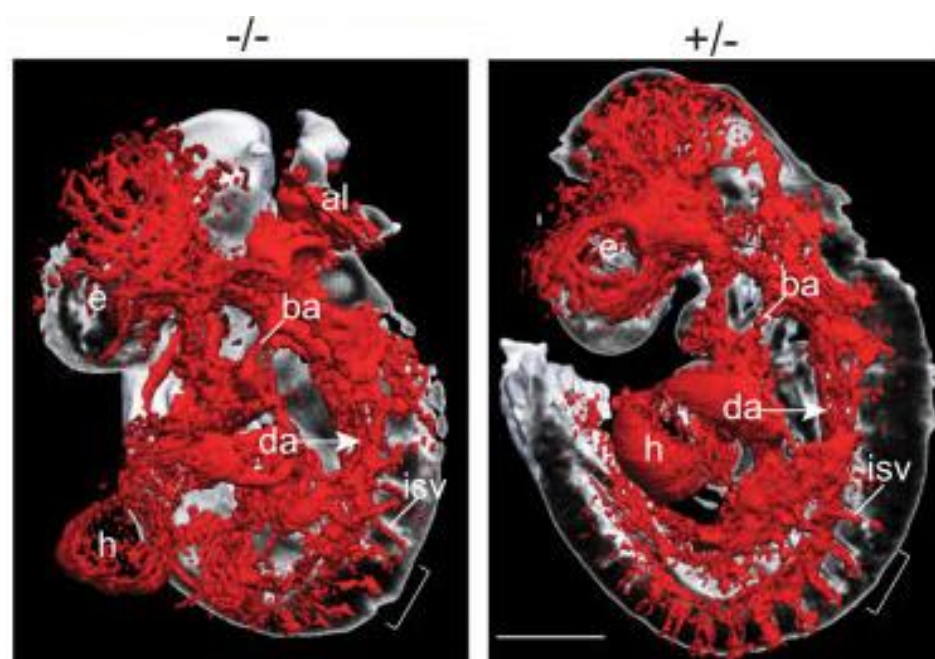


Figure 6: Optical projection tomography of vascular abnormalities in TIS11b<sup>-/-</sup> and TIS11b<sup>+/-</sup> embryos at E9.5 (Bell *et al.*, 2006). al=allantois, ba=brachial artery, da=dorsal aorta, e=eye, h=heart, isv=intersomitic vessels.

In addition, these embryos showed cardiac abnormalities as well as neural tube formation defects. Given the importance of VEGF in angiogenesis and the phenotypic similarity of TIS11b<sup>-/-</sup> embryos with VEGF-A-overexpressing ones, *Bell et al.* analysed VEGF levels in TIS11b<sup>-/-</sup> embryos. The authors demonstrated that VEGF levels were increased mainly due to activation of VEGF mRNA translation (*Bell et al., 2006*).

Induced TIS11b expression has been reported during mouse myoblast differentiation indicating a role of ZFP36L1 in myogenesis (*Busse et al., 2008*). The importance of TIS11b in adult tissue was evidenced by a conditional double TIS11b/TIS11d KO in the mouse thymus generated by *Hodson et al.* (*Hodson et al., 2010*). Thymus TIS11b/TIS11d-KO mice displayed defects in thymic development and acute lymphoblastic leukaemia six months after birth. Malignant transformation was not seen in conditional KOs of either TIS11b or TIS11d, suggesting overlapping functions of these two TIS11 proteins *in vivo*. Re-expression of TIS11b in primary tumor cells of the double KO led to death of cancer cells, supporting the physiological tumor-suppressor role of TIS11b *in vivo*. In this study, the oncogenic factor Notch1 was identified as a target of TIS11b. More recently, our team described a TIS11b-mediated post-transcriptional control of the mineralocorticoid receptor (MR) in renal cortical cells (*Viengchareun et al., 2014*).

### **2.1.3. TIS11d**

*Nie et al.* discovered in 1995 a third member of the TIS11 early response gene family, known as TIS11d, ZFP36L2, BRF2 or ERF-2 (*Nie et al., 1995*). Like TTP and TIS11b, TIS11d also destabilizes ARE-containing transcripts by promoting their deadenylation (*Johnson & Blackwell, 2002; Lai et al., 2002; Lai et al., 2003*). *Phillips et al.* have shown that TIS11d is a nuclear-shuttling protein like TTP and TIS11b (*Phillips et al., 2002*).

To decipher the biological role of TIS11d, *Ramos et al.* generated a partial TIS11d knock out mouse by deleting 29 N-terminal amino-acids (*Ramos et al., 2004*). This hypomorphic mutation resulted in a decreased TIS11d protein level as a major component of the *in vivo* phenotype without affecting TIS11d protein activity, localization and stability (*Ramos, 2012*).  $\Delta$ Zfp36l2 mice were viable, but homozygous females were infertile (*Ramos et al., 2004*). Furthermore, embryos in these mice did not progress beyond the two-cell stage, maybe due to the stabilization of maternal mRNAs, which cause the arrest in early embryonic development. *Ball et al.* proposed recently a more detailed explanation for the observed infertility in  $\Delta$ Zfp36l2 mice (*Ball et al., 2014*). Indeed, they identified the Luteinizing hormone receptor (LHR) mRNA as a target of TIS11d in ovaries and suggested that low levels of TIS11d in mutant mice are not sufficient to downregulate the LHR mRNA induced by Luteinizing hormone (LH) surge, thus resulting in

anovulation. In addition, TIS11d seems to be a critical modulator of definitive haematopoiesis as indicated by the phenotype of a complete TIS11d knock out (*Stumpo et al., 2009*). These mice are dying two weeks after birth due to haemorrhage and reduced number of hematopoietic progenitor cells in fetal liver. Microarray analysis of RNA derived from E14.5 WT or KO fetal livers demonstrated the upregulation of 239 transcripts in the KO liver. Among them are genes encoding for factors implicated in hematopoiesis such as Thrombospondin-1 (TSP1) and CXC-motif chemokines like CXCL1, CXCL4 and CXCL7. As mentioned earlier for TIS11b, TIS11d deletion in the thymus was found to promote acute lymphoblastic leukaemia (*Hodson et al., 2010*). In this study, microarray analysis of the whole thymus from wildtype and TIS11b/d KO mice showed a deregulated expression of more than 500 genes. Among them are genes implicated in lymphatic development and cancer. *Iwanaga et al.* reported a frame shift mutation of TIS11d gene in leukaemia patients (*Iwanaga et al., 2011*). Furthermore, TIS11d seems to be implicated in p53-induced apoptosis in human colorectal adenocarcinoma as suggested by increased TIS11d expression after p53 induction (*Jackson et al., 2006*). In this work, it was shown that TIS11d overexpression inhibits proliferation and promotes cell death.

#### **2.1.4. Zfp36l3**

More than one decade after the discovery of the TIS11 protein family, *Blackshear et al.* described a fourth member, called ZFP36L3 (*Blackshear et al., 2005*). ZFP36L3 shares the mRNA-destabilizing activity with TTP, TIS11b and TIS11d. ZFP36L3 seems to be permanently located in the cytoplasm possibly due to its large protein size or hydrophobic  $\alpha$ -Helices at the C-terminus (*Frederick et al., 2008*). By contrast to the other three TIS11 proteins, ZFP36L3 is exclusively expressed in the yolk sac and placenta of rodents (*Blackshear et al., 2005*). In addition, *Wu et al.* observed a low ZFP36L3 expression in murine adipocytes and macrophages (*Wu et al., 2008*). Interestingly, no orthologous was found in humans until now. Apart from the demonstrated ZFP36L3 expression during mouse embryogenesis and cornea maturation, the function of this TIS11 protein is still unclear (*Wu et al., 2008*).

## **2.2. Regulation of TIS11 protein expression**

TIS11 proteins are phylogenetically highly conserved (*Blackshear & Perera, 2014*). Orthologous to mammalian TTP, TIS11b and TIS11d were found in all studied vertebrates except birds, as well as in rodents, amphibians and fish.

### Induction of TIS11 protein expression

Expression of the TIS11 protein family is rapidly induced by different mitogens, hormones and stress (hypoxia, hypertonicity, UV radiation) due to the presence of specific response elements located in their respective promoters which are recognized by transcription factors (*Blackshear, 2002; Sinha et al., 2009; Sanduja et al., 2012; Viengchareun et al., 2014*). Compared to TTP, TIS11b and TIS11d display higher expression at the basal level, associated with a reduced response to stimuli. Different studies in mammals describe organ-specific expression of the three TIS11 proteins, indicating that the expression and regulation of these proteins is cell-type specific. Indeed, TTP, TIS11b and TIS11d respond differently to the same stimulus in one cell-type (*Carrick & Blackshear, 2007*). This differential regulation suggests that they are involved in different cellular processes. It is assumed that their tissue-specific expression and regulation contribute to their specificity of action in physiological and pathological processes (*Ciais et al., 2004*).

As already mentioned, TIS11 genes were discovered as early-response genes, almost undetectable in starved cells and rapidly expressed after cell activation by insulin, TPA, serum, etc. To study the serum-inducibility of TTP, the 5'-flanking region and the only intron of TTP gene (*Zfp36*) were analysed. Several promoter response elements which are bound by transcription factors such as SP1, activator protein 2 (AP2) or EGR-1 were found in the 5'-proximal region of TTP gene. In all cases, these consensus sequences behaved as classical enhancer elements as they could confer serum-inducibility, when cloned upstream of non-serum-inducible genes. *Lai et al.* demonstrated the requirement of the single intron for serum-induced TTP expression (*Lai et al., 1998*). In addition, the Transforming growth factor  $\beta$  (TGF $\beta$ ) stimulates TTP transcription through the binding of Smad3/4 to putative response elements in TTP promoter (Figure 7) (*Blackshear, 2002; Sanduja et al., 2012*). Sequence alignments of the promoter region of all three TIS11 proteins revealed the presence of a cyclic AMP response element (CRE) located exclusively in the TIS11b promoter. This motif was absent in the TTP or TIS11d promoter sequence, at least 2000 bp upstream of the transcription start site, suggesting that TIS11b, but not TTP or TIS11d expression is cAMP-regulated. Indeed, our lab found that ACTH induces TIS11b expression in adrenocortical cells through the binding of CREB transcription factor to a cyclic AMP response element located in TIS11b proximal promoter (unpublished data).

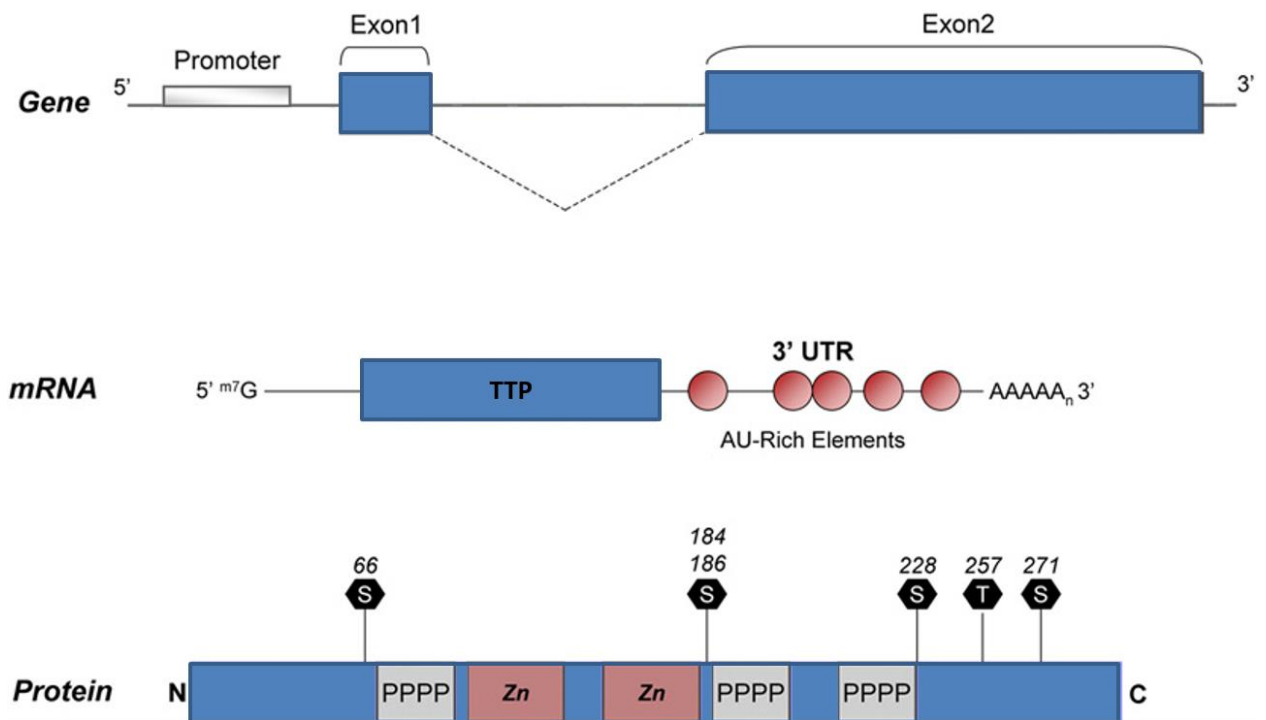


Figure 7: Schematic presentation of TTP gene, mRNA and protein. TTP gene consists of one intron and two exons (*Sanduja et al., 2012*). 3'UTR of TTP mRNA harbours several AU-rich elements. TTP protein structure displays three tetra-proline (PPPP) repeats, two CCCH zinc finger (Zn) domains and multiple serine/threonine phosphorylation sites.

### Expression of TIS11 proteins in normal human tissues

TIS11 proteins are detectable early in mice embryonic development as shown by the characterization of TIS11b KO embryos compared to the WT littermates (*Stumpo et al., 2004; Bell et al., 2006*). TIS11b is highly expressed in the chorion, yolk sac, neural tube, heart and brain. In human adults, *Carrick & Blackshear* detected an elevated mRNA level for the three members in lung, colon, pancreas, adrenal gland and ovaries, whereas TIS11 family mRNA expression was low in stomach, liver, heart and spleen (*Carrick & Blackshear, 2007*). It is mentioning that the three TIS11 proteins are not equally expressed in the same organ. For example, TIS11b is abundant in lung, colon, bladder, ovary, adrenal gland, pancreas and thymus, while weakly expressed in skeletal muscle, heart and brain. The levels of TIS11b and TIS11d are higher in the pancreas, adrenal gland and ovaries, compared to TTP (*Carrick & Blackshear, 2007*). The highest expression of TTP was observed in uterine cervix (*Carrick & Blackshear, 2007*). Unfortunately, an equivalent study to *Carrick & Blackshear* on TIS11 protein levels is still lacking.



### Autoregulation of TIS11 family mRNA expression

Interestingly, mRNA of all three TIS11 proteins contains AREs in the 3'UTR which regulate their mRNA stability (*Brooks et al., 2004*). Immunoprecipitation experiments demonstrated that TTP binds to its own mRNA in human monocytes. By using a reporter-gene assay, where TTP 3'UTR was fused to luciferase gene, it was confirmed that TTP regulates its own expression in a TTP 3'UTR-dependent manner. *Brooks et al.* identified three functional AREs in the TTP 3'UTR. This auto-regulatory negative feedback loop allows TTP expression to return rapidly to basal low levels 2-4h after induction (*Tchen et al., 2004*). Even if not demonstrated so far, a cross-regulation between TIS11 proteins has been suggested by *Stoecklin et al.* who found TIS11b/d mRNAs in a TTP-associated mRNA pool (*Stoecklin et al., 2008*).

### 2.3. Protein domains and truncation of TIS11 proteins

Sequence alignments suggest a higher homology between TIS11b and TIS11d compared to TTP. Based on this, a specific submotif PFAM PF04553 has been defined, pointing out the close relation of the N-terminal part of TIS11b and TIS11d. However, TIS11 proteins are sharing additional similarities regarding their structure (Figure 8).

TIS11 proteins contain a tandem CCCH zinc fingers, which are characterized by a 18 amino acid-spacer, an internal  $CX_8CX_5CX_3H$  (X = variable amino acids) motif and two highly conserved (R/K)YKTEL sequences. The tandem zinc finger domain (TZF), essential for mRNA-binding and nuclear localization, is evolutionary highly conserved and was found in proteins from yeast to humans. To investigate how the CCCH zinc fingers of TIS11 proteins interacts with class II AU-rich elements (5'-UUAUUUAUU-3'), *Hudson et al.* determined the NMR structure of TIS11d TZF domain (*Hudson et al., 2004*). Even in the absence of an mRNA, the TZF domain binds to two zinc atoms to form a pair of CCCH zinc fingers leading to the stabilization of the folded protein structure (Figure 9). Mutation of one zinc finger or of conserved amino acids in the TZF domain causes the loss of TTP-mRNA binding affinity (*Lai et al., 2002*). *Stoecklin et al.* observed the same effect when the first cysteine residues of both zinc fingers were replaced by an arginine (*Stoecklin et al., 2002*). Each of the CCCH zinc finger binds to separate 5'-UAUU-3' subsites of the ARE-containing single-strand mRNA, leading to a conformational change of the protein. mRNA recognition seems to be based on ARE sequence. Interestingly, the mRNA molecule interacts through a few nucleotides with the TIS11d TZF domain at one "face" of the protein, thus leaving the possibility of interactions between TIS11d and other factors modulating its mRNA binding affinity or activity. The protein-mRNA complex is further stabilized by interactions between RNA bases and conserved aromatic side chains of

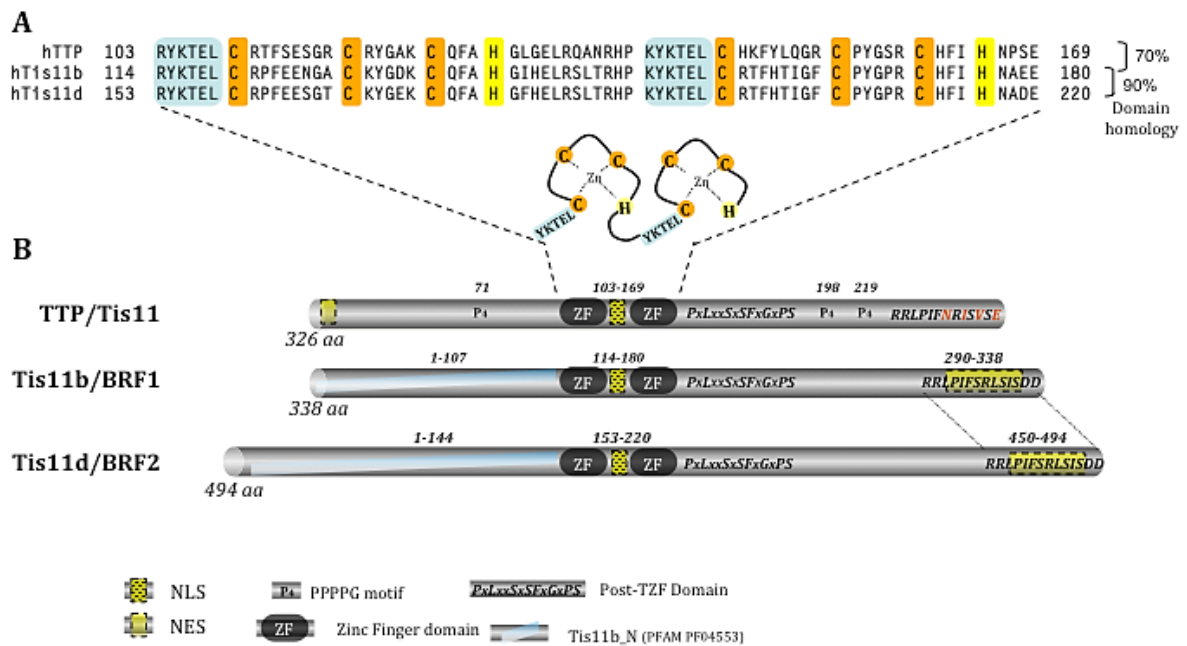


Figure 8: Structure similarities between human TIS11 proteins (Ciais *et al.*, 2013). (A) Sequence alignment of the tandem zinc finger (TZF) domain. (B) Common structural characteristics of the three TIS11 proteins, including the nuclear localization sequence (NLS) between the two zinc fingers (ZF), the nuclear export sequence (NES). See text for further descriptions.

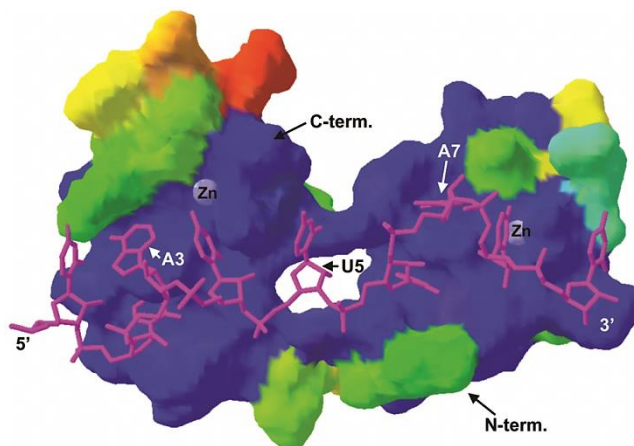


Figure 9: Predicted structure of human TTP tandem zinc finger (TZF) domain in a complex with 5'-UUAUUUAUU-3'-ARE (pink) based on original structure for TIS11d TZF domain (Carrick *et al.*, 2004; Hudson *et al.*, 2004). Colours illustrate amino acid similarities between TTP and TIS11d TZF (blue corresponds to high similarity > green > yellow > orange > red indicating low amino acid similarity).

the protein backbone. Taking into account the high degree of homology within the TIS11 protein family members, *Carrick et al.* proposed a model of the human TTP TZF peptide bound to an mRNA (Figure 9) (*Carrick et al., 2004*).

Closely to the TZF domain, a PxLxxSxSFxGxPS sequence motif was described for all three members (Figure 8B). The function of this domain remains largely unknown, but it contains a binding site for 14-3-3 proteins, at least for TTP and TIS11b (*Schmidlin et al., 2004; Stoecklin et al., 2004*). In between the two zinc fingers, a nuclear localization sequence (NLS) is present in all three human TIS11 proteins. On the other hand, a nuclear export sequence (NES) is located at the N-terminus of TTP, but present at the C-terminal end of TIS11b and TIS11d. Both NLS and NES are ensuring the nucleo-cytoplasmic shuttling of these proteins (*Murata et al., 2002; Phillips et al., 2002*). NLS-containing proteins are bound by nuclear import receptors like importin  $\alpha/\beta$  and transported into the nucleus in energy-dependent manner. NES-bearing proteins interact with the nuclear export receptor CRM-1. This interaction is specifically inhibited by the anti-fungal agent leptomycin B. As shown in Figure 10, the nucleo-cytoplasmic shuttling of TIS11 proteins is CRM-1 dependent (*Phillips et al., 2002*). Overexpressed TIS11 proteins fused to GFP are predominantly present in the cytoplasm whereas inhibition of CRM-1 dependent nuclear export leads to their nuclear localization. Interestingly, the fourth member ZFP36L3 remains cytosolic, even in the presence of leptomycin B, due to a non-functional NES and further modifications of the C-terminal part of the protein (*Frederick et al., 2008*). In general, subcellular localization of TIS11 proteins is cell type-dependent and regulated by external stimuli. For example, TTP is localized in the nucleus of starved fibroblasts and translocates into the cytoplasm immediately after serum stimulation (*Taylor et al., 1996*).

In contrast to the highly conserved CCCH tandem zinc finger (TZF) domain, the N- and C-terminal part differ much more between TTP, TIS11b and TIS11d, suggesting interactions with different partners as well as specific regulations of the family members. The only study describing the specific role of TIS11 protein domains in mRNA decay was performed by *Lykke-Andersen & Wagner* (*Lykke-Andersen & Wagner, 2005*). The authors focused mainly on TTP, but also showed some results for TIS11b. *Lykke-Andersen & Wagner* generated three truncated protein versions of TTP either by deleting the N-terminal part ( $\Delta$ NTD), or the C-terminal part ( $\Delta$ CTD), or both to keep just the TZF. The  $\Delta$ NTD mutant was expressed at similar levels as full-length TTP whereas the  $\Delta$ CTD mutant was expressed at 2- to 3-fold higher levels than full-length TTP. All protein forms were located in the cytoplasm. Protein activity and RNA-binding capacity were detected for both mutants although to a lower level compared to the full-length

## LMB

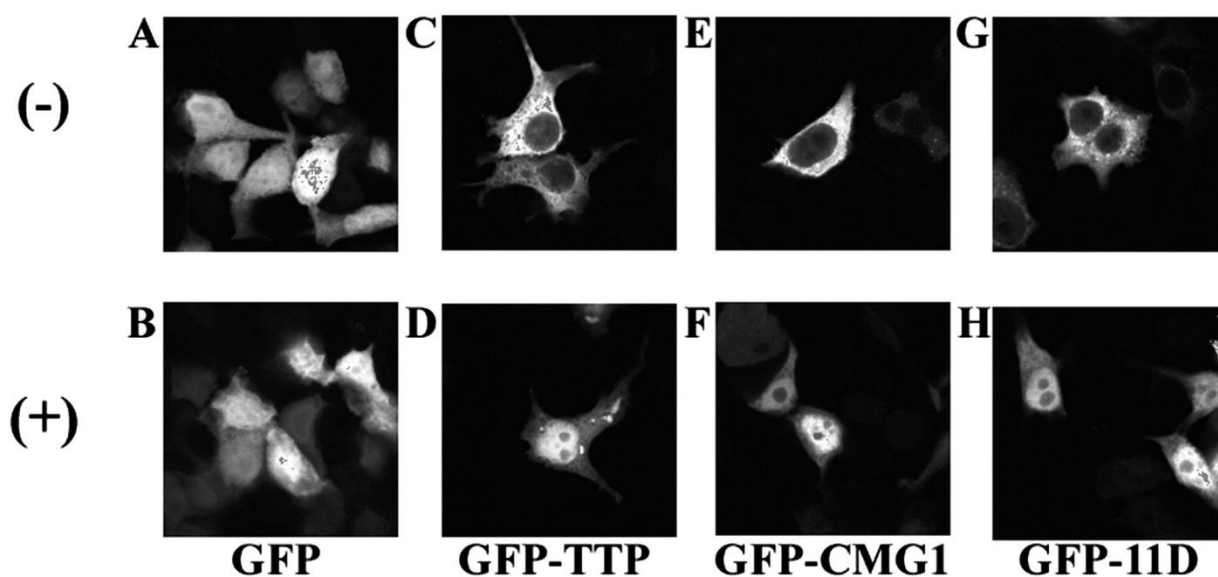


Figure 10: Subcellular localization of TTP, TIS11b (CMG1) and TIS11d (11D) in leptomycin B-treated HEK 293 cells (Phillips *et al.*, 2002).

protein, indicating an important role of both the N- and the C-terminus for an optimal mRNA decay activity. The TZF alone was able to bind RNAs, but unable to trigger mRNA decay, suggesting that RNA binding and mRNA decay activation are two separate functions of TIS11 proteins. Overexpression followed by co-immunoprecipitation experiments showed the interaction of the N- and the C-terminal domain of TIS11 proteins with factors implicated in decapping, deadenylation and exonucleolytic decay, supporting the idea that TIS11 proteins are linking mRNA to the RNA decay machinery (Lykke-Andersen & Wagner, 2005; Marchese *et al.*, 2010; Fabian *et al.*, 2013).

### 2.4. Functions of TIS11 proteins

Initially thought to be transcription factors because of their nuclear localization, rapid induction and TZF motif, TIS11 proteins are currently well identified as potent mediators of ARE-dependent mRNA decay. Even though mRNA destabilization remains their best-characterized role, TIS11 proteins seem to have alternative functions throughout the life of an mRNA. Keeping in mind that TIS11 proteins do not harbour intrinsic enzymatic activities, these proteins trigger mRNA decay via multiple protein-protein interactions (Table 6). TIS11 proteins could be considered as docking platforms between mRNAs and components of the mRNA decay machinery.

Interacting proteins	ID method	TTP interacting domain	Interacting proteins	ID method	TTP interacting domain
<i>Deadenylation</i>			<i>RNA surveillance</i>		
CAF1	OE-IP	C-terminus	Upf1	Y2H	
Ccr4	OE-IP	N-terminus	Upf2	Y2H	
Not1	OE-IP	C-terminus	<i>RNA helicases</i>		
<i>Decapping</i>			DDX5	OE-IP	
hDcp1a	IP, Y2H	C-terminus	DDX17	Y2H, OE-IP	zinc-finger
hDcp2	OE-IP	N-terminus	DHX9	OE-IP	
hEdc3	OE-IP	N-terminus	RHAU	Y2H	C-terminus
Hedls/hEdc4	OE-IP	N-terminus	<i>RNA binding proteins</i>		
<i>Exosome proteins</i>			AUF1	OE-IP	
Rrp4	OE-IP	N-terminus	AUF1 p45	OE-IP	zinc-finger
Rrp45	IP		CRD-BO/IGF2BP1	OE-IP	
Rrp43	Y2H		HuR	OE-IP	
Mtr3	Y2H		KSRP	IP	
<i>Histone deacetylases</i>			PABPC1/PABP1	Y2H, OE-IP	
HDAC1	OE-IP		PABP4	OE-IP	
HDAC3	OE-IP		TIAR	OE-IP	
HDAC7	OE-IP		hnRNPs	OE-IP	
<i>microRNA pathway</i>			<i>Signalling proteins</i>		
hAgo2/eIF2C2	OE-IP		MK2	KA	
hAgo4/eIF2C4	OE-IP		MK3	mouse-KO	
<i>Nuclear</i>			ERK2	KA	
PABPN1	OE-IP	TTP zinc-finger	JNK	KA	
PAP	OE-IP	TTP zinc-finger	p38	KA	
Hsp40	Y2H	C-terminus	PP2A	IP, OE-IP	C-terminus, pSer <sup>178</sup>
Nucelolin	Y2H, OE-IP	zinc-finger	MEKK1	IP	
Transportin	OE-IP		TRAF2	IP	
Nup214	Y2H, OE-IP		Protor-2	Y2H	C-terminus
<i>Proteasome-related</i>			<i>Transcription factors</i>		
Hsp70	Y2H, OE-IP	zinc-finger	Stat2	Y2H	
<i>RNases</i>			p65/NFkB	OE-IP	
hXrn1	IP, Y2H IP	N/C-terminus	<i>Phospho-TTP binding proteins</i>		
			14-3-3 proteins	Y2H, OE-IP	pSer <sup>52</sup> , pSer <sup>178</sup>

Table 6: TTP interaction partners (*Brooks & Blackshear, 2013*). IP, immunoprecipitation; KA, *in vitro* kinase assay; OE, overexpression; Y2H, yeast two hybrid; When the interacting domain in TTP is not indicated, this means that the full-length protein was used in the assays.

#### 2.4.1. Key players in mRNA decay

TIS11 proteins are binding mainly to class II ARE motifs such as AUUUA flanked by additional uridylate residues, through a tandem CCCH type zinc finger (*Brewer et al., 2004*). Mounting evidence indicates that the main function of TIS11 proteins is the recruitment of the deadenylation machinery on their target mRNA, leading to enhanced shortening of the poly(A) tail and subsequent mRNA destabilization. Three major deadenylation complexes are implicated in mammalian mRNA decay: Ccr4/Caf1/Not, PARN and Pan2/Pan3. Co-Immunoprecipitation experiments established the interaction between TTP and different deadenylases (*Lai et al.,*

2003). *Marchese et al.* demonstrated a phosphorylation-dependent interaction of TTP with the Caf1 deadenylase (*Marchese et al., 2010*). Very interestingly, a recent study demonstrated the direct interaction of TTP and Not1, the scaffold protein of the Ccr4-Not1 deadenylation complex (*Sandler et al., 2011; Fabian et al., 2013*). *In vitro*, TIS11 proteins could also induce PARN activity, while having no impact on ARE-lacking transcripts (*Lai et al., 2003*). However, the evidence of a direct interaction between TIS11 proteins and PARN is still lacking, suggesting an indirect recruitment of PARN via other proteins (*Lai et al., 2003; Lykke-Andersen & Wagner, 2005; Clement et al., 2011*). Neither a direct interaction between TTP and Pan2/Pan3 was detected so far, nor did Pan2/Pan3 suppression altered TTP-induced deadenylation (*Yamashita et al., 2005; Marchese et al., 2010*). By contrast, TTP is also an important actor in this process as it recruits components of the exosome to the deadenylated mRNA (*Chen et al., 2001*). Alternatively, deadenylated mRNAs could be also degraded via the 5'-3' decay pathway, requiring the removal of the m7G-cap at the 5'-end. Co-immunoprecipitation experiments identified Dcp2 and the 5'-3' exonuclease Xrn1 as interaction partners of TTP (*Fenger-Gron et al., 2005; Lykke-Andersen & Wagner, 2005; Hau et al., 2007*).

#### **2.4.2. Alternative functions of TIS11 proteins during mRNA lifecycle**

TIS11 proteins are regulating almost all steps of the mRNA life cycle, from biogenesis to degradation (Figure 11) (*Ciais et al., 2013*).

#### **mRNA transcription and 3'-end processing**

TIS11 proteins were first thought to be transcription factors due to their TZF domain. Two studies support this idea which is still controversial. TTP fused to the DNA-binding domain of the transcription activator protein GAL4 induced transcription of a GAL4-responsive luciferase reporter gene (*Murata et al., 2000*). Experiments using truncated TTP forms suggested an N-terminal localization of the major transactivation region. Interestingly, this TTP activity seems to be controlled by Protein kinase C (PKC). On the other hand, GST pull-down assays showed a direct interaction between TIS11b and the Hepatocyte nuclear transcription factor 1 $\alpha$  (HNF1 $\alpha$ ) (*Dudziak et al., 2008*). Luciferase reporter assays revealed a reduction of HNF1 $\alpha$ -mediated transactivation by TIS11b.

In the 3'-end mRNA processing context, binding of Cth2, a yeast homologue of TIS11 proteins, to AREs near the poly(A) signal of the immature mRNA resulted in 3'-extended, unstable transcripts (*Prouteau et al., 2008*). Deletion mutants of Cth2 suggest that control of nuclear polyadenylation is predominantly restricted to the N-terminal part of the protein. A study of our

lab described for the first time the function of TIS11b in 3'-end maturation of mammalian mRNAs (*Desroches-Castan et al., 2011*). In endothelial cells, TIS11b does not affect the expression of Dll4, an important angiogenesis regulator, by regulating mRNA stability. TIS11b binds to an ARE located in a weak poly(A) signal at the 3'-end of Dll4 mRNA. This interaction perturbs the correct 3'-end maturation and causes 3'-readthrough transcripts, leading to mRNA degradation via nuclear surveillance processes. A recent high-throughput analysis identified a binding of TTP to PABPN1, a regulator of polyadenylation (*Su et al., 2012*). Nuclear interaction of the two proteins inhibited polyadenylation and further expression of well-known TTP targets, such as GM-CSF, TNF $\alpha$  and IL-10.

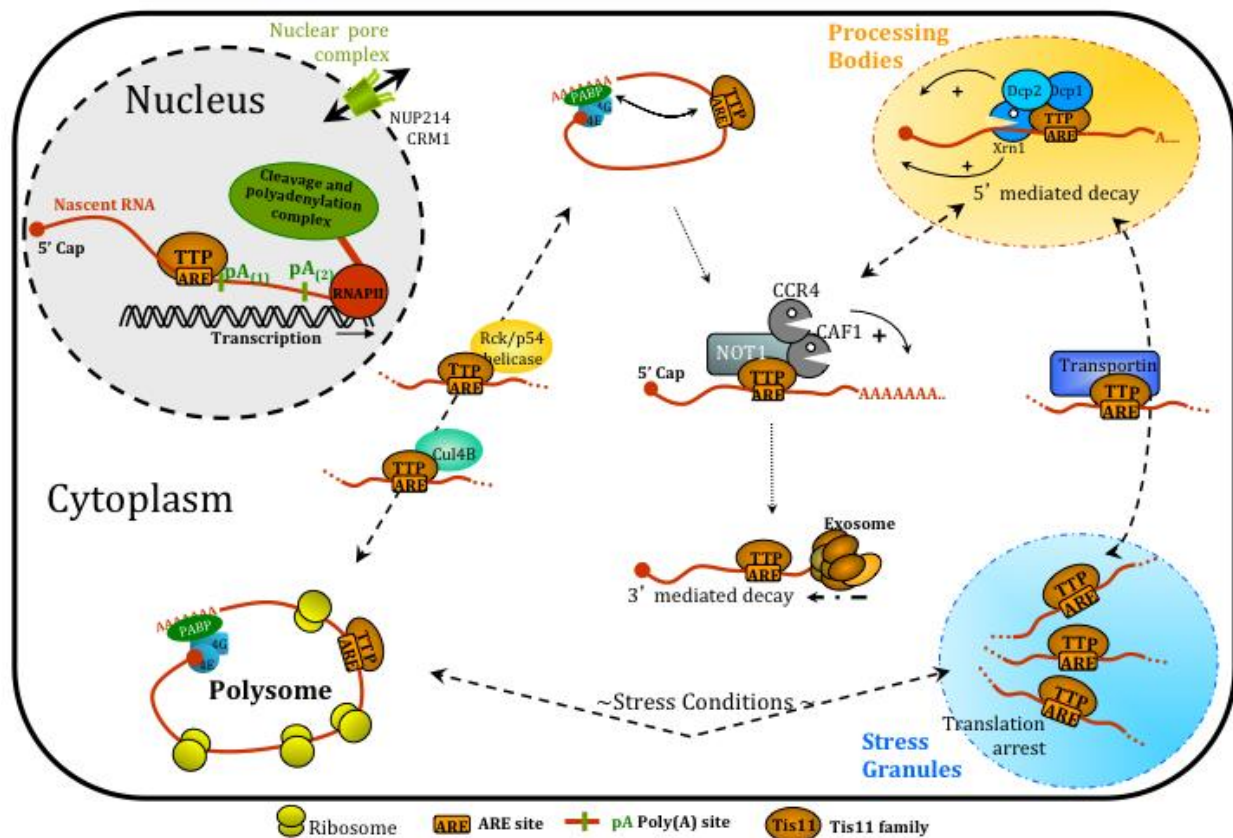


Figure 11: Multiple functions of TIS11 proteins during mRNA life cycle have been shown (*Ciais et al., 2013*). TTP and related proteins regulate 3'-end maturation and poly(A) site selection in the nucleus. In some circumstances binding to ARE-containing transcript by TIS11 proteins can take place in the nucleus, followed by translocation of the protein-mRNA-complex into the cytoplasm via the interaction between TTP and the nuclear pore complex. After destabilization of the circularized mRNA, TIS11 proteins recruit components of the mRNA decay machinery leading to a 5' or 3' decay of the transcript. TTP and related proteins are described as components of processing bodies and stress granules. TIS11 proteins are implicated in the formation of processing bodies and in the mRNA-transfer from processing bodies to stress granules via their interaction with transportin. TTP and related proteins modulate also AU-rich element-mediated translation by interacting with Cul4B or Rck/p54 helicase.

## mRNA transport and/or subcellular localization

Following transcription, mRNAs are released from the nucleus into the cytoplasm in order to be translated, stored or degraded. Discrete cytoplasmic foci, like processing bodies (P-bodies), were described as subcellular compartments containing mRNA decay proteins as well as TTP and related proteins (*Kulkarni et al., 2010*). *Franks & Lykke-Andersen* have reported that TTP and TIS11b can nucleate P-body formation on ARE-containing mRNAs (Figure 12) (*Franks & Lykke-Andersen, 2007*). Because mRNA decay enzymes could be limiting factors, mRNAs are sequestered in P-bodies to avoid their translation until they are degraded. In addition, *Stoecklin et al.* have shown a localization of TIS11 proteins in stress granules (SG) in response to stress (*Stoecklin et al., 2004*). They proposed that TIS11 proteins are transferring mRNAs from SGs to P-bodies for translation repression and degradation (*Kedersha et al., 2005*). TTP contributes to this dynamic trafficking via its interaction with transportin, a molecule which controls nucleocytoplasmic transport of macromolecules (*Chang & Tarn, 2009*).

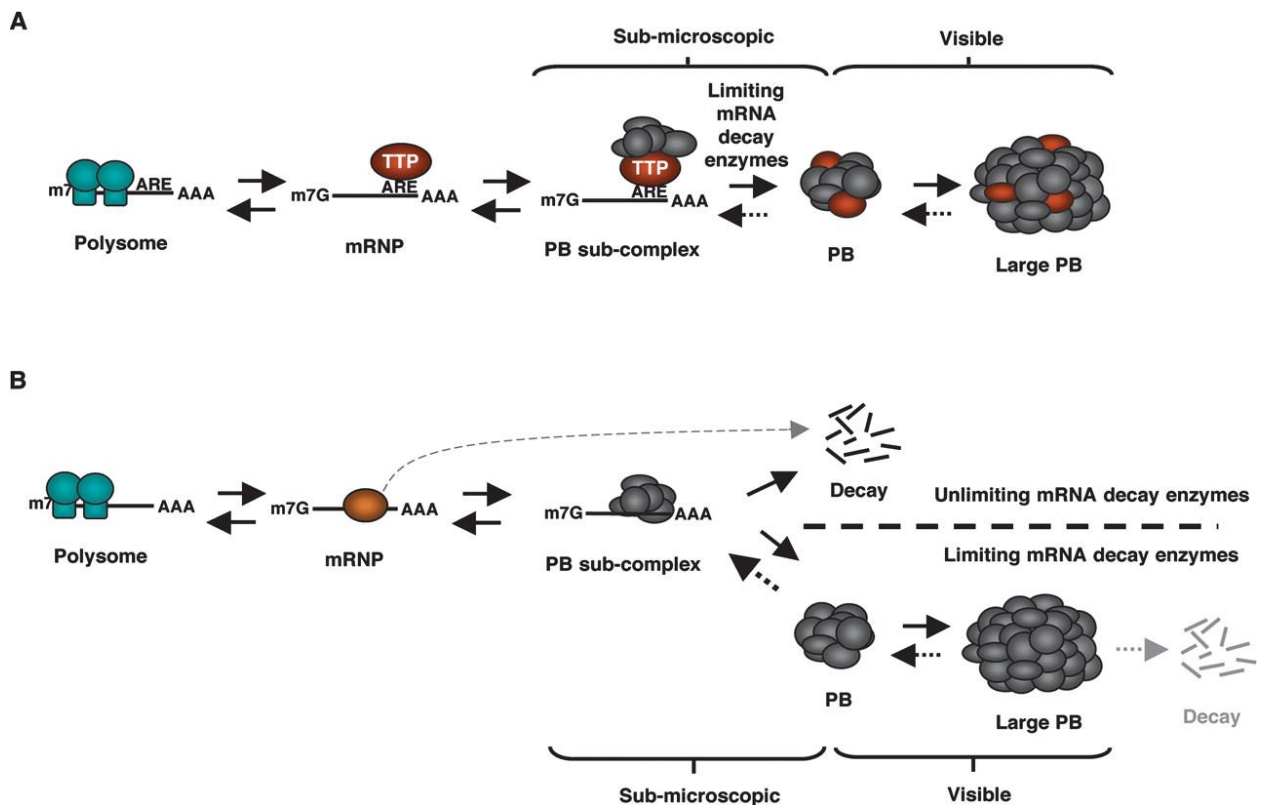


Figure 12: Subcellular localization of mRNAs is regulated by TIS11 proteins (*Franks & Lykke-Andersen, 2007*). (A) TTP and TIS11b initiate the formation of submicroscopic P-bodies (PB-subcomplex), when bound to their AREs. If mRNA decay enzymes are limiting, PB-subcomplexes aggregate to form microscopically visible P-bodies. (B) When mRNA decay enzymes are nonlimiting, mRNAs are efficiently degraded without aggregating into PB. Under conditions in which mRNA degrading enzymes are limiting, PB subcomplexes form larger PBs to silence mRNA translation until degradation of the transcripts.



### mRNA translation

Several studies have tried to decipher the role of TIS11 proteins in translation. First of all, TIS11 proteins are interacting with other ARE-BPs, such as TIA-1, TIAR-1 and HuR, all associated with translational control (*Espel, 2005*). HuR seems to replace TTP to initiate TNF $\alpha$  translation (*Tiedje et al., 2012*). Second, TTP and polysomes are co-localized after LPS-stimulation of macrophages (*Rigby et al., 2005*). A recent study described the association between TTP and the helicase RCK resulting in translational repression (*Qi et al., 2012*). Polyribosome fractionation experiments in the same study suggested that TTP shifts its mRNA targets to lighter fractions, thus ending in translational repression. Another report identified Cullin 4B (Cul4B), a scaffolding component of an ubiquitin E3 ligase complex, as potential TTP-interacting protein (*Pfeiffer & Brooks, 2012*). The stable knock down of Cul4B in macrophages led to reduced LPS-induced TTP expression. Interestingly, even if less expressed, TTP was more active in those cells as indicated by shorter TNF $\alpha$  mRNA half-life. The authors observed less TNF $\alpha$  mRNA loaded onto polysomes in the same cellular context. In addition, a co-localization of Cul4B and TTP in P-bodies and exosome granules was shown. The authors conclude that Cul4B is required to associate TTP/TNF $\alpha$ -mRNA complexes with the polysome to initiate mRNA translation. Reduced Cul4B expression shifts TNF $\alpha$ -mRNA from polysomes towards mRNA decay.

*Bell et al.* provided further convincing evidence for a role of TIS11 proteins in the regulation of translation (*Bell et al., 2006*). Increased VEGF in TIS11b<sup>-/-</sup>-derived fibroblasts was due to enhanced translational efficiency rather than mRNA stabilization.

Although, these studies indicate that TIS11 protein function in translational regulation is more complex than anticipated and needs further characterization.

### MiRNA-regulated pathways

There is an emerging role of TTP as novel regulator of miRNA-dependent post-transcriptional regulation. Like TTP, miRNAs are regulating gene expression by modulating translation of target mRNAs or inducing their degradation. As already mentioned, 3'UTRs are docking sites of both miRNA and RNA-binding proteins. Furthermore, *Jing et al.* demonstrated that TTP cooperate with miR-16 in the control of TNF $\alpha$  mRNA stability (*Jing et al., 2005*). The authors hypothesize that the binding of TTP to the ARE promotes miR-16 interaction with a 3'UTR sequence outside the ARE. MiR-16 associated factors such as the endoribonuclease Argonaute 2 (AGO2) are further mediating TTP-induced mRNA decay. In contrast to this synergistic effect of TTP and miR-16, *Ma et al.* provided a model, where TTP and miRNAs could antagonize each other (*Ma et al., 2010*). The seed sequence of miR-466l is complementary to the AUUUA

pentamer, which is the characteristic ARE sequence of TTP. The authors found increased expression of IL-10, a well-known TTP target, in miR-466l-overexpressing macrophages. The authors suggested that accumulation of miR-466l leads to increased assembly of the RNA-induced silencing complex (RISC) at the ARE located in the 3'UTR of IL-10 mRNA, thus preventing the interaction of TTP with IL-10 transcript and further mRNA decay. This results in the stabilization of IL-10 mRNA and subsequently increased IL-10 expression. *Kim et al.* provided a novel mechanism by which TTP positively regulates the biogenesis of miRNA *let-7* by downregulating Lin28 expression (*Kim et al., 2012a*). Restoring TTP expression in ovarian cancer cells increased mature *let-7* levels which inhibited the expression of *let-7* target gene CDC34, a protein implicated in cell proliferation. Thereby TTP destabilizes Lin28 mRNA, a negative regulator of *let-7* biogenesis, rather than interacting directly with *let-7*. The authors demonstrated the direct TTP binding to an ARE located in the 3'UTR of Lin28. Recently, *Bhattacharyya et al.* demonstrated that TTP is implicated in miR-155 biogenesis in the context of cystic fibrosis (CF) (*Bhattacharyya et al., 2013*). In primary epithelial cells isolated from CF patients, high levels of miR-155 induce the overexpression of IL-8 which is a TTP target (*Suswam et al., 2008*). Restoring TTP expression in those cells caused a marked decrease of mature miR-155 levels. miRNA expression profiling of these TTP-overexpressing cells led to the identification of miR-1 induction. The positive correlation between TTP and miR-1 expression and the low abundance of mature miR-155 led the authors to hypothesize that TTP induces miR-1, which further inhibits the biogenesis of miR-155 and subsequent expression of the pro-inflammatory cytokine IL-8.

## **2.5. Regulation of TIS11 protein activities**

TIS11 proteins are target of extensive post-translational modifications, especially phosphorylations (*Cao et al., 2007b*). Several signalling pathways target TIS11 proteins, such as ERK MAPK, p38 MAPK, JNK, Glycogen synthase kinase-3  $\beta$  (GSK3 $\beta$ ), cAMP-protein kinase A (PKA), PKB/AKT and PKC pathways. Figure 13 represents kinase signalling pathways regulating TTP and TIS11b protein expression, localization, degradation and activity (*Ciais et al., 2013*). In addition, ubiquitination of phosphorylated TIS11 proteins was observed (*Schichl et al., 2011*).

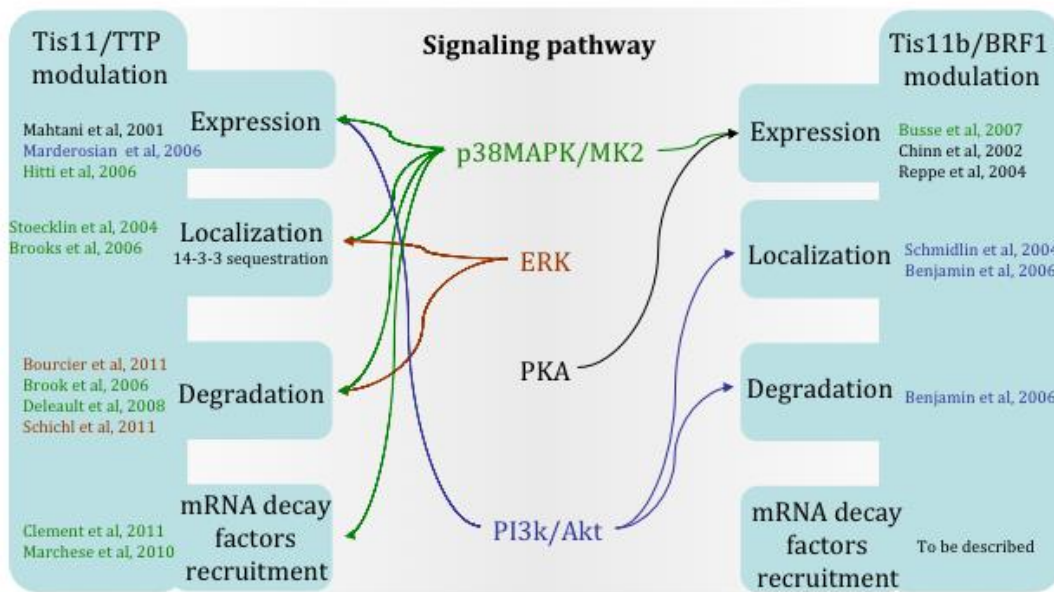


Figure 13: Signalling pathways acting on TTP and TIS11b (Ciais et al., 2013).

### 2.5.1. Post-translational modifications – phosphorylation

TIS11 proteins exhibit larger molecular weight on SDS gels than their predicted size. This effect is due to hyperphosphorylation of these proteins (Cao et al., 2004). Several putative phosphorylation sites are present within the protein sequence. They are highly conserved between species. Indeed, mass spectrometric and site-directed mutagenesis identified Ser<sup>66</sup>, Ser<sup>184</sup>, Ser<sup>186</sup>, Ser<sup>228</sup>, Thr<sup>257</sup> and Thr<sup>271</sup> as major phosphosites in human TTP protein, which correspond to Ser<sup>58</sup>, Ser<sup>176</sup>, Ser<sup>178</sup>, Ser<sup>220</sup>, Thr<sup>250</sup> and Thr<sup>274</sup> in mouse TTP (Cao et al., 2006). Very recently, a major phosphopeptide containing Ser<sup>90</sup> and Ser<sup>93</sup> of the human TTP protein was published (Cao et al., 2014). *In vitro* phosphorylation assays revealed that a number of protein kinases including ERK2, p38 MAPK, MK2, PKA, PKB/AKT and PKC directly phosphorylate TIS11 proteins. ERK1 and GSK3 $\beta$  are predicted to target TIS11 proteins based on *in silico* studies. The serine-threonine protein phosphatase (PP2A) promotes TTP dephosphorylation to complete the signalling circuit (Sun et al., 2007). It was initially hypothesized that phosphorylation of TIS11 proteins attenuates the mRNA-binding affinity of these proteins, leading to the stabilization of their target mRNAs. *In vitro* experiments confirmed that unphosphorylated TTP binds mRNA with a higher affinity (Hitti et al., 2006). However, several studies showed that mRNA binding was not impaired, when TIS11 proteins are phosphorylated by several kinases (Schmidlin et al., 2004; Sun et al., 2007; Maitra et al., 2008; Marchese et al.,

2010; Clement et al., 2011). Both expression and phosphorylation of TIS11 proteins are influenced by p38 MAPK activity. This pathway is stabilizing both TTP mRNA and protein (Tchen et al., 2004; Brook et al., 2006; Hitti et al., 2006). Busse et al. reported that p38 MAPK activity is required for TIS11b expression during myogenesis (Busse et al., 2008). The p38 MAPK pathway is a major regulator of ARE-mediated mRNA decay. The phosphorylation of TTP at Ser<sup>52</sup> and Ser<sup>178</sup> by the p38 MAPK and its downstream target MK2 allows TTP interaction with cytosolic 14-3-3 proteins which inhibits TTP-mediated mRNA decay and protects it from proteasomal degradation in the cytosol (Figure 14) (Chrestensen et al., 2004; Stoecklin et al., 2004). The same effects are achieved by PKB/AKT phosphorylation of TIS11b at Ser<sup>92</sup> and Ser<sup>203</sup> (Schmidlin et al., 2004; Benjamin et al., 2006). Furthermore, 14-3-3 binding to phosphorylated TTP prevents its localization in stress granules, where untranslated mRNAs are stored together with translation initiation factors (Stoecklin et al., 2004). In contrast, localization of TTP in P-bodies is phosphorylation-independent. MK2-induced phosphorylation of TTP is counterbalanced by the phosphatase PP2A, which is competing with 14-3-3 proteins for TTP binding. PP2A was shown to dephosphorylate TTP at Ser<sup>178</sup>, thus re-activating TTP-mediated mRNA decay (Sun et al., 2007; Sandler & Stoecklin, 2008). It is worth mentioning that the same phosphosites regulate TIS11 protein stability and TIS11-mediated mRNA decay activity, revealing an unexpected link between these two processes.

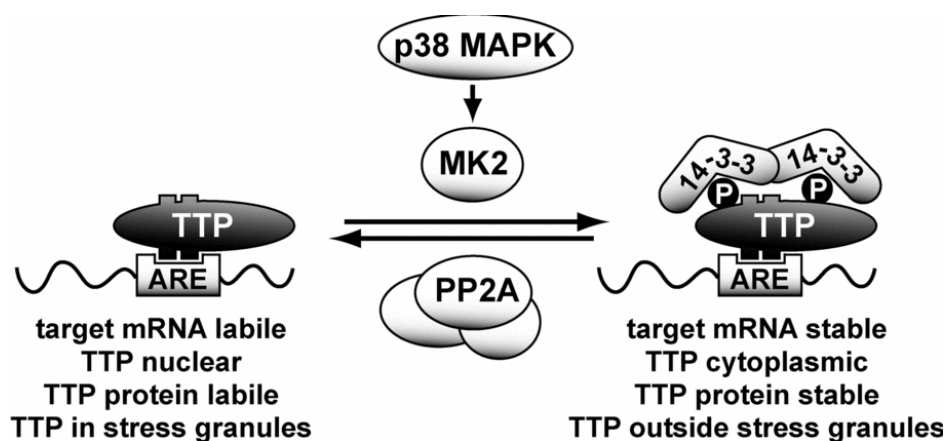


Figure 14: Central role of the p38-MAPK–MK2–PP2A–TTP axis in the regulation of TTP protein activity, stability and localization (Sandler & Stoecklin, 2008).

Phosphorylation of TIS11 proteins alters their interaction with other proteins such as components of the mRNA decay machinery. It was recently shown that MK2-mediated phosphorylation of TTP results in an inhibition of TTP-Caf1 interaction and a consequent blockade of TTP-mediated deadenylation of ARE-containing mRNAs (*Marchese et al., 2010; Clement et al., 2011*). In contrast, *Maitra et al.* reported that the phosphorylation of TIS11b at Ser<sup>54</sup>, Ser<sup>92</sup> and Ser<sup>203</sup> by MK2 did not impair the recruitment of the mRNA decay factors, suggesting that post-translational modifications of TIS11b protein occur after RNA-binding and interaction with components of the mRNA decay machinery (*Maitra et al., 2008*). Nothing is known so far concerning the impact of phosphorylation of TIS11b on its interaction with mRNA decay factors.

A few studies investigated TIS11 protein phosphorylation by the ERK/MAPK pathway. *Tan & Elowitz* observed that TIS11b expression in mouse embryonic stem cells is ERK-dependent (*Tan & Elowitz, 2014*). Activation of ERK by Fibroblast growth factor (FGF)-treatment caused a cytosolic accumulation of all three TIS11 proteins. However, inhibition of MEK1/2, an upstream activator of ERK, led to a rapid decrease of TTP, TIS11b and TIS11d mRNA expression. While low TIS11b mRNA levels were maintained, TTP and TIS11d mRNA expression were recovered after 10 h incubation with MEK1/2 inhibitor. These changes in mRNA levels were reflected at the protein level. Those results suggest that the inhibition of ERK signalling pathway elicit a transient response of TTP and TIS11d but a sustained response of TIS11b in mouse embryonic stem cells. *Bourcier et al.* reported that ERK-mediated phosphorylation of TTP enhances its proteasomal degradation which leads to stabilization of CXCL8 mRNA, an important cytokine in angiogenesis and tumor progression (*Bourcier et al., 2011*). *Deleault et al.* showed that the activation of either p38 MAPK or ERK inhibits TTP-mediated destabilization of TNF $\alpha$  mRNA (*Deleault et al., 2008*). Interestingly, the combined activation of p38 MAPK and ERK was even more efficient in this context.

**In conclusion,** the TIS11 protein family is phylogenetically highly conserved and is expressed from yeast to humans. In mammals, three members, TTP, TIS11b and TIS11d are known. Under quiescent conditions, they are weakly expressed while different external stimuli induce rapid and transient TIS11 family gene expression. TIS11 proteins promote ARE-mediated mRNA decay in eukaryotic cells. In addition, emerging evidence reveals involvement of this protein family in other steps of the mRNA life cycle.

Common structural properties were described for the different members. TIS11 proteins harbour a highly conserved tandem CCCH zinc finger domain (TZF) which enables these proteins to bind to AREs in the 3'UTR of target mRNAs. In addition, the nuclear-cytoplasmic shuttling of TIS11 proteins is regulated by a leucine-rich nuclear export sequence as well as a nuclear localization sequence. Studies with truncated TIS11 proteins revealed that the TZF alone is able to bind to mRNAs but that the mRNA-destabilizing activity is dependent on the presence of either the N- or C-terminal domains. TIS11 proteins function as binding platforms for enzymes involved in decapping, deadenylation and exonucleolytic decay.

TIS11 proteins target and destabilize the same ARE-containing mRNAs *in vitro*, but play a unique role *in vivo*. Specific mouse knock out models highlighted the importance of TIS11 proteins in inflammation, angiogenesis and cancer. Depletion of TIS11 proteins leads to the abnormal stabilization of short-lived mRNAs encoding for cytokines, chemokines and other key factors in these diseases. Among others, TNF $\alpha$ , GM-CSF, VEGF, c-myc, c-fos, COX-2, IL-6, IL-8, Dll4 and HIF-1 $\alpha$  have been described as TIS11 protein targets.

TIS11 protein sequences exhibit high abundance of phosphosites indicating post-translational regulation of these proteins. Indeed, TTP and related proteins are targets of different kinases which regulate their protein stability, localization and their ability to recruit components of the mRNA decay machinery. A two-phase model describing the rapid adaptation of cells to external stimuli has been proposed: (1) Activated kinases phosphorylate TTP at critical serines, leading to inactive cytoplasmic TTP (in complex with 14-3-3 proteins) and cytokine mRNA stabilization, (2) Following this “on” phase, the phosphatase PP2A is active and dephosphorylates TTP, thereby resulting in the rapid degradation of target mRNAs and the shutdown of cytokine expression.

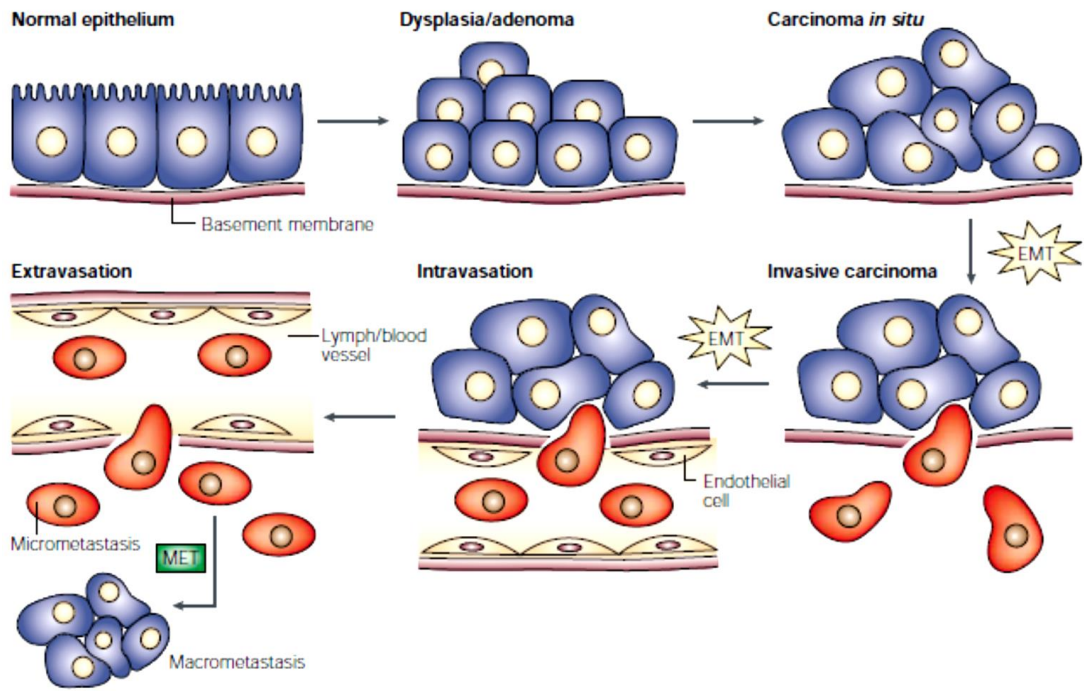


Figure 15: Different phases of tumor development (Thiery, 2002). EMT, epithelial-mesenchymal transition; MET, mesenchymal-epithelial transition.

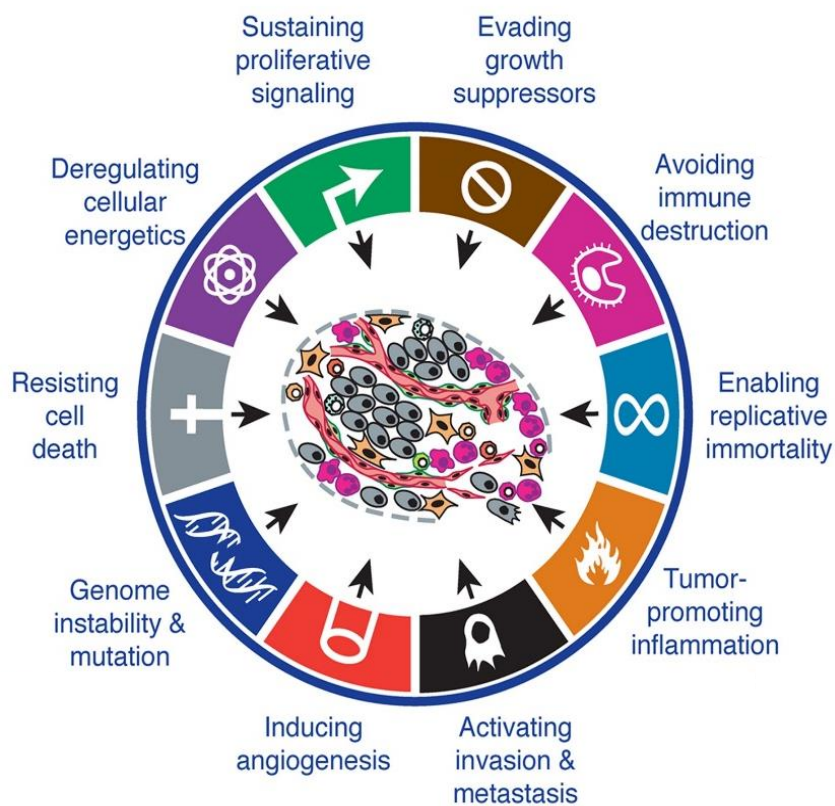


Figure 16: Hallmarks during tumor progression (Hanahan & Weinberg, 2011).

## Chapter 3      The multi-step development of human cancer

Tumorigenesis could be classically divided in initiation, promotion and progression (*Barrett, 1993*). It is a dynamic, multi-step process, where each step presents a genetic or epigenetic alteration which drives forward the progressive transformation of normal cells into proliferating cancer cells (Figure 15). Carcinogenesis is driven by gain-of-function mutations of oncogenes and loss-of-function mutations of tumor suppressor genes. However, recent investigations have shown the pivotal role of the tumor microenvironment within this process. Tissue homeostasis is normally regulated by the balance between cell division and programmed cell death (apoptosis). The rapid and uncontrolled proliferation can lead to benign tumors, which may turn malignant. Like every normal tissue, tumors need to be connected to the blood vessel system to be supplied with oxygen and nutrients. During the “angiogenic switch”, cancer cells start to induce blood vessel formation mainly by the secretion of VEGF. Sustained angiogenesis enables tumor cells to disseminate after going through the epithelial-mesenchymal transition (EMT). A minority of circulating tumor cells could proceed towards metastasis. In order to develop and evolve to malignant and invasive status, cancer cells have to acquire almost all key features defined as “Hallmarks of cancer” by *Hanahan & Weinberg* (Figure 16) (*Hanahan & Weinberg, 2011*). During the course of tumor progression a permanent interaction occurs between cancer cells and their environment consisting of several cell types. Cancer cells are building their niche by inducing angiogenesis, inflammation, immune responses and metabolic changes in their surroundings. On the other hand, the tumor microenvironment holds the potential to limit cancer progression (*Barcellos-Hoff et al., 2013*). The complex crosstalk between cancer cells and the tumor microenvironment will be discussed further in this chapter.



### 3.1. The hallmarks of cancer

To better understand and describe the complexities of cancer, *Hanahan & Weinberg* rationalize this neoplastic disease to a manageable number of underlying principles (*Hanahan & Weinberg, 2000; Hanahan & Weinberg, 2011*). The authors believe that almost all human cancer types are sharing the same acquired capabilities during the transformation of normal cells into malignant tumor cells. Cancer cells need to gain these functions to survive, proliferate and disseminate. *Hanahan & Weinberg* propose ten hallmarks: sustaining proliferative signalling (III), evading growth suppressors (IV), resisting cell death (V), enabling replicative immortality (VI), inducing angiogenesis (VII), activating invasion and metastasis (VIII), which enable tumor progression and metastatic dissemination. These traits are underlined by genome instability/mutation (I) and tumor-promoting inflammation (II) (Figure 16). During the last years, re-programming of cellular metabolism in tumor tissue (IX) as well as the escape of cancer cells from attacks and elimination by the immune system (X) appeared to be functionally important for cancer progression. It needs to be strengthened that these functional entities are rather distinct biological pathways than a complex network. Furthermore, many factors, implicated in these processes could act as tumor-promoting or -inhibiting agents, depending on age, immune and inflammatory response as well as on genetic and metabolic state of the organism. The mentioned hallmarks, which are essentially modified during carcinogenesis, will be briefly illustrated in the next paragraphs.

#### 3.1.1. Enabling hallmark: Genome instability and mutation

Alterations of the genome are the basis of several hallmarks in cancer progression. Abnormal chromosome structures and numbers as well as irregular mitosis in cancer cells have been described long time ago.

In normal tissue, mutations are rare because of several highly efficient and redundant cellular mechanisms, which detect and resolve DNA damage. Cancer cells need several mutations to orchestrate tumorigenesis. The “mutator hypothesis” indicates that genome instability is already present in precancerous lesions and drives tumor progression by accelerated spontaneous mutation rates. Therefore, cancer cells increase their sensitivity to mutagenic agents or induce changes in genome surveillance circuits that normally force DNA-damaged cells into senescence or apoptosis (*Negrini et al., 2010*). High-throughput sequencing of human cancers revealed that *TP53* tumor suppressor (encodes p53) and mitotic DNA damage checkpoint genes, classical oncoproteins such as the epidermal growth factor receptor (EGFR) and the small GTPase RAS, or tumor suppressor proteins like cyclin dependent kinase inhibitor 2A (CDKN2A) and the

phosphatase and tensin homologue deleted on chromosome 10 (PTEN) were the most frequently mutated genes. The clonal expansion and advantageous selection of certain mutations are enabling the acquisition of other hallmarks and consequently are promoting tumor progression.

Telomeres are nucleoprotein structures which protect eukaryotic chromosome ends that are progressively shortened during each round of DNA replication. Loss of p53 tumor suppressor function in cancer cells abrogates cell cycle arrest and apoptosis in case of critically short telomeres. Lasting DNA replication generates karyotypic instability associated with deletion of chromosomal segments in tumor cells (*Artandi & DePinho, 2010*).

Genome instability can also be acquired through epigenetic mechanisms such as DNA methylation and histone modifications (*Berdasco & Esteller, 2010*).

### **3.1.2. Enabling hallmark: Tumor-promoting inflammation**

In 1863, *Rudolf Virchow*, observed a tumor infiltration by inflammatory cells and tumor growth at sites of chronic inflammation (*Balkwill & Mantovani, 2001*). Historically, it was thought that the tumor-associated immune response takes place to eliminate malignant cells as inflammation is a normal physiological host response to tissue damage or infection. An unresolved inflammatory reaction can evoke chronic inflammation, which is currently accepted as enabling hallmark during carcinogenesis. Indeed, 25 % of cancers are associated with “smouldering” inflammation and in almost all tumors inflammatory cells and inflammatory mediators such as cytokines or chemokines are present in the tumor microenvironment (*Mantovani et al., 2008*). Initially it was thought that tumor-associated inflammation is an attempt of the host immune system to eradicate the tumor. During the last decades, experimental findings revealed a paradoxical tumor-promoting effect of immune cell infiltration of tumor tissue. Like genome instability, inflammation contributes to several hallmarks of cancer progression as it increases the risk of cancer initiation, enhances proliferation and resistance to apoptosis, stimulates angiogenesis and tissue remodelling as well as cell invasion and metastasis (*Mantovani et al., 2008*).

To better describe the connection between cancer and inflammation two pathways, an intrinsic and an extrinsic one, are proposed (Figure 17): First, the intrinsic pathways driven by the activation of oncogenes, like Ras, Myc and Ret or the inactivation of tumor suppressors, such as the Von Hippel Lindau factor (VHL), PTEN and TGF $\beta$ , induces the production of tumor-promoting inflammatory cytokines, chemokines and growth factors building-up an inflammatory tumor microenvironment to attract inflammatory cells. In pancreatic carcinoma patients, pancreatitis and mutations in the gene encoding K-RAS are frequently found.

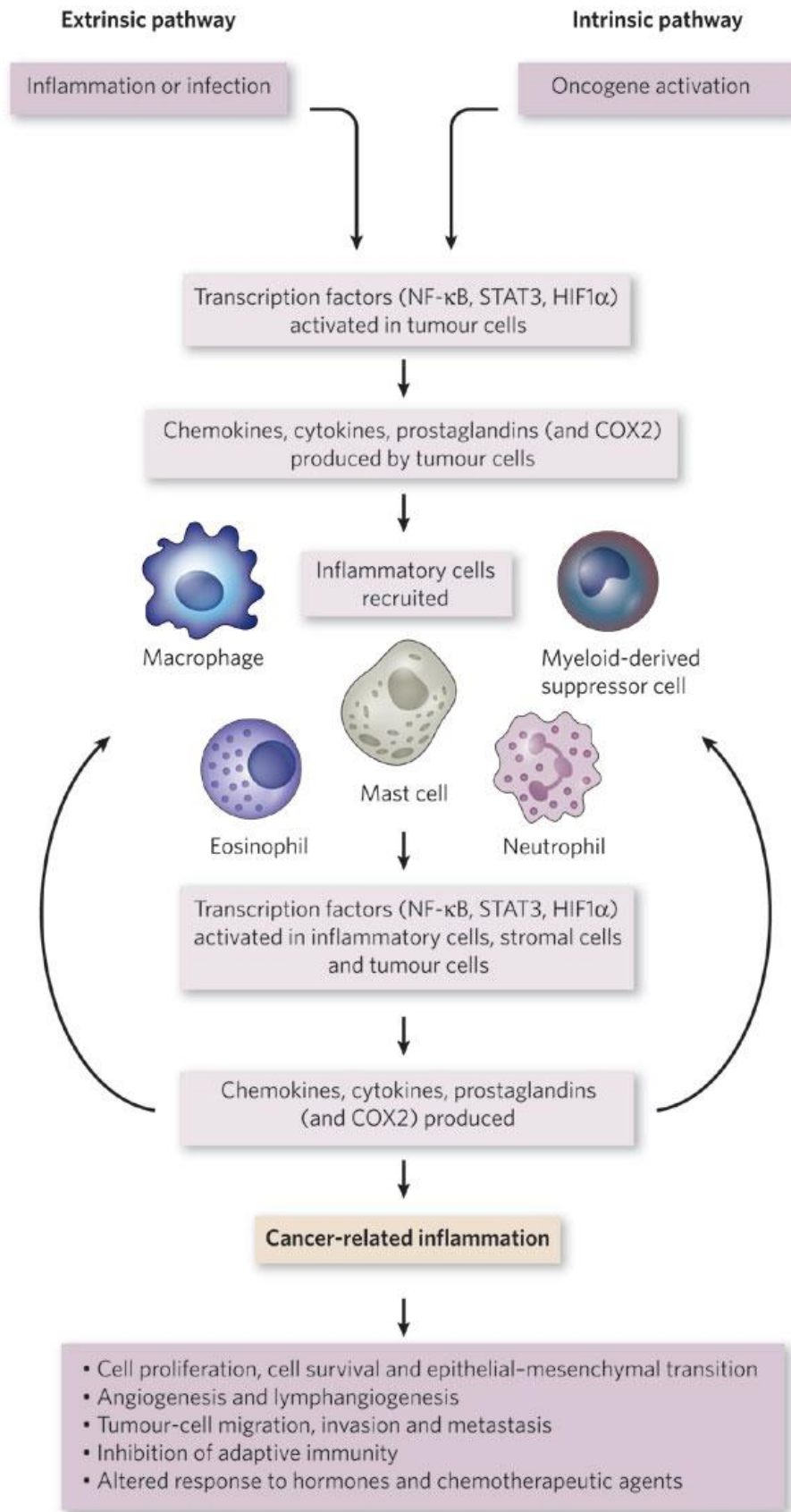


Figure 17: The intrinsic and extrinsic pathways that describe the link between cancer and inflammation (Mantovani et al., 2008).

Indeed, *Guerra et al.* demonstrated the development of pancreatic carcinomas in adult mice with chronic pancreatitis, when the K-Ras oncogene was activated (*Guerra et al., 2007*). In a murine breast cancer model, the inactivation of type II TGF $\beta$  receptor, which inhibits TGF $\beta$  action, increases the production of CXCL5 and CXCL12 (Stromal-derived factor 1 (SDF-1)) chemokines to attract myeloid-derived suppressor cells in the tumor. These cells are known to suppress the immune response and to facilitate the formation of metastasis. Second, the extrinsic pathway that trigger chronic inflammation and path the way for cancer includes for example infections with *Helicobacter pylori*, which is associated with gastric cancer and gastric mucosal lymphoma (*Yoshida et al., 2014*).

Various cell types are implicated in the inflammatory process, which will be discussed in more detail with regard to the tumor microenvironment. Briefly, inflammation activates various protein kinases, such as Janus-activated kinase (JAK), PI3K/AKT or MAPKs, and transcription factors like STAT family members, NF- $\kappa$ B, AP-1 or HIF-1 $\alpha$ , leading to the secretion of cytokines and chemokines by immune and stromal cells. These soluble factors, forming a complex network, mediate cell-to-cell communication and chronic inflammation in the tumor stroma. The next paragraph will focus on some cytokines and chemokines known to play an important role in cancer. Their balance and the presence of their respective receptors are regulating pro- or anti-tumoral effects of the tumor environment (*Zamarron & Chen, 2011*).

### **3.1.2.1. Cytokines**

**TNF $\alpha$** , the prototypical pro-inflammatory cytokine, seems to have a double-edged role in cancer. High TNF $\alpha$  concentrations can induce an anti-tumoral effect in murine sarcomas (*Havell et al., 1988*). In contrast, low levels of this cytokine are associated with tumor induction as TNF $\alpha$  induces production of reactive oxygen and nitrogen species, which causes DNA damage and further mutations (*Balkwill, 2006*). The oncogenic Ras seems to be the switch between these two contradictory roles of TNF $\alpha$  (*Cordero et al., 2010*). In addition, the TNF $\alpha$  response depends on the producing-cell type in the tumor microenvironment. While high TNF $\alpha$  levels deriving from cancer-associated immune cells increase patient survival, TNF $\alpha$  secretion of the tumor stroma showed lower survival rates in lung cancer patients (*Ohri et al., 2010*).

**TGF $\beta$**  is an immune-suppressing and anti-inflammatory cytokine, which activates SMAD-dependent transcription (*Massague, 2008*). Its role in tumor development is stage-dependent. Early in tumorigenesis, TGF $\beta$  functions as tumor suppressor whereas in late stages, TGF $\beta$  induces invasion and EMT (*Morrison et al., 2013*). As IL-6, TGF $\beta$  promotes angiogenesis in cancer (*Wikstrom et al., 1998*).

**IL-6**, another pro-inflammatory and tumor-promoting cytokine, acts downstream of Ras and induces activation of transcription factors, such as STAT3 or NF- $\kappa$ B (Hodge *et al.*, 2005; Grivennikov & Karin, 2010). Like TNF $\alpha$ , IL-6 facilitates tumorigenesis by inhibiting apoptosis and promoting proliferation as well as angiogenesis (Hodge *et al.*, 2005). In addition, both cytokines are positively contributing to the metastatic cascade (Kim *et al.*, 2009).

Another anti-inflammatory cytokine is **IL-10** which is secreted by tumor cells as well as by almost all immune cells of the tumor microenvironment (Gastl *et al.*, 1993). IL-10 has anti-tumoral properties by inhibiting NF- $\kappa$ B and related pro-inflammatory cytokine expression (Schottelius *et al.*, 1999). In addition, IL-10 expression in tumor cells inhibits metastasis and tumor angiogenesis (Thiery, 2002; Kohno *et al.*, 2003). Via its immunosuppressive effect, IL-10 facilitates immune surveillance escape of tumor cells (Hamidullah *et al.*, 2012).

Figure 18 illustrates the complex role of cytokines in carcinogenesis (Landskron *et al.*, 2014). Immune response, caused by tissue injury or infection, activates expression and release of pro-inflammatory mediators such as TNF $\alpha$ , IL-6 and IL-8 by immune cells. These factors are able to induce the breakdown of the epithelial barrier and promote immune cell infiltration (Wu *et al.*, 2014). Chronic inflammation and pro-inflammatory cytokines like TNF $\alpha$  cause production of free radicals. This cellular stress could initiate tumor development due to DNA damages and the activation of signalling pathways promoting cell survival and proliferation through AKT, ERK1/2 and HIF-1 $\alpha$ . TGF $\beta$  supports EMT and therefore the malignant transformation of cancer cells. Other cytokines and chemokines such as IL-10, IL-17 and IFN $\gamma$  are involved in angiogenesis and pro-tumoral macrophages polarization. Tumor growth and finally invasion of malignant cells is supported by pro-inflammatory cytokines and other factors, that induce proliferation, inhibit apoptosis and promote angiogenesis (mainly through VEGF and IL-8) as well as EMT. IL-10 and TGF $\beta$  aid the tumor to escape from immune surveillance. Finally, attracted immune cells (tumor-infiltrating leukocytes, tumor-associated macrophages and cancer-associated fibroblasts) are contributing to further tumor growth, metastasis and the maintenance of an immunosuppressive tumor microenvironment.

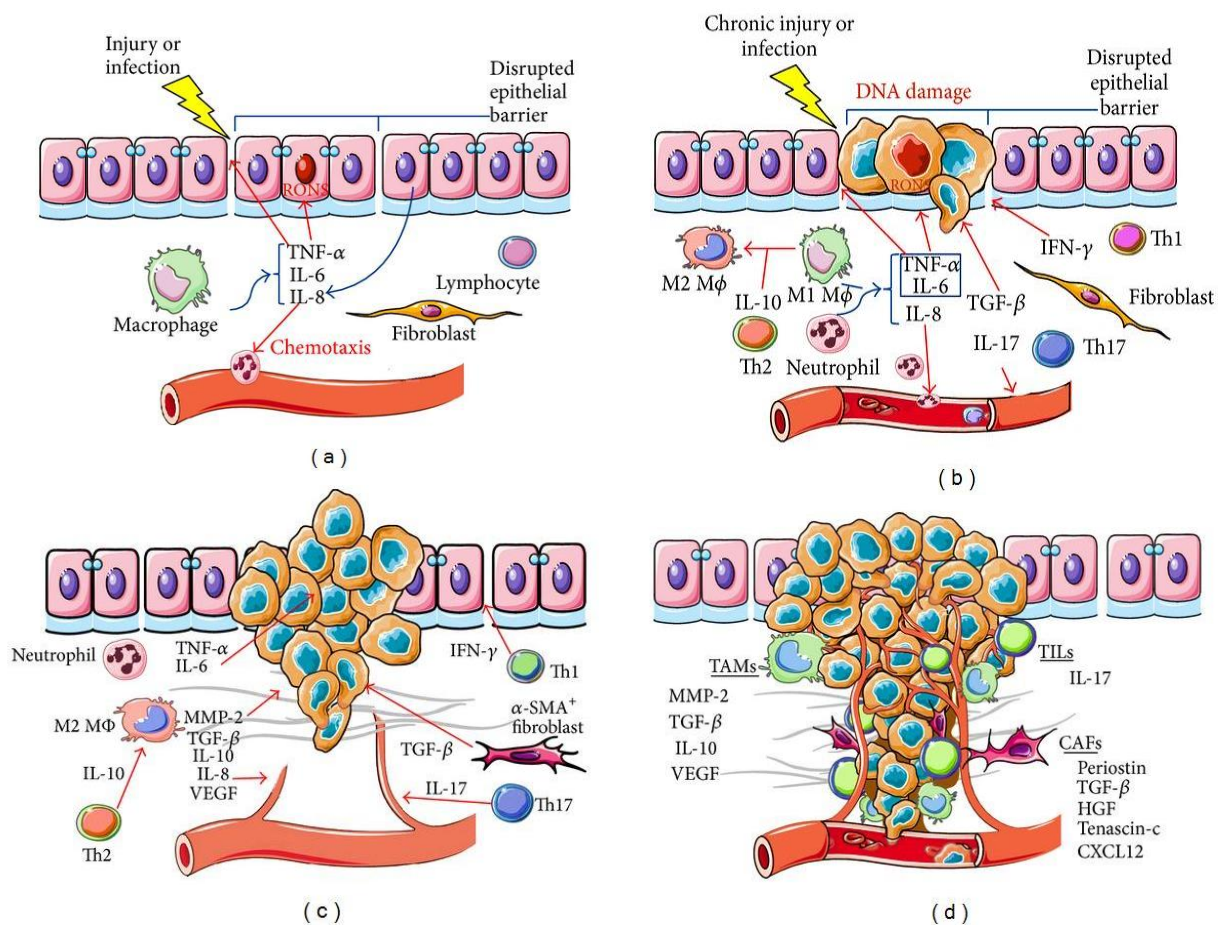


Figure 18: The complex role of cytokines and chemokines during cancer initiation and progression (Landskron *et al.*, 2014).

(a) Induced expression of pro-inflammatory cytokines such as  $\text{TNF}\alpha$ , IL-6 and IL-8 due to tissue injury or infection leading to the disruption of the epithelial barrier and the infiltration of inflammatory cells.

(b) Chronic inflammation and pro-inflammatory mediators such as  $\text{TNF}\alpha$  can cause DNA damage through RONS, which initiates tumor formation.  $\text{TGF}\beta$  activates EMT to induce malignant transformation. Lymphocyte-derived  $\text{IFN}\gamma$ , IL-10 and IL-17 promote further epithelial barrier disruption, transition of macrophage phenotype from M1 to M2 and angiogenesis.

(c) Pro-inflammatory cytokines can induce tumor growth and invasion by stimulating cell proliferation, reducing apoptosis, enhancing EMT and inducing angiogenesis via the major action of VEGF and IL-8. IL-10 and  $\text{TGF}\beta$  facilitate tumor immune evasion as they are anti-inflammatory cytokines.

(d) Immunosuppressive environment of the tumor is maintained by TAM-, TIL- and CAF-derived factors implicated in tumor growth and metastasis.

CAF, cancer-associated fibroblast; CXCL12, CXC-motif chemokine 12 (SDF-1, stromal-derived factor 1); HGF, hepatocyte growth factor;  $\text{IFN}\gamma$ , interferon  $\gamma$ ; IL, interleukin; MMP, matrix-metalloprotease; M1 M $\phi$ , macrophage phenotype 1; M2 M $\phi$ , macrophage phenotype 2; TAM, tumor-associated macrophage;  $\text{TGF}\beta$ , tumor growth factor  $\beta$ ; Th, T-helper cell; TIL, tumor-infiltrating leukocyte;  $\text{TNF}\alpha$ , tumor necrosis factor  $\alpha$ ; RONS, reactive oxygen/nitrogen species; VEGF, vascular endothelial growth factor.

### 3.1.2.2. Chemokines

In addition to cytokines, chemokines and their related receptors are key components in cancer-related inflammation (*Mantovani et al., 2010*). Based on their structure, chemokines are classified in four subfamilies: C, CC, CXC and CX3C chemokines. Chemokines by definition are small molecules that regulate directional migration of cells via specific G-protein coupled receptors. Under physiological conditions, chemokines are either controlling “homing” of leukocytes or are activated during the inflammatory response. In cancer, chemokines are especially associated with the recruitment of macrophages in the tumour tissue even though a broader range of function has been described more recently (*Mantovani et al., 2010*).

CCL2 (Monocyte-chemoattractant protein 1 (MCP-1)), CCL5 and CCL7 are produced by the tumor and stromal cells. They represent major attractants of macrophages and their concentration correlates with metastasis and a consequent poor prognosis in breast cancer (*Luboshits et al., 1999; Ueno et al., 2000; Karnoub et al., 2007; Soria & Ben-Baruch, 2008*). CCL2 also regulates M2 polarization and survival of TAMs. CXCR3 ligands, such as CX3CL1 (Fractalkine) or CXCL10 (Interferon  $\gamma$ -induced protein 10 (IP-10)), recruit natural killer cells and lymphocytes in tumor tissue and are related to anti-tumoral responses in murine lymphoma, melanoma, renal cell carcinoma and leukaemia models (*Lavergne et al., 2003; Saudemont et al., 2005; Pan et al., 2006; Wendel et al., 2008*).

Beside their main function as recruiters of immune cells, chemokines are also inducing angiogenesis through their receptors on endothelial cells (ECs) or indirectly through pro-angiogenic factor-producing leukocytes (Figure 19) (*Strieter et al., 2006; Palacios-Arreola et al., 2014*). Pro-angiogenic chemokines such as CXCL1, CXCL2, CXCL3 (Fractalkine), CXCL5, CXCL6 and CXCL8 (IL-8) are promoting blood vessel formation via CXCR1 and CXCR2 on the membrane of ECs. For example, CXCR2 signalling induces MMP2/9 activity that increases the release of VEGF and FGF by degrading the extracellular matrix (*Li et al., 2005*). CXCL8 (IL-8) is considered to be the most potent inducer of angiogenic processes such as chemotaxis, stress fibre assembly, EC proliferation and tube formation. CXCR4 and its ligand CXCL12 (SDF-1) are promoting migration and proliferation of ECs in human ovarian cancer as well as tube formation and VEGF release (*Kryczek et al., 2005*). Anti-angiogenic chemokines such as CXCL4, CXCL9, CXCL10, CXCL11 and CXCL14 inhibit EC proliferation and migration via CXCR3 (*Strieter et al., 2004*). CCL2 attracts TAMs which secrete pro-angiogenic factors (*Ueno et al., 2000; Soria & Ben-Baruch, 2008*).

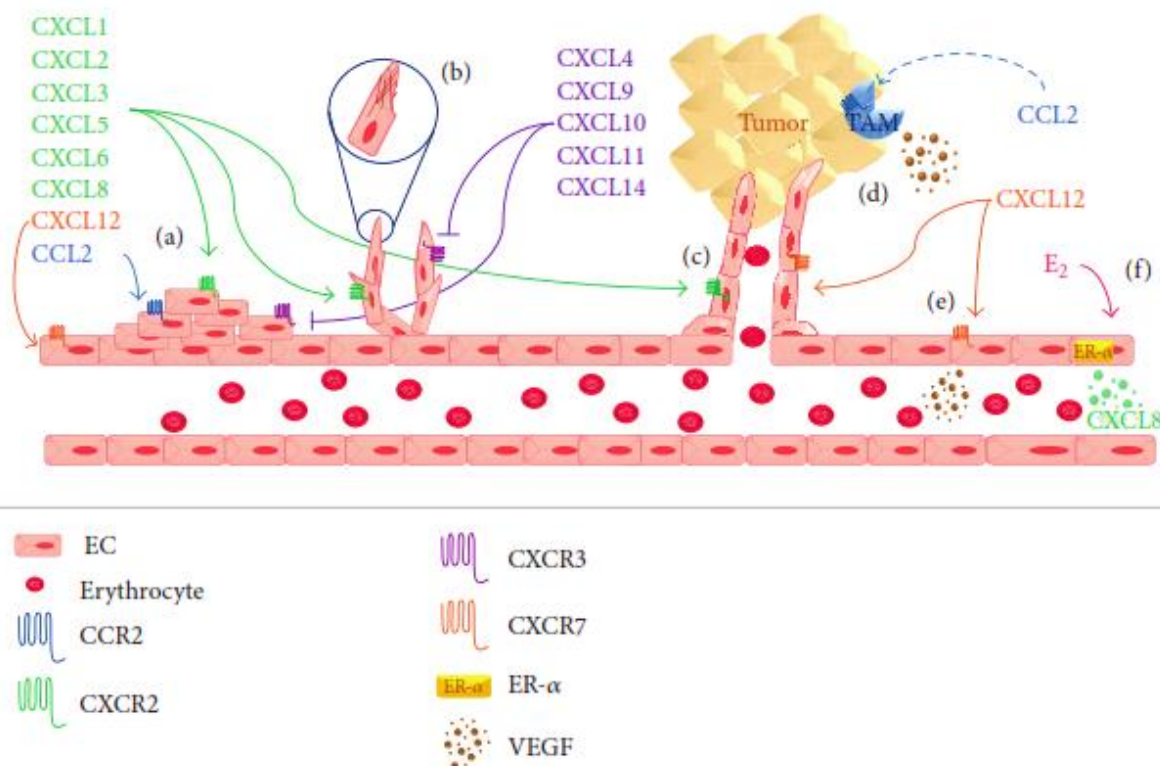


Figure 19: Involvement of chemokines in angiogenesis of breast cancer (*Palacios-Arreola et al., 2014*). Chemokines bind to CXCR2 and promote (a) proliferation of ECs, (b) assembly of stress fibres and (c) tube formation. Anti-angiogenic chemokines act via CXCR3 and inhibit these processes. CCL2 induces EC proliferation and increases TAM infiltration, which release pro-angiogenic factors such as VEGF. CXCL12 binds to CXCR7 and induces EC proliferation as well as (e) VEGF expression of these cells. (f) CXCL8 secretion by ECs is stimulated via the E<sub>2</sub>-estrogen receptor  $\alpha$  axis.

CXCR4 is the most frequently overexpressed chemokine receptor in cancer and is involved in tumor progression as well as metastasis in breast cancer (*Muller et al., 2001*). Therefore it is not surprising that the CXCL12-CXCR4 axis is the most studied one. This axis regulates processes including chemotaxis, migration and adhesion (Figure 20) (*Mukherjee & Zhao, 2013*). Recently, *Chen et al.* reported a significantly higher metastasis rate and shorter overall- and disease-free survival in patients with CXCR4-high-expressing triple negative breast cancer (*Chen et al., 2013*). CXCL12 secreted by cancer-associated fibroblasts (CAFs) binds to CXCR4 on the membrane of tumor cells to initiate their proliferation and tumor growth. CXCR4-expressing tumor cells leave the primary tumor and migrate towards a CXCL12 gradient. CXCL12 secreted by potential metastatic sites attracts tumor circulating cells leading to the onset of metastasis formation (Figure 20) (*Yang et al., 2005*).



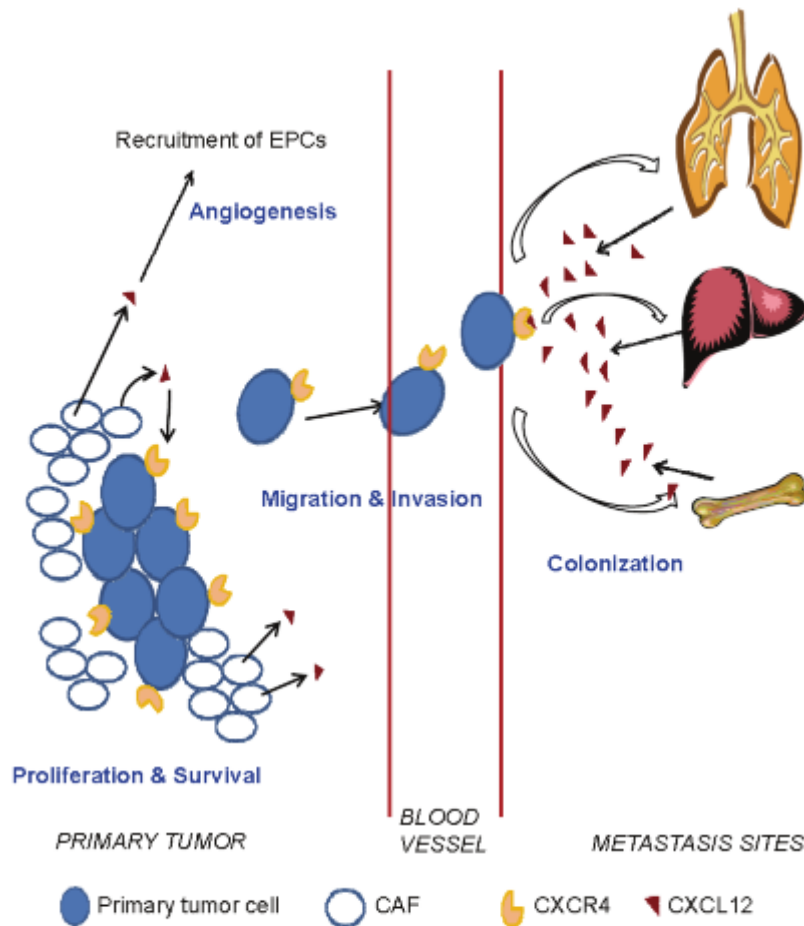


Figure 20: Interaction of CXCR4 with its ligand CXCL12 in primary tumor and metastasis of breast cancer (Mukherjee & Zhao, 2013). CXCL12 secreted by cancer-associated fibroblasts (CAFs) binds to CXCR4 on the membrane of tumor cells to initiate their proliferation and migration towards a CXCL12 gradient of metastasis sites leading to the onset of metastasis formation. EPC, endothelial progenitor cells.

### 3.1.3. Hallmark: Sustaining proliferative signalling

Sustained chronic proliferation is the crucial trait of cancer cells. Cell proliferation is tightly controlled in healthy tissue and normally induced by external stimuli, like growth factors. Cancer cells are able to overcome this stimuli-dependence by several alternative ways: these cells may produce their own growth factors and corresponding cell surface receptors resulting in an autocrine activation of cancer cell proliferation (Witsch *et al.*, 2010). Alternatively, cancer cells may stimulate growth factor secretion of cells within the surrounding tumor stroma. In addition, tumor cells often show somatic mutations of proliferation stimuli-transmitting receptors or downstream components of the signalling pathway, leading to a constitutive activation of the receptor and the subsequent signalling cascade. Indeed, in 40 % of human melanomas the B-RAF protein is mutated leading in constitutive signalling of RAF via the MAPK pathway (Davies & Samuels, 2010). Furthermore, the “oncogene-induced DNA replication stress model”

indicates that either the activation of oncogenes due to mutations positively influences cell growth or the loss of p53 DNA damage checkpoint function enables cancer cells to overcome p53-induced apoptosis or senescence leading to sustained cell proliferation (*Negrini et al., 2010*). Another example for the disruption of negative-feedback mechanisms involves PTEN phosphatase. Loss-of-function mutations in PTEN increase PI3K signalling and promote tumorigenesis (*Yuan & Cantley, 2008*).

#### **3.1.4. Hallmark: Evading growth suppressors**

In addition to the induction of lasting growth-stimulatory signals, cancer cells need to develop insensitivity against anti-growth signals to gain the capability to proliferate uncontrolled. Cellular growth is normally limited by growth inhibitors located in the surrounding environment, in the extracellular matrix or on the surface of neighbouring cells. These factors interrupt cell division as they cause an arrest in the interphase of the mitosis. Key players within this regulation are the retinoblastoma proteins (RB) and the tumor suppressor protein p53.

The RB proteins bind mainly to E2F transcription factors altering the expression of E2F target genes implicated in cell cycle progression, angiogenesis, cell death and genome stability and functions as tumor suppressor (*Burkhardt & Sage, 2008*). It is believed that RB proteins are inactivated in almost all human cancers. While RB proteins are mainly transducing external signals, p53 is sensitive to intracellular signals alerting for DNA damage and suboptimal growth conditions. p53 is able to induce cell cycle arrest to overcome these stress situations or is capable to promote apoptosis in case of irreparable damage. Mutations of RB protein and p53 pathways enable tumor cells to proliferate as they circumvent the action of these two critical gatekeepers of the cell cycle.

“Contact inhibition”, normally ensuring tissue homeostasis, is abrogated in tumors. Merlin, the cytoplasmic *NF2* gene product, is coupling cell-surface adhesion molecules such as E-Cadherin to transmembrane growth factor receptors like EGFR. Thus, Merlin strengthens cell-cell contacts and sequesters growth factor receptors signalling (*Curto et al., 2007*). LKB1 epithelial polarity protein could overcome the mitogenic effect of Myc oncogene expression. Loss of LKB1 destabilizes epithelial integrity and enables Myc-dependent transformation of epithelial cells (*Hezel & Bardeesy, 2008*). However, it remains to be determined how frequent contact inhibition is altered in human cancers.

### **3.1.5. Hallmark: Resisting cell death**

Apoptosis, the programmed cell death, is a cleaning process to eliminate mutated cells in normal tissue. Insufficient cell survival factor and p53 signalling are also promoting apoptosis. During tumorigenesis or as result of anti-cancer therapy, cancer cells are exposed to multiple apoptosis-inducing stresses, due to elevated oncogene signalling and DNA damage caused by hyperproliferation (*Kelly & Strasser, 2011*).

The apoptotic machinery consists of upstream regulators and downstream effectors. Extra- or intracellular death-inducing signals activate the caspase cascade leading to proteolysis and later phagocytosis of the cell. The balance between regulators and effectors is controlled by pro- and anti-apoptotic members of the Bcl-2 protein family. Tumor cells need to overcome this regulatory circuit to survive. Therefore, loss of p53 tumor suppressor function, overexpression of anti-apoptotic regulators or survival signals, like IL-3 or Bcl-2 and downregulation of pro-apoptotic factors are frequent in cancer. In general, attenuated apoptotic activity correlates with aggressiveness of tumors and resistance to therapy.

Necrotic cells release their content, for example IL-1 $\alpha$ , in the tumor microenvironment, thus attracting potential tumor-promoting inflammatory immune cells and inducing proliferation (*Grivennikov et al., 2010*).

Autophagy is a cell survival mechanism caused by different stress such as nutrient deficiency. For example, HIF-1 $\alpha$ -induced expression of its target genes Bcl2/E1B 19 kDa-interacting protein 3 (BNIP3L) and BNIP3-like proteins is required for hypoxia-induced autophagy (*Mazure & Pouyssegur, 2009*). The autophagic program induces the formation of intracellular vesicles enveloping cell organelles such as mitochondria and ribosomes, allowing the breakdown of these catabolite-rich cellular compartments. In this way, cancer cells are recycling metabolites to survive in nutrient-limited environments. In contrast, autophagy seems also to be a barrier during tumor progression, as mice bearing inactivated alleles of components of the autophagy machinery are more sensitive to cancer (*White & DiPaola, 2009*). Paradoxically, radiotherapy and certain cytotoxic drugs induce autophagy and thereby facilitate tumor survival as well as reversible dormancy of cancer cells pointing at the contradictory effects of these cancer therapies. These mechanisms may promote the persistence and eventual relapse of some tumors in response to conventional anti-cancer therapies.

### **3.1.6. Hallmark: Enabling replicative immortality**

Life-span of normal cells is limited by senescence and later cell death. Cancer cells can tackle this cell fate via a transition called immortalization. A main characteristic of this process is the re-activation of the telomerase, an enzyme which inhibits the shortening of the telomeres by adding telomere repeat sequences to the end of the chromosome. Telomerase activity is correlated with resistance of tumor cells to the induction of senescence and cell death. During the last years, certain studies have shown delayed telomerase activation during cancer progression and proposed that cancer cells first acquire tumor-promoting mutations then activate subsequently the telomerase to stabilize the mutant genome (*Artandi & DePinho, 2010*).

### **3.1.7. Hallmark: Inducing angiogenesis**

Vascularization of the tumor tissue is essential as it guarantees the supply with oxygen and nutrients which is essential for neoplastic growth. The activation of quiescent vasculature to form new vessels is an early event in tumorigenesis which is called the “angiogenic switch”. Because of the great importance of angiogenesis during cancer progression, as well as the already mentioned link between TIS11b protein and this process, the following paragraphs will introduce tumor angiogenesis in more detail.

#### **3.1.7.1. Physiological angiogenesis**

During embryogenesis, angioblasts differentiate into ECs leading to the *de novo* formation of a vascular network (vasculogenesis) followed by the differentiation into arteries and veins. Subsequent vessel sprouting (angiogenesis) ensures the expansion of the vasculature. Angiogenesis is defined as the formation of new blood vessels from pre-existing ones (*Folkman, 1971*). In adults, mature vasculature becomes quiescent, but ECs retain high plasticity to keep the ability to sense and respond to angiogenic signals. Angiogenesis is activated during wound healing or menstrual cycle. The formation of new blood vessels is controlled by the balance between pro- and anti-angiogenic factors including growth factors, extracellular matrix proteins, proteases and adhesion molecules (Figure 21). Among others, the VEGF protein family and its related receptors plays a pivotal role in angiogenesis. *In vivo* mouse models demonstrated that the deletion of one allele as well as a modest overexpression of VEGF-A cause embryonic lethality due to defective vascularization (*Carmeliet et al., 1996; Ferrara et al., 1996; Miquerol et al., 2000*). This phenotype show that slight variations of VEGF-A expression leads to deleterious consequences during early development.

## VEGF proteins

The prototype of angiogenesis-promoting factors is the vascular endothelial growth/permeability factor (VEGF/VPF), which was discovered in 1989 (Connolly *et al.*, 1989; Ferrara & Henzel, 1989). Five different members of the VEGF family are known so far: VEGF-A, VEGF-B, VEGF-C, VEGF-D and the Placenta growth factor (PLGF). VEGF proteins form mainly homodimers although heterodimers of VEGF-A and PLGF exist. The different VEGF proteins have non-redundant roles *in vivo*. Due to the important biological function of VEGF proteins their expression is tightly controlled at all stages of gene expression (Arcondeguy *et al.*, 2013). VEGF proteins exist as soluble and matrix-bound isoforms.

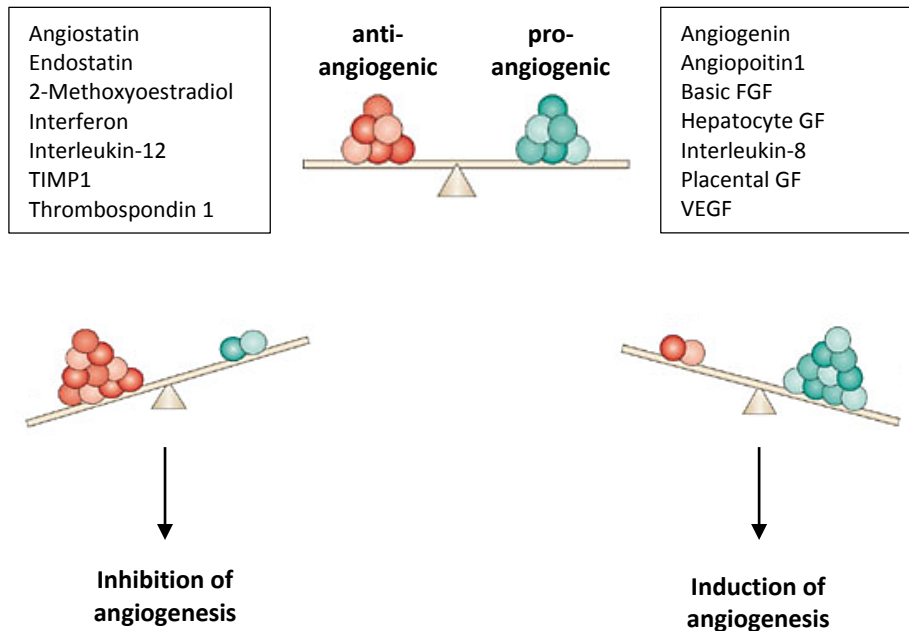


Figure 21: A balance between pro- and anti-angiogenic factors controls angiogenesis.

## Regulation of VEGF expression

*VEGF-A* is encoded at chromosome 6 and its gene sequence contains eight exons separated by seven introns (Figure 22) (Vincenti et al., 1996). *VEGF-A* expression is tightly controlled at the transcriptional level. Several consensus-binding sites for transcription regulators such as AP-1 and SP-1 are present in the promoter region of *VEGF-A*. These transcription factors are dependent on external stimuli like hormones, growth factors, inflammatory cytokines, tumor suppressors/oncogenes and hypoxia (Pages & Pouyssegur, 2005). Here, hypoxia is of great interest as it is a main driver of tumor angiogenesis. Indeed, functional hypoxia response elements (HRE) located in the 5' flanking region of *VEGF-A*, are target of HIF-1 and HIF-2 (Blancher et al., 2000).

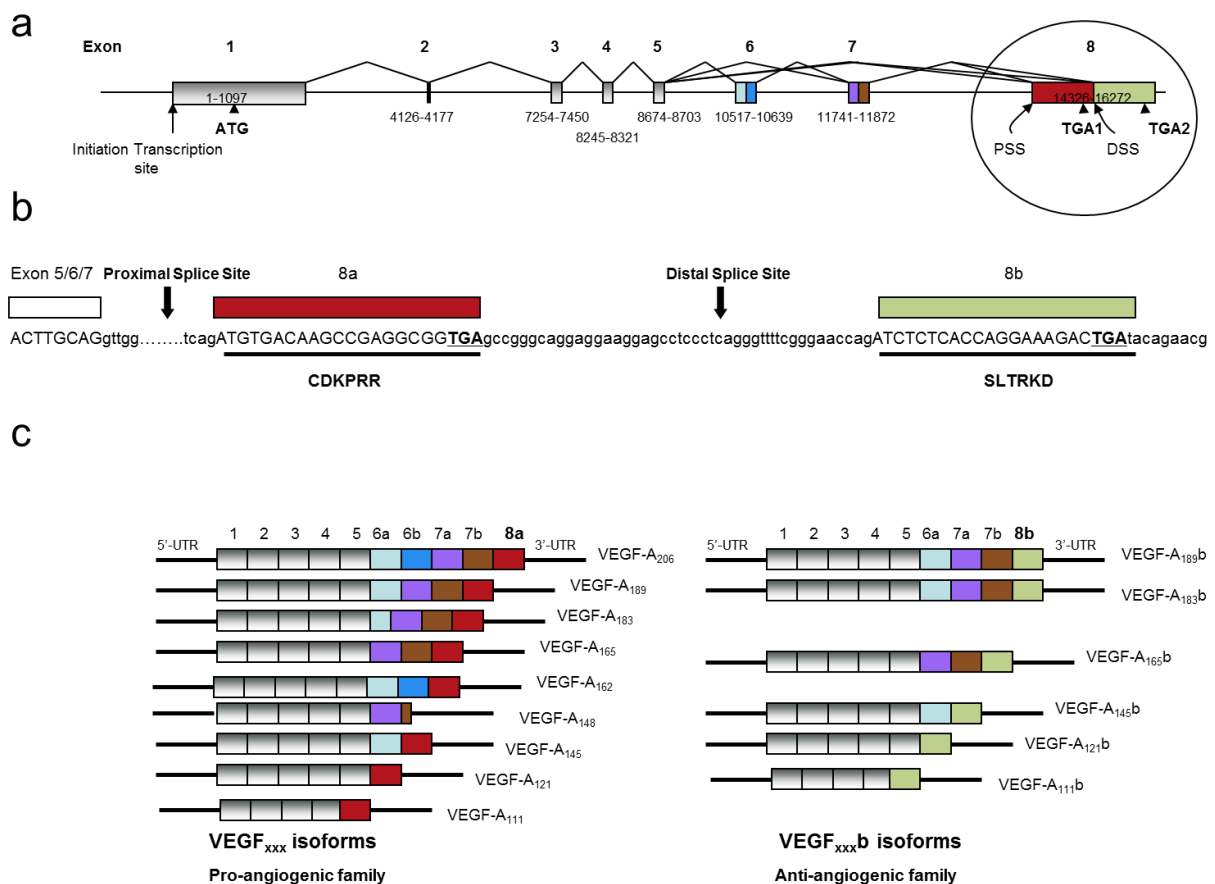


Figure 22: Regulation of VEGF-A expression by alternative splicing (Eymin et al., 2014). (a) Schematic illustration of the human *VEGF-A* gene containing eight exons spared by seven introns. (b) Focus on exon 8 located at the C-terminal part of the *VEGF-A* gene. Alternative splicing of exon 8 using the proximal splice site (PSS) or distal splice site (DSS) generates either VEGF<sub>xxx</sub> or VEGF<sub>xxx</sub>b subfamilies. (c) Overview about the different isoforms of VEGF<sub>xxx</sub> (pro-angiogenic) or VEGF<sub>xxx</sub>b (anti-angiogenic) subfamilies.

At the post-transcriptional level, alternative splicing and processing further increase the complexity of the VEGF protein family (*Houck et al., 1991*). Due to alternative splicing processes, nine splice variants for VEGF-A, denoted as VEGF<sub>xxx</sub> (VEGF<sub>111</sub>, VEGF<sub>121</sub>, VEGF<sub>145</sub>, VEGF<sub>148</sub>, VEGF<sub>162</sub>, VEGF<sub>165</sub>, VEGF<sub>183</sub>, VEGF<sub>189</sub> and VEGF<sub>206</sub>), have been described so far in humans (*Ferrara, 2004*). These isoforms differ by the presence or absence of exon 6a, exon 6b and exon 7. Most of the cells produce VEGF<sub>121</sub>, VEGF<sub>165</sub> and VEGF<sub>189</sub>. Due to different binding affinities for their cognate receptors, VEGF-A isoforms are associated with different functions. In addition, exon 8 contains an alternative splicing site, which generates further VEGF-A splice variants, namely VEGF<sub>xxx</sub>b (*Bates et al., 2002; Harper & Bates, 2008*). VEGF<sub>xxx</sub>a and VEGF<sub>xxx</sub>b display the same length on the protein level, but differ in six C-terminal amino acids. This difference leads to a changed tertiary structure and function of the VEGF-A protein. Indeed, VEGF<sub>xxx</sub>b isoforms bind to, but do not activate the VEGF receptor and are therefore described to act as anti-angiogenic molecules (*Kawamura et al., 2008; Delcombel et al., 2013*). Except the placenta, VEGF<sub>xxx</sub>b isoforms are assumed to form more than half of the total VEGF-A protein in normal non-angiogenic tissues. However, in several tumors such as melanoma, prostate, kidney and colon cancer, VEGF<sub>xxx</sub>b isoforms are downregulated leading to the predominant expression of VEGF<sub>xxx</sub> isoforms. Reduced VEGF<sub>xxx</sub>b expression is associated with metastasis formation in colorectal cancer (*Diaz et al., 2008*). For example, overexpression of VEGF<sub>165</sub>b in human prostate tumor cells inhibited cancer cell-mediated migration and proliferation of ECs *in vitro* as well as tumor growth *in vivo* (*Rennel et al., 2008*). However, a simultaneous increase of VEGF<sub>xxx</sub>b and VEGF<sub>xxx</sub> isoforms was reported for breast cancer tissue and colon cancer (here associated with less microvessel density) (*Tayama et al., 2011; Grepin et al., 2012*). However, in non-small lung cancer cells (NSCLC), VEGF<sub>165</sub>b protein expression is heterogeneous and high levels are significantly related to lymph node metastases (*Eymin et al., 2014*). Very interestingly, the same study reported elevated VEGF<sub>165</sub>b in NSCLC, treated with the anti-angiogenic monoclonal anti-VEGF antibody bevacizumab. This effect was even more pronounced when these cells were treated with the chemotherapeutic agent cisplatin. In addition, VEGF<sub>165</sub>b-overexpressing cells were less sensitive to cisplatin- or hypoxia-induced apoptosis. SiRNA-based specific neutralization of VEGF<sub>165</sub>b reversed bevacizumab-mediated invasive phenotype of lung adenocarcinomas in mice and inhibited tumor growth. Altogether, these results indicate that the role of VEGF<sub>xxx</sub>b seems to be more complex than initially thought. Furthermore, post-transcriptional control of VEGF-A expression includes the regulation of its mRNA stability. In normoxic conditions, VEGF-A mRNA is highly labile due to the combined action of destabilizing elements located in the 5' and 3'UTR. Under hypoxia, VEGF-A transcripts are

stabilized. VEGF-A mRNA stability is mainly regulated by multiple AREs located in the 3'UTR of the mRNA (Levy *et al.*, 1995). ARE-binding proteins such as AUF-1 and the TIS11 protein family bind to these sequence motifs and induce mRNA decay (Ciais *et al.*, 2004). The action of destabilizing ARE-proteins is antagonized by ELAV/Hu proteins targeting either the same AREs or specific sequences leading to the stabilization of VEGF-A mRNA (Cherradi *et al.*, 2006). VEGF-A cDNA harbours two major alternative polyadenylation sites suggesting further regulation of VEGF-A expression. Several mechanisms ensuring the translational control of VEGF-A transcript have been described. In the 5'UTR of the VEGF-A mRNA, three functional in-frame alternative CUG start codons and two functional internal ribosome entry sites (IRES) were found, indicating alternative cap-independent translation of the transcript in addition to the classical initiation of translation in eukaryotes (Huez *et al.*, 1998). Finally, numerous studies have reported that VEGF-A is a target of miRNAs which bind to VEGF mRNA 3'UTR. MiRNA-regulated VEGF-A expression is often associated with translation inhibition. *Dicer*-KO mice which show impaired miRNA biogenesis died at E12.5 to E14.5 due to retarded development and defective angiogenesis (Yang *et al.*, 2005).

### Canonical VEGF–VEGF receptor signalling

VEGF is mainly acting on endothelial cells (ECs) in a paracrine manner (Koch & Claesson-Welsh, 2012). However, autocrine VEGF signalling is also essential for ECs survival (Lee *et al.*, 2007; Domigan *et al.*, 2014).

VEGF proteins activate several signalling pathways through their binding to VEGF receptor tyrosine kinases. Three VEGF receptors, named VEGFR1 (Flt1), VEGFR2 (Flk1) and VEGFR3 (Flt4), are described (Figure 23). VEGFR1 is the most abundant in monocytes and macrophages. VEGFR2 is mainly expressed in vascular endothelial cells, whereas VEGFR3 is predominantly present on lymphatic endothelial cells.

In general, the binding of VEGF to its cognate receptor expressed on the plasma membrane of the recipient cell promotes receptor homo- or heterodimerization. This conformational change activates the receptor and leads to the auto- or trans-phosphorylation of tyrosine residues of the receptor dimer itself as well as of downstream signal transducers. Finally, the activated signalling cascades will provoke a biological response such as EC proliferation, migration and organization into functional vessels. It is of note that the different properties of the VEGF proteins and the expression pattern of the VEGF receptors as well as the availability of co-receptors such as Neuropilin1/2 modulate the different biological outcomes of VEGF–VEGFR



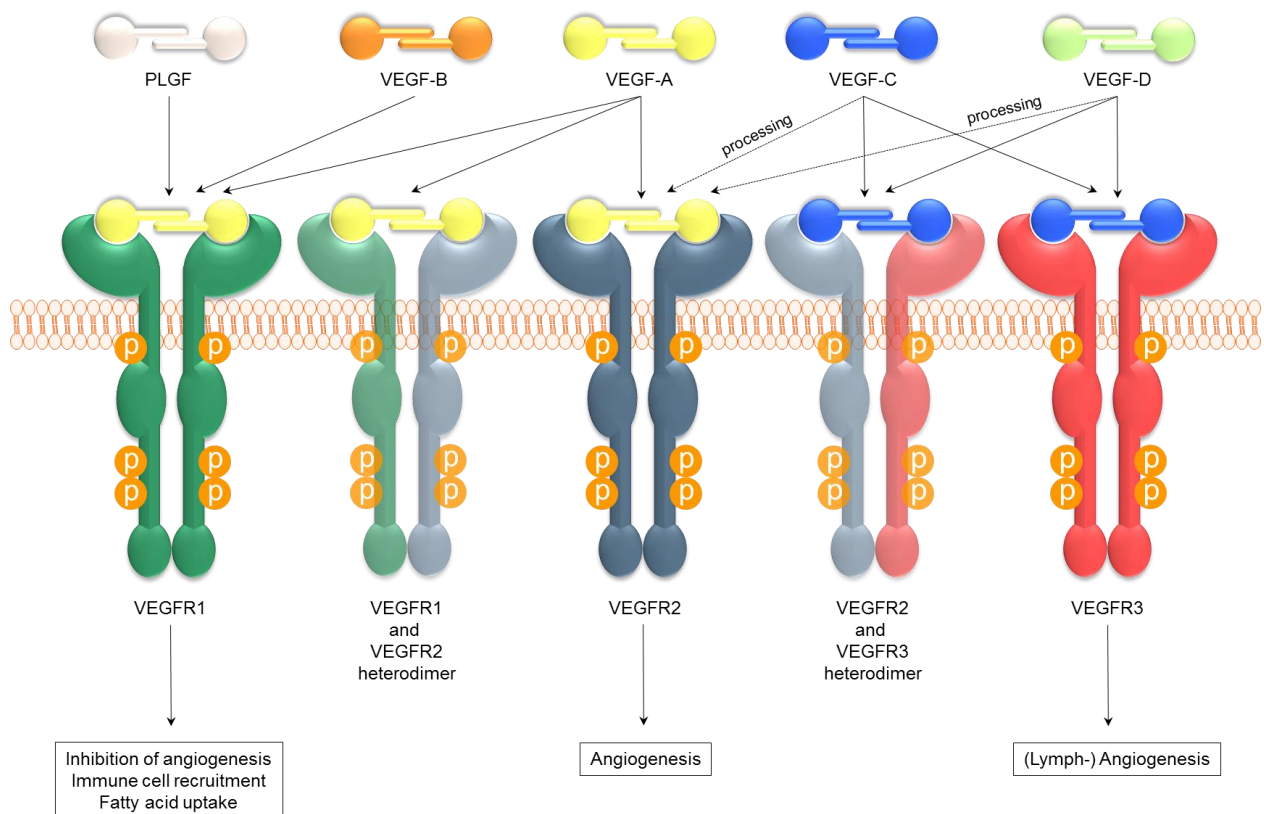


Figure 23: The binding specificities of VEGF proteins and VEGFR signalling complexes. PLGF, VEGF-B, VEGF-A, VEGF-C and VEGF-D bind with different affinities to three tyrosine kinase receptors (VEGFR1-3). These receptors form homo- and heterodimers. Dimerization of VEGFR with their co-receptors Neuropilin 1/2 are not illustrated. Activation (Indicated phosphorylation sites are exemplary.) of VEGFR due to ligand binding induces intracellular signalling cascades leading to several biological responses such as immune cell recruitment, fatty acid uptake, and (lymph-) angiogenesis depending on the VEGFR subtype.

signalling. In addition, the activation of distinct signalling pathways highly depends on which tyrosine residue of the VEGFRs is phosphorylated upon receptor activation. Neuropilin1/2 (Nrp1/2) are known to form heterodimers with VEGFR modulating the biological function of VEGFRs. While Nrp1 seems to be the preferred co-receptor for VEGFR1/2, Nrp2 associates with VEGFR3 (Fuh *et al.*, 2000). Furthermore, integrins are known to modify VEGFR signalling. For example, VEGFR2/integrin  $\alpha\beta3$  interaction is crucial for active angiogenesis (Gluzman-Poltorak *et al.*, 2001).

VEGFR1 binds VEGF-A, VEGF-B and PLGF (Koch & Claesson-Welsh, 2012; Jeltsch *et al.*, 2013). This receptor is not essential for EC function, but inhibits VEGFR2 signalling as it entraps VEGF-A due to a high affinity for this ligand. Therefore VEGFR1 is a negative regulator of angiogenesis. On the other hand, VEGFR1 modulates indirectly EC function as it recruits

monocytes which secrete angiogenic factors. Furthermore, VEGFR1 is implicated in the transport of fatty acids.

VEGFR2 is the predominant VEGF receptor expressed on the plasma membrane of vascular ECs as well as their progenitors and is crucial for ECs cell differentiation, proliferation, migration and tube formation as well as survival. VEGFR2-deficient mouse embryos die at E8.5 due to an early defect in the development of haematopoietic and endothelial cells, indicating the importance of this receptor during development and angiogenesis (*Shalaby et al., 1995*). In addition, this phenotype is similar to that of VEGF-A knock out mice (*Carmeliet et al., 1996; Ferrara et al., 1996*). VEGFR2 binds VEGF-A (with a lower affinity than VEGFR1), VEGF-C and VEGF-D. VEGF-A acts as a mitogen for ECs as it induces cell proliferation through VEGFR2-activated RAS/RAF/ERK/MAPK pathway. Several VEGFR2-activated signalling cascades, including the p38 MAPK pathway, promote EC migration towards high VEGF-A concentrations. Activation of the VEGFR2/PI3K/PKB pathway regulates the survival of ECs. As its alternative name indicates, VEGF (Vascular permeability factor (VPF)) promotes vascular permeability through VEGFR2 activity.

VEGFR3 is the main regulator of lymphendothelial cell function. This receptor is also involved in angiogenesis during embryonic development but becomes later a key regulator of lymphangiogenesis. VEGFR3 binds to VEGF-C and to VEGF-D. Lymphoedemas are observed in humans with loss-of-function VEGFR3 mutations. VEGFR3 is also present on the plasma membrane of macrophages.

VEGFR signalling is followed by a rapid clearance of the activated receptor from the cell membrane or alternatively by dephosphorylation of the receptor tyrosine residues. For example, the VEGFR2-ligand complex is ubiquitinated and internalized into endosomes, then is degraded in lysosomes.

### Mechanisms of physiological angiogenesis

Several angiogenic mechanisms leading to the formation of new blood vessels are described. Intussusception, where vessels split from pre-existing ones due to the penetration of smooth muscle cells through the endothelial cell layer, is the most rapid angiogenic process (*Burri et al., 2004*). Second, circulating bone marrow-derived endothelial precursor cells (CEPs) home to sites where angiogenesis is active such as tumors and support the formation of new blood vessels (*Patenaude et al., 2010*). A third mechanism, named sprouting angiogenesis, activates ECs of existing vessels to invade in the surrounding tissue. Sprouting angiogenesis is a multi-step process which could be roughly divided into an initial activation phase followed by the

maturation of the new vessel (Figure 24) (Carmeliet & Jain, 2011; Potente et al., 2011). During the activation phase, pro-angiogenic factors favour vasodilation and increase vascular permeability. In addition, matrix-metalloproteases degrade the perivascular basement membrane to allow ECs migration in response to integrin signalling. The formed “sprout” contains leading “tip cells” which migrate towards the pro-angiogenic factors gradient and trailing “stalk cells” which proliferate leading to elongation and branching of the new vessel. The degree of sprouting is negatively regulated by Dll4 (secreted by “tip cells”) and its receptor Notch. Notch negatively regulates the sprouting of ECs by repressing VEGFR2 expression in “stalk cells”, if their neighbours already acquired the “tip cell” phenotype. ECs continue to self-organize into tubular structures forming the vessel lumen. During the maturation phase, ECs stop migrating and proliferating (“phalanx cell” phenotype). The new vessels are stabilized by the reconstitution of the basement membrane and Platelet-derived growth factor (PDGF)-induced pericyte recruitment

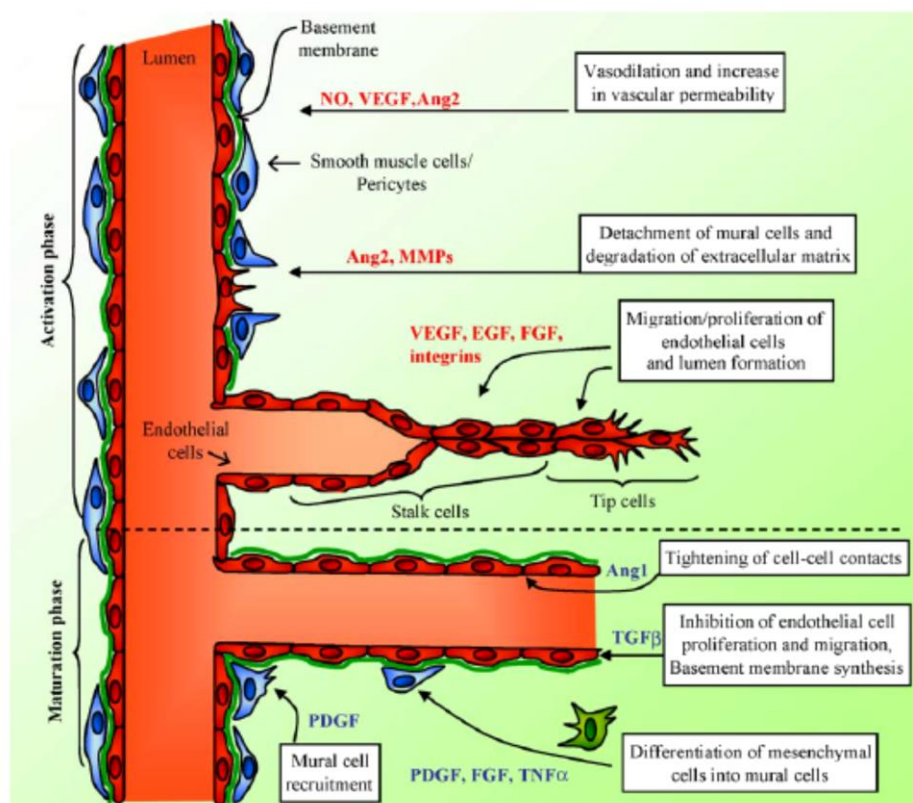


Figure 24: The multi-step process of angiogenesis including the activation phase, the sprouting and the maturation phase (David et al., 2009). NO, nitric oxide; VEGF, vascular endothelial growth factor; Ang2, angiopoietin 2; Ang1, angiopoietin 1; MMPs, matrix-metalloproteinases; EGF, epidermal growth factor; FGF, fibroblast growth factor; TGFβ, tumor growth factor β; PDGF, platelet-derived growth factor; TNFα, tumor necrosis factor α.

or their differentiation from mesenchymal progenitors. Finally, the development of tight and adherens junctions between ECs and/or pericytes results in vessel quiescence and establishment of the circulation. Pro- and anti-angiogenic factors are involved in every step of the sprouting angiogenesis. VEGF and FGFs stimulate the proliferation and migration of ECs. Angiopoietin 1 (Ang1) induces vascular quiescence via the receptor Tie-2, but is antagonized by Ang2 which activates angiogenesis. PDGF is mainly implicated in the vessel maturation. Transforming growth factor  $\beta$  (TGF $\beta$ ) is involved in ECs cell function, in the differentiation of mesenchymal cells and in the regeneration of the basement membrane. Although different signalling pathways are implicated in angiogenesis, the VEGF-VEGFR1/2 signalling as well as the Dll4-Notch signalling axis are the most important in this process.

Lymphangiogenesis, the formation of lymphatic vessels from pre-existing ones, seems to function similarly to angiogenesis, even if the molecular mechanisms are less explored (*Tammela & Alitalo, 2010; Stacker et al., 2014*). Lymphatic vessels derive originally from veins where ECs start to express lymphatic markers like PROX-1. Lymphangiogenesis is induced in response to VEGF-C/VEGFR3 signalling. Lymphatic vessels are essential for survival in embryogenesis as well as in adults where they regulate tissue fluid homeostasis, immune cell trafficking and fatty acid uptake. Lymphangiogenesis appears concomitantly to angiogenesis in inflammation, wound healing and tumor metastasis (*Cao, 2005*).

### **3.1.7.2. Tumor angiogenesis**

Like normal tissue, tumor cells need to be connected to the vascular network as they require oxygen and nutrients as well as a possibility to evacuate metabolic waste and carbon dioxide. The process of tumor-associated neovascularization is addressing these needs. This fundamental process was firstly described by *Judah Folkman* in 1971 (*Folkman, 1971*). Since then, extensive research on tumor angiogenesis can be recorded.

### The angiogenic switch

Avascular tumor growth is limited to 1-2 mm<sup>3</sup> due to the limit of oxygen diffusion (*Bergers & Benjamin, 2003; Carmeliet & Jain, 2011*). Cancer cells induce angiogenesis, through the “angiogenic switch” to evolve beyond this size (Figure 25). In addition to this initial thought, recent data showed an early implication of this event during tumor initiation, highlighting the importance of this process in pre-malignant conditions (*Raica et al., 2009*). Finally, sustained angiogenesis will pave the way for the dissemination of cancer cells.

Oncogene signalling as well as intratumoral hypoxia-induced HIF-1 $\alpha$  expression promotes synthesis of a large panel of pro-angiogenic factors including molecules that regulate the maintenance and destruction of the perivascular milieu as well as factors which stimulate ECs proliferation and migration (*Bergers & Benjamin, 2003; Benazzi et al., 2014*). These factors are expressed by tumor cells, but also by several cell types of the tumor microenvironment (macrophages, blood cells, etc.). Some of them are briefly described below.

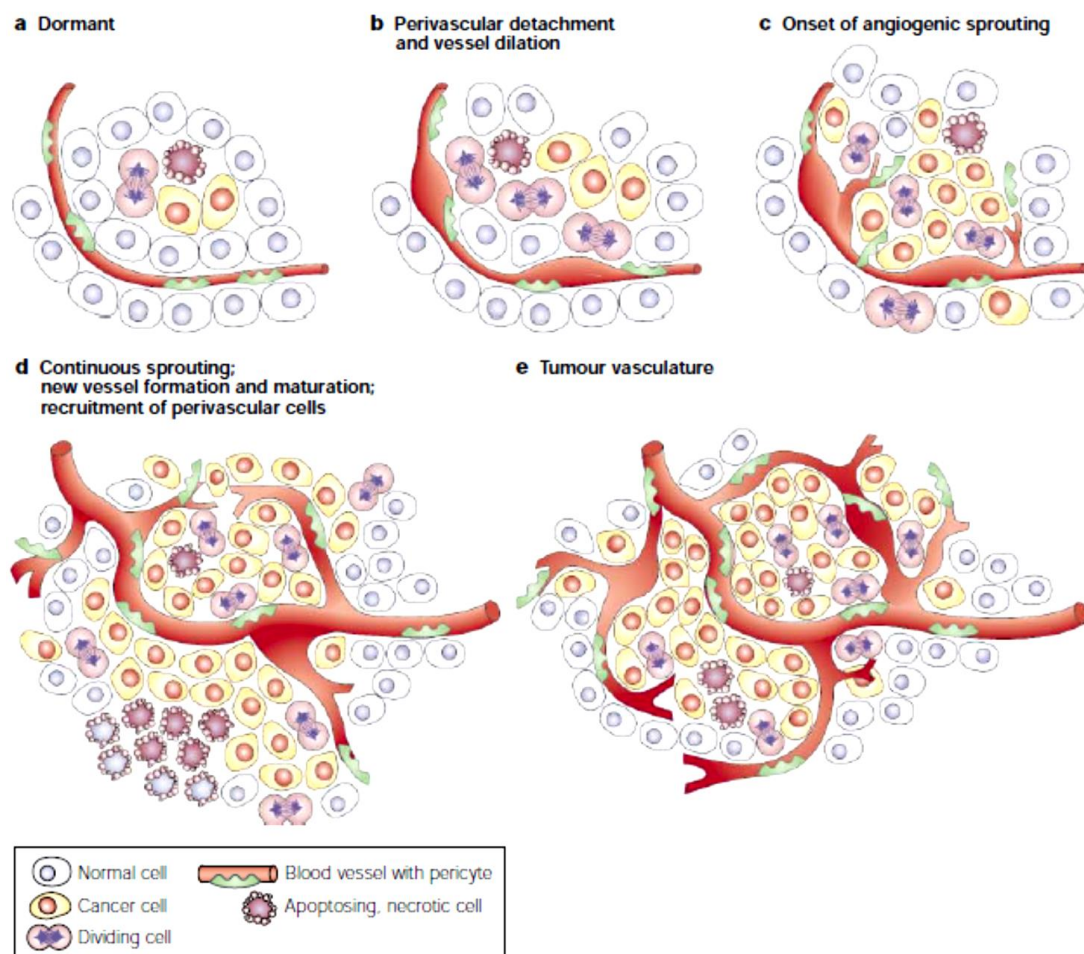


Figure 25: The angiogenic switch during tumor development (*Bergers & Benjamin, 2003*).

VEGF is expressed by epithelial cells, endothelial cells, stromal cells and tumor cells as well as cancer stem cells, suggesting that paracrine and autocrine VEGF signalling induces tumor angiogenesis. QPCR studies revealed distinct expression patterns of the VEGF<sub>xxx</sub> isoforms in different types of tumors (*Zygalaki et al., 2007*). For example, VEGF<sub>165</sub> and VEGF<sub>121</sub> are the predominant VEGF isoforms in lung and colon cancer. The expression of VEGF<sub>xxx</sub>b isoforms in tumors is still controversial (*Eymin et al., 2014*). In colorectal cancer, VEGF<sub>165b</sub> suppression was correlated to invasion and metastases (*Diaz et al., 2008*). By contrast, a significant correlation between high levels of VEGF<sub>165b</sub> and lymph node metastases in non-small lung cancer cells was recently reported (*Eymin et al., 2014*).

VEGFR2 is the main receptor involved in tumor angiogenesis. *Millauer et al.* demonstrated that the expression of a dominant-negative mutant of VEGFR2 in endothelial cells inhibits glioblastoma tumor growth and vascularization *in vivo* (*Millauer et al., 1994*). Interestingly, Nrp1 has been detected also on human pancreatic adenocarcinoma cells which do not express VEGFR2 (*Gray et al., 2005*). It has been postulated that VEGF may act as a bridge between tumor cell-expressed Nrp1 and VEGFR2 on a neighbouring EC. This so-called transactivation may contribute to tumor angiogenesis, in which tumor cells would provide not only VEGF but also Nrp1 to attract VEGFR2-expressing ECs (*Soker et al., 2002*). The basic fibroblast growth factor (bFGF) is produced by tumor cells, macrophages and other cell types of the tumor microenvironment. bFGF stimulates major steps of tumor angiogenesis such as ECs proliferation, migration, and extracellular matrix degradation. Ang2 induces together with VEGF host vessel permeability, microvascular dilation and vessel sprouting. Ang2 and VEGFR2 expression remains high in late stage tumor development to maintain the angiogenic plasticity in tumors (*Vajkoczy et al., 2002; Baeriswyl & Christofori, 2009*). SDF-1 secreted by tumor cells attracts stromal and immune cells. Those cells create a tumor environment which favours angiogenesis by producing VEGF and other pro-angiogenic cytokines such as IL-8 (*Squadrito & De Palma, 2011*). Furthermore, stromal cell-derived MMPs release sequestered VEGF due to the remodelling of the ECM (*Kessenbrock et al., 2010*).

The above described sprouting angiogenesis is the most important mechanism of tumor vascularization, but in contrast to normal vasculature, tumor vessels lack maturation. Other models of tumor blood vessel formation are summarized in Figure 26 (*Zhu et al., 2011; Benazzi et al., 2014*). Intussusception; (2) vessel co-option (mosaic vessel formation), where tumor cells hijack pre-existing blood vessels and integrate them into the tumor vasculature, or (3) vascular mimicry, when cancer cells form vessel-like structures, are described as alternatives (*Paulis et al., 2010*). In addition, (4) bone marrow-derived vascular progenitor cells are recruited to tumors

by different cytokines where they differentiate into pericytes or endothelial cells (Fang & Salven, 2011). Tumors can use all of these mechanisms to form new blood vessels, even simultaneously.

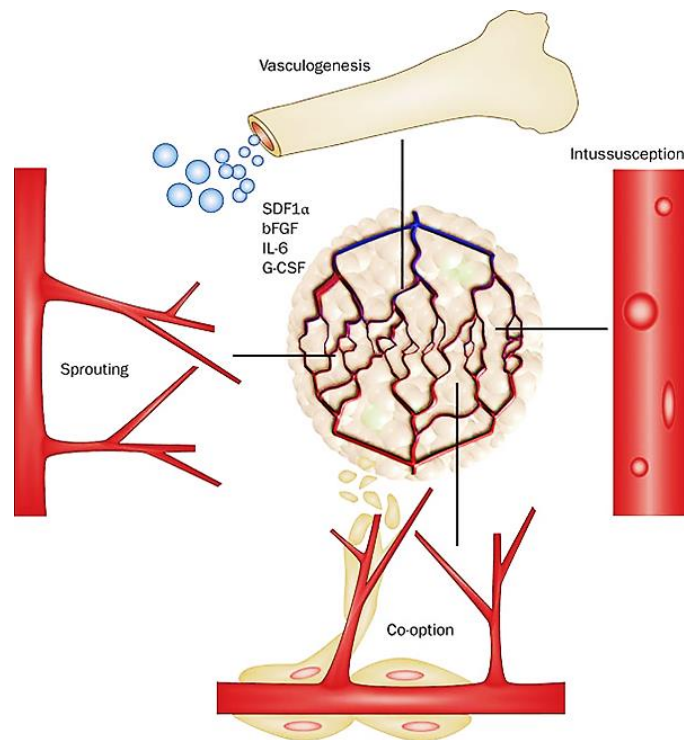


Figure 26: Schematic representation of tumor angiogenesis including sprouting angiogenesis, vasculogenesis (bone marrow-derived endothelial progenitor cells are recruited to the tumor by different factors such as the stromal-derived factor 1 $\alpha$  (SDF-1 $\alpha$ ), the basic fibroblast growth factor (bFGF), IL-6 and G-CSF), intussusception and vessel co-option (Zhu *et al.*, 2011).

### Tumor vasculature

By contrast to normal tissues, tumors lose the tightly regulated balance between pro-and anti-angiogenic factors. This balance normally induces rapid maturation and stabilization of new blood vessels. Tumor blood vessels fail to become mature and quiescent, thus allowing sustained angiogenesis in tumor tissue.

Instead of a hierarchical organization, tumor vasculature is typically aberrant and dysfunctional with a chaotic, heterogeneous organization (Figure 27, 28) (Jain, 2005; Goel *et al.*, 2011). Vessels are varying from large lumen to tortuous and compressed capillaries and can even have dead ends. Differentiation of tumor vessels into arteriols, capillaries or venules often fails. Normal vessels are lined with a monolayer of quiescent “phalanx” ECs, which are polarized and aligned in the direction of the blood flow for optimal perfusion. ECs in tumor vessels have lost this polarity, leading to an irregular shape and weak tight/adherens junctions between ECs.

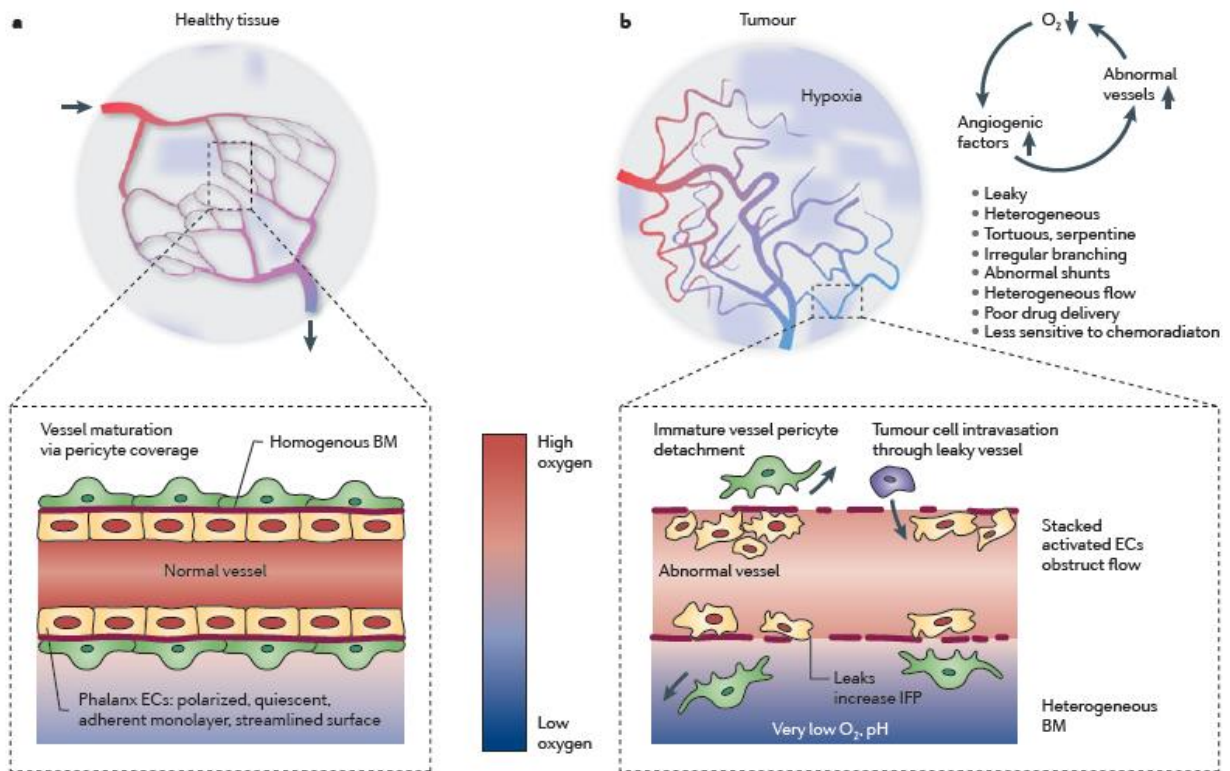


Figure 27: Tumor vasculature is abnormal in their structure and function (*Carmeliet & Jain, 2011*). (a) Organization and normal vessel structure in healthy tissue. (b) Abnormal pattern and structure as well as function of tumor vessels lead to hypoxic regions within the tissue. BM, basement membrane; EC, endothelial cell; IFP, interstitial fluid pressure.

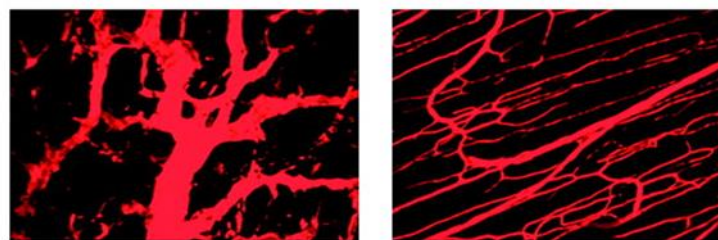


Figure 28: Abnormalities of tumor microvasculature (*Jain, 2005; Goel et al., 2011*). Vasculature in human colon cancer in mice (left) versus healthy skeletal muscle (right).

Those ECs are poorly aligned and contain multiple fenestrations. Furthermore, activated ECs in tumors undergo endothelial-to-mesenchymal transition and move away from their origin. Healthy quiescent ECs are engulfed by pericytes which stabilize the vessels. Tight contacts between ECs and pericytes and the embedding of both cell types in the basement membrane reduce vessel permeability. In tumors, pericytes are often less differentiated, leading to a loosely



coverage of the vasculature. In addition, the basement membrane shared by ECs and pericytes is either abnormally thick or thin and loosely associated to both cell types.

All these abnormal structural characteristics cause heterogeneous perfusion of the tumor tissue as blood flow could be either rapid in some vessels or stagnant in others. Consequently, diffusion of oxygen, nutrients and drugs is highly heterogeneous within a tumor, leading to hypoxic regions within the tissue. Those regions could not be reached by chemotherapy as the deleterious effect of this treatment depends on reactive oxygen species. Furthermore, leakiness of tumor vessels leads to haemorrhage. The absence of functional lymphatic vessels results in increased interstitial pressure due to insufficient drainage of the tissue (*Choi et al., 2013*). The function of immune cells can also be impaired by the abnormal tumor vasculature. To compensate for the oxygen and nutrients demand, angiogenesis is further stimulated in tumor cells. Permanent secretion of pro-angiogenic factors leads to excessive vessel branching and restarts this vicious cycle. Sustained angiogenesis is supported by the hypoxic and inflammatory tumor microenvironment which favours the selection of more aggressive tumor cells and promotes metastasis. This aspect will be further discussed in more detail below. Altogether, the above listed tumor vasculature abnormalities favour cancer progression and facilitate the formation of metastasis. Furthermore, structural characteristics of tumor vessels can influence responsiveness to anti-cancer therapies.

### **3.1.8. Hallmark: Activating invasion and metastasis**

Dissemination of cancer cells was believed to be a late event in tumorigenesis. During the last years, several studies strengthened the observation that this process takes place very early in cancer development. Invasion and the formation of metastasis is a multi-step process. This hypoxic-driven cascade is initiated by the loss of cell-to-cell or cell-to-ECM adhesion. Cells locally invade and intravasate into nearby blood and lymphatic vessels, then are transferred through the lymphatic and blood system. By a process called extravasation, tumor cells escape the circulation and enter distant organs to form micrometastasis. After an adaptation of these invasive cells to the foreign tissue microenvironment, a process also termed colonization, macroscopic tumor metastasis start to grow (*Nguyen et al., 2009*). Two main intracellular processes are implicated in the described single-cell invasion-metastasis cascade: epithelial-mesenchymal transition (EMT) and its reversion, the mesenchymal-epithelial transition (MET) (Figure 29). During EMT, epithelial cells acquire the ability to invade and disseminate as well as to resist apoptosis (*Yilmaz & Christofori, 2009*). This cellular program is orchestrated by the induced expression of various transcription factors, such as Snail, Slug, Twist and Zeb1/2. Their action leads to the loss of adherens junctions (translocation of  $\beta$ -catenin to the nucleus,

repression of E-Cadherin and induction of N-Cadherin) and the associated transition from a polygonal/epithelial to a spindly/fibroblastic morphology which confers the cell the ability to migrate. Within this step, extracellular matrix-degrading enzymes, mainly matrix-metalloproteases (MMPs), are expressed and secreted to facilitate invasion by remodelling the ECM. Once arrived at their final destination, tumor cells need to revert EMT to become again non-invasive. Colonization is not a cancer cell-autonomous event. The support of stromal cells, which form the tissue microenvironment and cancer cell homing, is indispensable for this adaptation. The communication between cancer cells and stromal or immune cells in their surrounding is crucial for effective invasion and metastasis formation.

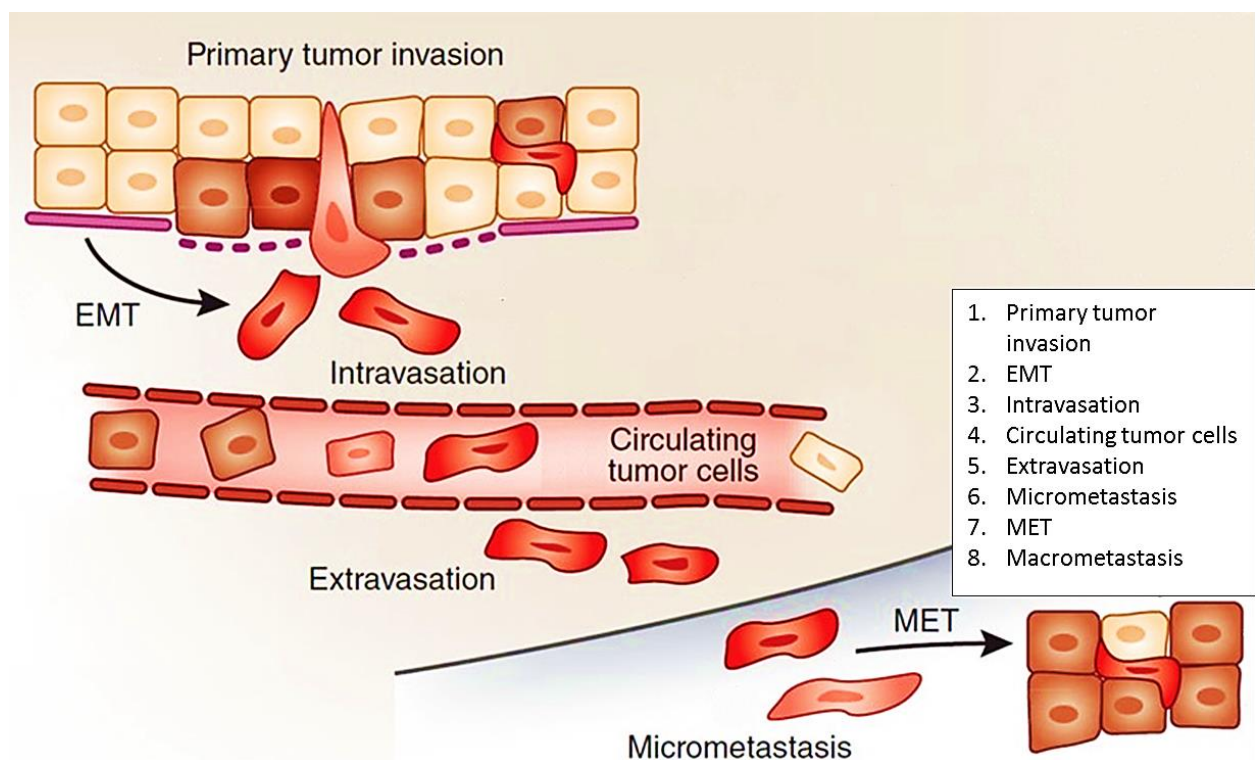


Figure 29: The invasion-metastasis cascade (Thompson & Haviv, 2011).

### 3.1.9. Emerging hallmark: Deregulating cellular energetics

The high proliferation rate of tumor tissue requires important amounts of energy. The most striking alterations of tumor metabolism include enhanced glycolysis rate, elevated glutaminolytic flux, increased amino acid and lipid metabolism, induced mitochondrial biogenesis and elevation of pentose-phosphate pathway as well as macromolecule biosynthesis (Phan et al., 2014).

In normal cells, glucose is metabolized into pyruvate which is further converted to acetyl-CoA. The Krebs cycle, fuelled by acetyl-CoA, generates NADH and FADH<sub>2</sub> which provide electrons

to the mitochondrial respiratory chain. Normal cells prioritize glycolysis, which generates two ATP per glucose molecule, only in oxygen-limited conditions. In contrast, cancer cells metabolize glucose via glycolysis even under normoxic conditions (Warburg effect) and via aerobic respiration (*Vazquez et al., 2010*).

Low effective glycolysis need to be compensated in highly proliferating tumor tissue. Therefore, cancer cells increase expression of glucose transporters such as glucose transporter 1 to 4 (GLUT1-4) to augment cellular glucose uptake (*Hsu & Sabatini, 2008*). Additionally, the hypoxic areas and activation of tumor oncogenes within the tumor are forcing the upregulation of glucose transporters and the expression of enzymes involved in glycolysis. Major oncogenes such as Ras and Myc as well as HIF-1 $\alpha$  are known to induce glycolysis (*Jones & Thompson, 2009*). In this context, HIF-1 $\alpha$  transcriptional activity is de-repressed due to the inactivation of the histone deacetylase Sirt6, a protein which is implicated in calorie restriction and longevity (*Longo & Fontana, 2010*). While c-myc activates transcription of glycolytic enzymes under normoxia, HIF-1 $\alpha$  promotes expression of glycolytic genes under hypoxia. Thus, cancer cells drive glycolysis permanently to respond to their high energy need. p53, known to inhibit glucose uptake by negatively influencing expression of glucose transporters, for example, is often silenced in cancer (*Phan et al., 2014*).

In cancer cells, most pyruvate, the end-product of glycolysis, is further processed to lactate instead of acetyl-CoA, mainly due to the overexpression of the lactate dehydrogenase (LDHA). It seems that a tumor consists in two cell subpopulations: one, which is metabolizing glucose and secreting lactate via the MCT4 transporter and the second which uses this lactate as a main carbon source for their energy metabolism (*Kennedy & Dewhirst, 2010*). This symbiosis enables cancer cells to ensure the availability of a carbon source even in the presence of restricted nutrient supply due to less functional and leaky tumor vasculature. Furthermore, lactate secretion lowers the pH in tumor surrounding tissue which further activates degradation of the extracellular matrix by metalloproteases, facilitating invasion and metastasis (*Bonuccelli et al., 2010; Parks et al., 2011; Parks et al., 2013*). Tumor cells itself are responding to this massive acidosis by HIF-1 induced expression of H<sup>+</sup>/monocarboxylate transporter 4 (MCT4) and carbonic anhydrase IX/XII expression (*Chiche et al., 2009*). These two pH-regulating systems ensure tumor cell survival in low pH-microenvironment. Pyruvate conversion into lactate causes less formation of reactive oxygen species, thus protecting cancer cells from oxidative stress. Beside ATP as a source of energy, glycolysis provides important intermediates which serve as substrates for several other biosynthetic pathways. In addition to their role in the energy

metabolism, glycolytic enzymes are implicated in cancer survival, metastasis, invasion, chromatin remodelling and others (*Kim & Dang, 2005*).

Cancer cells use also glutaminolysis to fuel their cellular bioenergetics demand and metabolism. Metabolizing glutamine into glutamate or  $\alpha$ -ketoglutarate fuels the Krebs cycle which in turn provides intermediates of the Krebs cycle that could be used for the synthesis of lipids, cholesterol and amino acids. Glutaminolysis is often upregulated in cancer cells due to c-myc overexpression (*Wise et al., 2008*).

### **3.1.10. Emerging hallmark: Evading immune destruction**

Historically, an infiltration of tumors by immune cells was associated with a good prognosis, thus supporting the importance of the immune system against tumor progression. Paradoxically, enhanced tumor formation was found in immunodeficient mice and mouse models which were lacking, either natural killer cells or CD8<sup>+</sup> cytotoxic T lymphocytes or CD4<sup>+</sup> T<sub>H</sub> helper cells (*Kim et al., 2007; Teng et al., 2008*). Transplantation experiments in mice revealed the “immunoediting” process of cancer cells: Immunogenic cancer cell clones are eliminated in immunocompetent tumor-bearing mice. The remaining weakly immunogenic cancer cells could form secondary tumors when transplanted in immunocompetent or immunodeficient hosts. In contrast, cancer cells derived from primary tumors grown in immunodeficient mice were not edited and kept their immune-sensitive characteristics. Therefore, transplantation of those cells in immunocompetent recipients is rejected (*Kim et al., 2007; Teng et al., 2008*). In tumors, the expression of immune-checkpoint proteins can be dysregulated leading to resistance to the host-immune system. Cancer cells or components of the tumor microenvironment overexpress inhibitory ligands and receptors that regulate T-cell function in normal tissue. The two immune-checkpoint receptors that have been the most studied in this context, are the cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) and the programmed cell death protein 1 (PD1) (*Pardoll, 2012*). Both are inhibitory receptors on T-cell activity. Tumors exploit the CTLA4 pathway to diminish T-cell proliferation, infiltration and activation. On the other hand, tumors can evade the host immune attack via the PD1 pathway. PD1 ligands present on the tumor cell membrane induce the PD1 pathway in T-cells leading to the inactivation of these immune cells. In addition, cancer cells can manipulate tumor-host immunological interactions for example by secreting TGF $\beta$  or other immunosuppressive factors to inhibit immune cells (*Yang et al., 2010*). This complex network will be detailed as part of the tumor microenvironment paragraph. Tumors are also able to attract immune cells, which will

form a tumor-promoting microenvironment. As the evidence for an anti-tumor immunity is still lacking, immunoevasion remains as an emerging hallmark in cancer progression.

**3.2. The cancer niche or tumor microenvironment**

In contrast to the long-lasting idea that a tumor is a relatively homogenous population of cancer cells, tumors are more and more recognized as complex organs containing several cell types as well as blood and lymphatic vessels (Figure 30). Given the intratumoral heterogeneity, the understanding of cancer cell molecular mechanisms alone is not sufficient anymore to treat this life-threatening disease efficiently. During the last decade, cancer research started to focus also on tumor-associated cells, which create the “tumor microenvironment”, as well as on the crosstalk between malignant cancer cells (epithelial parenchyma of carcinomas) and the tumor

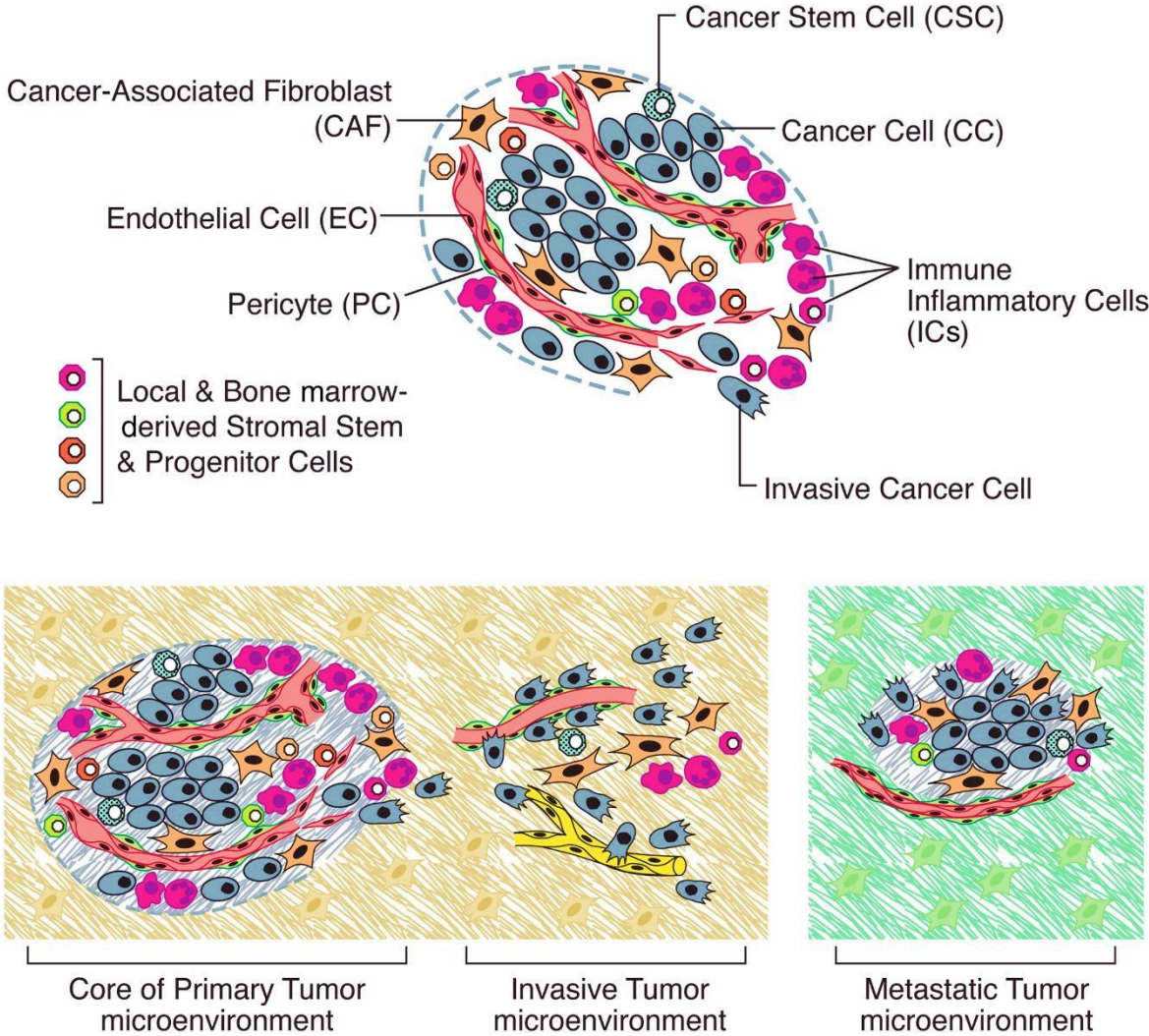


Figure 30: Complexity of the interactions between tumor cells and cells of the tumor microenvironment (Hanahan & Weinberg, 2011).

stroma. The importance of the tumor microenvironment is strengthened by the functional contribution of several cell types to different hallmarks during cancer progression. In a more provocative way, beside cellular tumor-promoting epi-/genetic alterations, the construction of the cancer niche is essential for tumor cell survival, proliferation and gain of malignant potential (*Barcellos-Hoff et al., 2013*). Cells of the tumor stroma are attracted from normal tissue by cancer cells or derive from the bone-marrow as progenitor cells. Autocrine and paracrine signals between cancer cells and their surroundings lead to the reprogramming of the tumor microenvironment to support tumor initiation, promotion and progression (*Quail & Joyce, 2013*). On the other hand, reprogramming the capacities of the tumor microenvironment could limit cancer progression and could be consequently an interesting target for cancer therapy. Tumor stroma is composed of non-malignant cells such as cancer-associated fibroblasts (CAF), tumor-associated macrophages (TAM) and neutrophils (TAN) as well as mesenchymal stem cells and the ECM. The following paragraphs will give a brief overview about major cell types located in the tumor stroma and how they are influencing the biology of cancer cells.

### **3.2.1. Cell types of the tumor microenvironment**

#### **3.2.1.1. Cancer stem cells**

Over the last decades, the existing clonal evolution model, which assumes that each cell within a tumor has the same potential to form new neoplastic lesions by gaining genetic and epigenetic changes, was completed by another model, suggesting that just a subpopulation, termed cancer stem cells (CSCs), has the ability to self-renew, differentiate and regenerate tumors (*O'Connor et al., 2014*). Therefore, the discovery of CSCs and the plasticity of tumors suggest that a genetically homogeneous subpopulation within cancer tissue could be phenotypically heterogeneous due to various differentiation states of these cells. The origin of CSCs remains still unclear. CSCs are the driving force in carcinogenesis based on their capability of self-renewal and multi-lineage differentiation through symmetric or asymmetric cell division. To study the degree of stemness of tumor cells that drives carcinogenesis, tissue of a primary tumor was dissociated and cell solutions were diluted. Isolated single cancer cell were inoculated in immunodeficient mice and tumor appearance was monitored. By using this functional assay, CSCs could be identified in leukaemia as well as in several solid tumors (*Bonnet & Dick, 1997; Al-Hajj et al., 2004*). It seems that EMT-related processes are supporting self-renewal capabilities of CSCs to ensure their clonal expansion at distant tissue sites after dissemination. The threat posed by CSCs in cancer is strengthened by their chemoresistance as

well as by the fact that they can stay dormant to cause cancer relapse several years after diagnosis and successful treatment (*Singh & Settleman, 2010*).

### **3.2.1.2. Immune inflammatory cells**

Infiltrating immune cells, especially tumor-associated macrophages (TAMs) and tumor-associated neutrophils (TANs), are increasingly recognized as constituents of the tumor stroma. Tumor cells attract mature immune inflammatory cells very early in tumor development by releasing factors like the Colony-stimulating factor 1 (CSF-1) and Monocyte-chemoattractant protein 1 (MCP-1). In addition, tumors recruit undifferentiated myeloid progenitor cells from the bone marrow, which will differentiate into macrophages or neutrophils.

To simplify, immune cells are polarized to two extreme phenotypes by tumor-derived factors and intratumoral hypoxia, which act in contradictory ways (either tumor-promoting or tumor-antagonizing). Activated TANs, TAMs (M2-subtype) as well as other immune cells release tumor-promoting factors, like VEGF, EGF, FGF2, chemokines and cytokines (IL-1, IL-8, TNF $\alpha$ ) as well as pro-invasive/angiogenic matrix-degrading enzymes (MMP-2/7/9/12, COX-2) to facilitate key events during tumor progression, such as proliferation, angiogenesis and invasion, and generate an immunosuppressive microenvironment (*Mantovani et al., 2008*). In contrast, M1 macrophages are anti-tumoral and pro-inflammatory. However, the simple M1/M2 model of macrophages polarization seems to be more complicated than estimated. *Xue et al.* propose a “spectrum model” with at least nine distinct macrophage activation programs depending on environmental stimuli (*Xue et al., 2014*). In addition, TAMs are involved in EMT and cancer stem cell regulation.

### **3.2.1.3. Cancer associated fibroblasts**

Cancer-associated fibroblasts (CAFs) are a dominant, heterogeneous cell population of the tumor stroma (*Ostman & Augsten, 2009*). Their phenotype is distinct from that of normal fibroblasts. Several subpopulations of CAFs are known: resident fibroblasts, bone marrow-derived progenitor cells (myofibroblasts) and trans-differentiating fibroblasts (*Zeisberg et al., 2007; Mishra et al., 2008*). Transdifferentiation of stromal fibroblasts into CAFs is mediated by TAM-secreted TGF $\beta$ , PDGF and bFGF (*Elenbaas & Weinberg, 2001*). CAFs secrete several extracellular matrix components, such as fibronectin, collagen as well as MMPs, and are therefore involved in the remodelling of the established stroma in advanced tumors (*Bhowmick et al., 2004; Kalluri & Zeisberg, 2006; Mueller et al., 2007*). In addition, CAFs themselves produce and secrete VEGF, MCP-1 and several growth factors, promoting angiogenesis and

inflammation (Sotgia *et al.*, 2012). Consequently, appearance of CAFs in tumors is associated with pathological fibrosis as well as growth and invasion of tumor cells as they are initiating angiogenesis and the recruitment of myeloid cells.

### 3.2.2. Heterotypic signalling coordinates cells of the tumor stroma

Tumor cells communicate with their environment via direct cell-to-cell contacts, secreted paracrine-acting factors and exosomes (van Niel *et al.*, 2006). Barcellos *et al.* proposed a concept of a dynamic and evolving cancer niche during tumorigenesis (Barcellos-Hoff *et al.*, 2013). Co-evolution of malignant cells and their environment by intense exchanges will result in successful tumor progression (Figure 31, upper part).

Early neoplastic lesions start to recruit stromal cells to form a first tumor stroma, which responds reciprocally to force malignant phenotype of cancer cells. The critical signal for cancer niche construction are still unknown but CXCL12 (SDF-1) and TGF $\beta$  seem to be important candidates (Moses & Barcellos-Hoff, 2011). Cancer cells feed back signals towards the stroma to reprogram cells of the tumor microenvironment thus inducing angiogenesis and immune surveillance escape. Within this step, immune cells are recruited to expand the tumor niche (Tu *et al.*, 2008; Quante *et al.*, 2011). In addition, fibroblasts as the main source of growth factors, cytokines and ECM components, are contributing to this phase. The lower part of Figure 31 illustrates the heterotypic signalling between tumor cells and their dynamic environment and points out the importance of these complex interactions during cancer progression. TGF $\beta$  and CXCL12 are crucial in recruitment of CAFs and bone marrow-derived mesenchymal stem cells (MSCs) (Quante *et al.*, 2011). Exosomes produced by the primary tumor are transferring pro-inflammatory molecules, proteins and miRNAs to activate CAFs, recruit MSCs, alter immune response and prepare premetastatic sites (Szajnik *et al.*, 2010; Webber *et al.*, 2010; Peinado *et al.*, 2012). Oncogenic alterations of cancer cells promote their ability to induce angiogenesis and consequently the maturation of the cancer niche. In the pancreas, for example, Myc-induced IL-1 $\beta$  expression mediates VEGF-A activation and distribution from the ECM (Shchors *et al.*, 2006). In mammary tumors, CSF-1 induces angiogenesis in tumor tissue and thus promotes tumor progression. Genetic deletion of CSF1 delayed the transition of primary tumors to invasive, metastatic carcinomas *in vivo* (Lin *et al.*, 2001). This process was reversed by overexpressing CSF-1. After having reached distant organs, cancer cells need to repeat reciprocal heterotypic signalling to change the naive, normal tissue environment in order to form macrometastasis (Figure 31, lower part). However, the existence of “metastatic niches” – tumor environments, which are susceptible for freshly seeded cancer cells – has been recently described



as an early event in tumor development (Peinado et al., 2011). Pre-existing hospitable stroma could be intrinsic to the tissue or induced by circulating factors secreted by the primary tumor. Tumor-promoting inflammatory cells play an important role in this process. In relation to the development of novel targeted cancer therapies, the comprehension of the dynamic crosstalk between cancer cells and components of their environment will be essential.

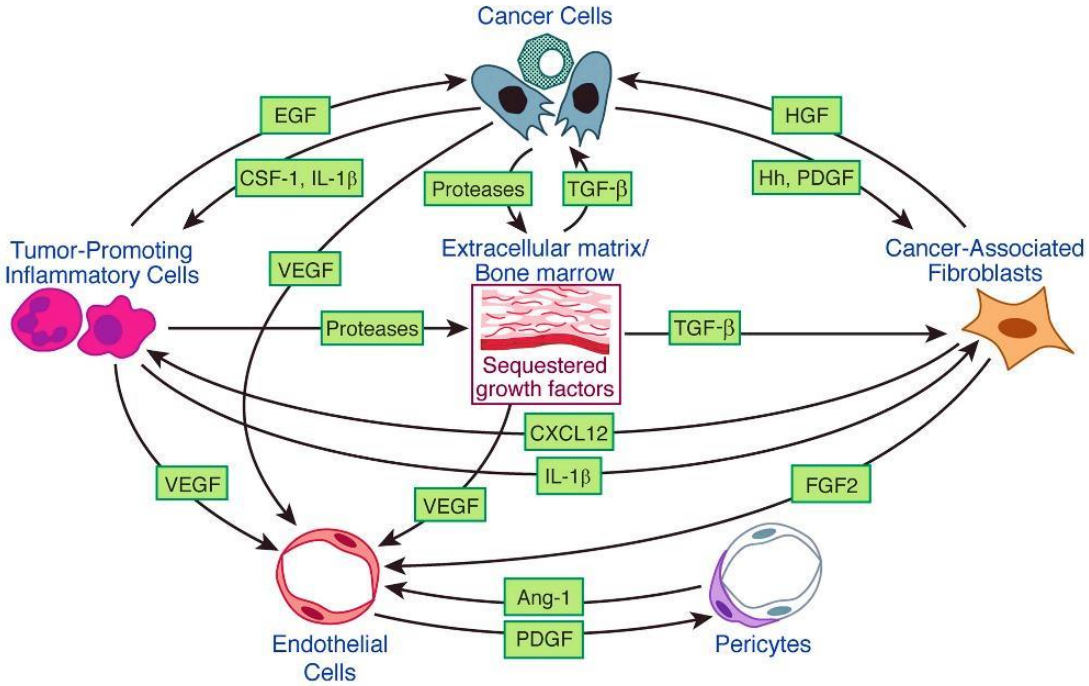
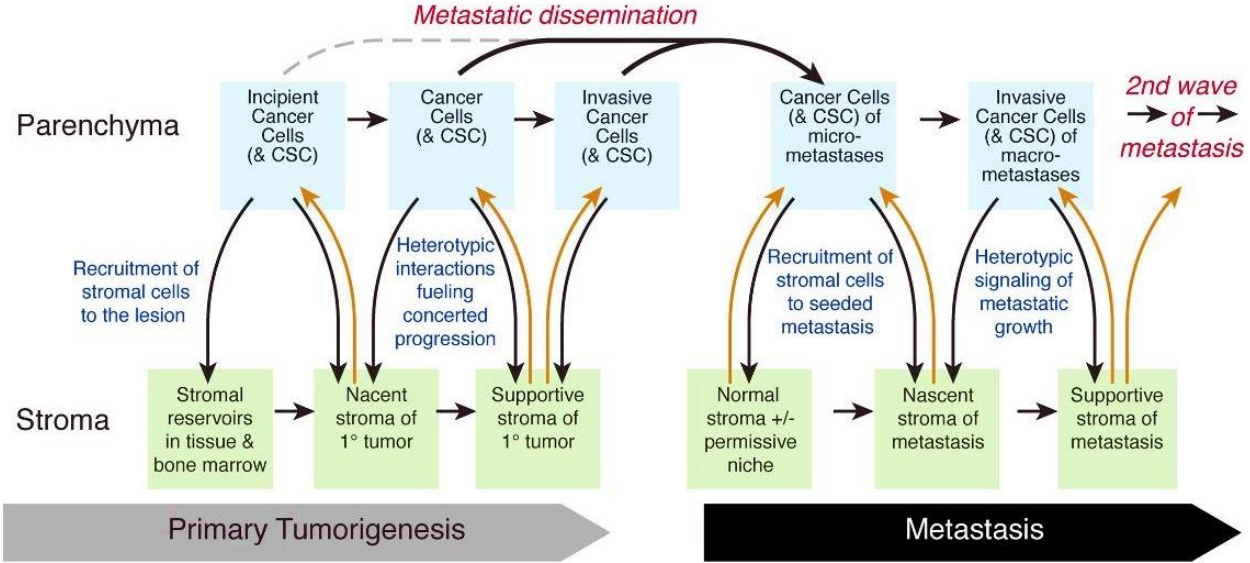


Figure 31: Signalling network of cancer cells and the tumor microenvironment during tumorigenesis (Hanahan & Weinberg, 2011).

### 3.2.3. Hypoxia

The high proliferative capacity of tumor cells moves away cancer cells from oxygen- and nutrient-carrying blood vessels. In addition, the abnormal tumor vasculature is inefficient to overcome this default. Instead of cell death, this deficient environment favours cell survival and migration of tumor cells through the major action of the hypoxia-inducible factor (HIF). This transcription factor is activated under low oxygen levels and regulates the transcription of various genes implicated in tumor cell metabolism, angiogenesis and EMT. Therefore, hypoxia signalling is involved in almost every step of tumorigenesis and impacts tumor cells and each cell type of the tumor microenvironment (*Ruan et al., 2009; De Bock et al., 2011; Casazza et al., 2014*).

In various but not in all cancers, hypoxia is associated with therapy resistance and disease progression as it favours the most invasive tumor cells.

#### HIF-1 signalling

HIF-1 is a dimer consisting of the oxygen-sensitive HIF-1 $\alpha$  subunit and the constitutively expressed HIF-1 $\beta$  subunit (Figure 32) (*Semenza, 2013*). Under normoxic conditions, HIF-1 $\alpha$  is hydroxylated by oxygen-dependent prolyl-hydroxylase domain proteins (PHD). The Von Hippel Lindau tumor suppressor protein (VHL) binds to hydroxylated HIF-1 $\alpha$  which induces the further ubiquitination of the protein by the E3 ubiquitin ligase and targets HIF-1 $\alpha$  for proteasomal degradation. In addition to oxygen, the cellular metabolic status modulates HIF-1 $\alpha$  stability as PHD proteins use the TCA cycle intermediate  $\alpha$ -ketoglutarate as substrate. Under hypoxia, hydroxylation of HIF-1 $\alpha$  does not occur, leading to the stabilization of the protein. HIF-1 $\alpha$  dimerize with HIF-1 $\beta$  enabling HIF-1 transcriptional activity and the regulation of adaptive cell responses to hypoxia, including cell proliferation, cellular metabolism and angiogenesis.

Like HIF-1 $\alpha$ , HIF-2 $\alpha$  is also overexpressed in cancer cells (*Talks et al., 2000*). The overexpression of HIF-2 $\alpha$  in TAMs was reported and correlated with high tumor grade and poor prognosis. Strikingly, HIF-1 $\alpha$  has been shown to function as a tumor promoter in cancer associated fibroblasts, and as a tumor suppressor in breast cancer cells, suggesting that HIF-1 $\alpha$  activity is cell-type- and compartment-specific (*Chiavarina et al., 2010*).

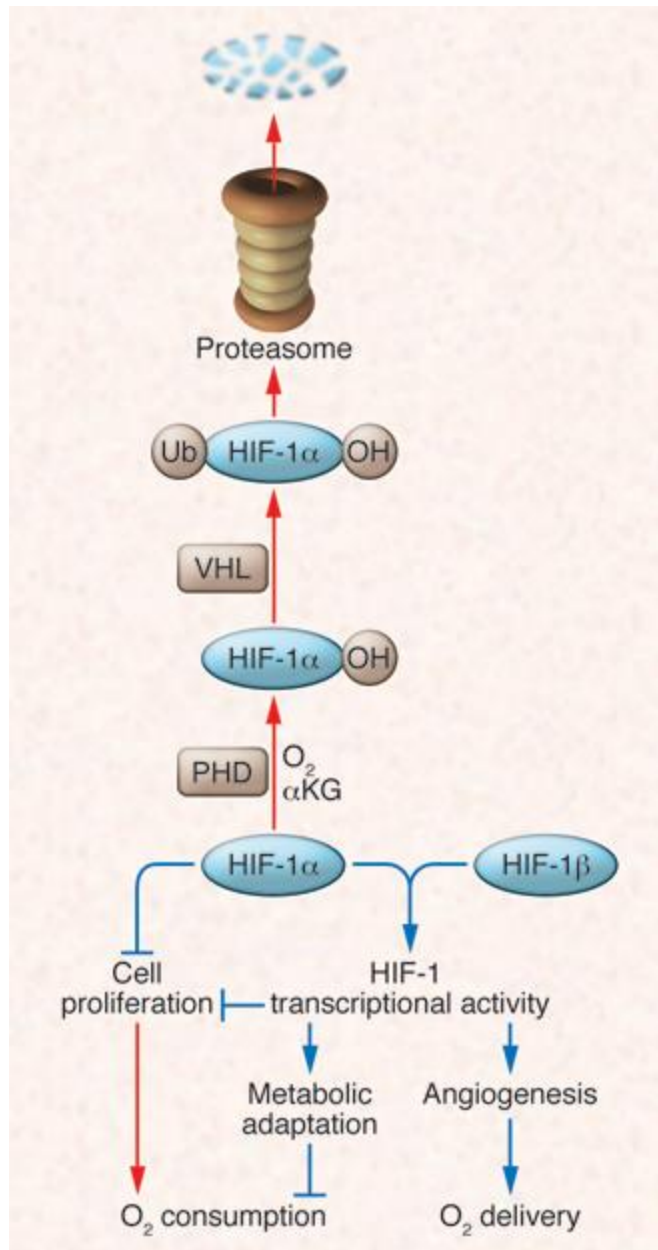


Figure 32: Oxygen-dependent regulation of HIF-1 $\alpha$  stability and HIF-1 signalling (Semenza, 2013). Further details are explained in the text.  $\alpha$ KG,  $\alpha$ -ketoglutarate; HIF-1 $\alpha/\beta$ , hypoxia-inducible factor  $\alpha/\beta$ ; PHD, prolyl-hydroxylase domain proteins; Ub, ubiquitin; VHL, Von Hippel Lindau factor.

### Effect of hypoxia on tumor cells

Under hypoxia, HIF-1 induces the transcription of VEGF and other pro-angiogenic cytokines, thereby stimulating angiogenesis and vascular remodelling to improve oxygen delivery and tissue perfusion (Forsythe *et al.*, 1996; Rey & Semenza, 2010). Moreover, HIF-1 activates transcription of several genes favouring glucose uptake and metabolism as well as factors implicated in pH regulation (Brahimi-Horn *et al.*, 2011). Thus, HIF-1 ensures energy supply by enhancing glycolysis and enables cancer cells to survive in an acidic environment at the same time. On the other hand, HIF-1 inhibits the proliferation of cancer cells by inducing cell cycle

arrest. Due to HIF-1 transcriptional activity, Myc expression and its activity as driver of cell proliferation are repressed (*Koshiji et al., 2004*). HIF-1 $\alpha$  directly blocks DNA replication via non-transcriptional mechanisms (*Hubbi et al., 2013*). Hypoxia is associated with EMT as HIF-1 induces the expression of the E-Cadherin repressors Twist1 and Snail (*Hill et al., 2009*). HIF-1 enhances also the expression of matrix remodelling enzymes such as lysyl-oxydase (LOX) and metalloproteases that degrade cell-matrix interactions to facilitate migration and invasion of cancer cells (*Pouyssegur et al., 2006*). In addition, HIF-1 upregulates the c-met proto-oncogene, the chemokine receptor CXCR4 and the autocrine motility factor (AMF), all known to be implicated in metastasis and invasion (*Pennacchietti et al., 2003*).

### Effect of hypoxia on CAFs, TAMs and TANs in tumors

Even though the exact mechanisms underlying the CAF phenotype are still unknown, hypoxia-induced expression of specific genes which are determining this phenotype were described. Indeed, Caveolin-1 is inhibited by HIF-1 signalling. Loss of Caveolin-1 induces a CAF phenotype of mammary fibroblasts and enhances tumor growth when these cells were co-injected with breast cancer cells in mice (*Chiavarina et al., 2010*). Interestingly, HIF-1 $\alpha$ -deletion in fibroblasts before the onset of breast tumors accelerated tumor growth in mice (*Kim et al., 2012b*). HIF-1 $\alpha$ -induced expression of TGF $\beta$  and SDF-1 alone or in combination drives a CAF phenotype (*Toullec et al., 2010; Kim et al., 2012b*).

The infiltration of tumor tissue by myeloid cells, mainly macrophages and neutrophils, is also a hypoxia-driven process. Low oxygen levels induce IL-8 expression, a cytokine, which attracts neutrophils (*Kunz et al., 1999*). As tumor associated macrophages are polarized either to tumor-suppressive M1 macrophages or tumor-promoting M2-macrophages, one can hypothesize that macrophages change their phenotype depending on the hypoxic status of the tumor during cancer progression (*Mantovani et al., 2008*). At early stages, when intratumoral hypoxia is low, M1-like phenotype promotes anti-tumor immunity. With the progressive increase of hypoxia during cancer evolution, macrophages acquire the pro-tumor M2 phenotype driven by cytokines of the tumor stroma (*Saccani et al., 2006*).



**In conclusion**, the hallmark concept of *Hanahan & Weinberg* is a useful model to describe and understand the complexity of cancer disease. This awareness is essential for the development of efficient anti-tumoral therapies in the future (*Hanahan & Weinberg, 2011*).

During tumor initiation, promotion and progression, cancer cells need to acquire the following capabilities to survive, proliferate and finally to disseminate: sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis. These traits are supported by genome instability/ mutation and tumor-promoting inflammation, re-programming of cellular metabolism in tumor tissue as well as the escape of cancer cells from elimination by the immune system.

During the last decades, the tumor microenvironment appeared as a key driver of cancer progression as it paves the way for survival, proliferation and invasion of cancer cells. Intratumoral hypoxic conditions as well as tumor-secreted chemoattractants recruit endothelial, immune and stromal cells in their surroundings, which create a complex and dynamic signalling network to promote the disease.

*Hanahan & Weinberg* propose beside the validation of defined hallmarks, to further investigate the mechanisms of invasion and metastasis, the role of metabolic changes (aerobic glycolysis) as well as the effect of immune surveillance in tumors as future directions in cancer research. Further understanding of the tumorigenic process will help to refine existing cancer therapies and to discover new molecular targets for the development of alternative therapeutic approaches.

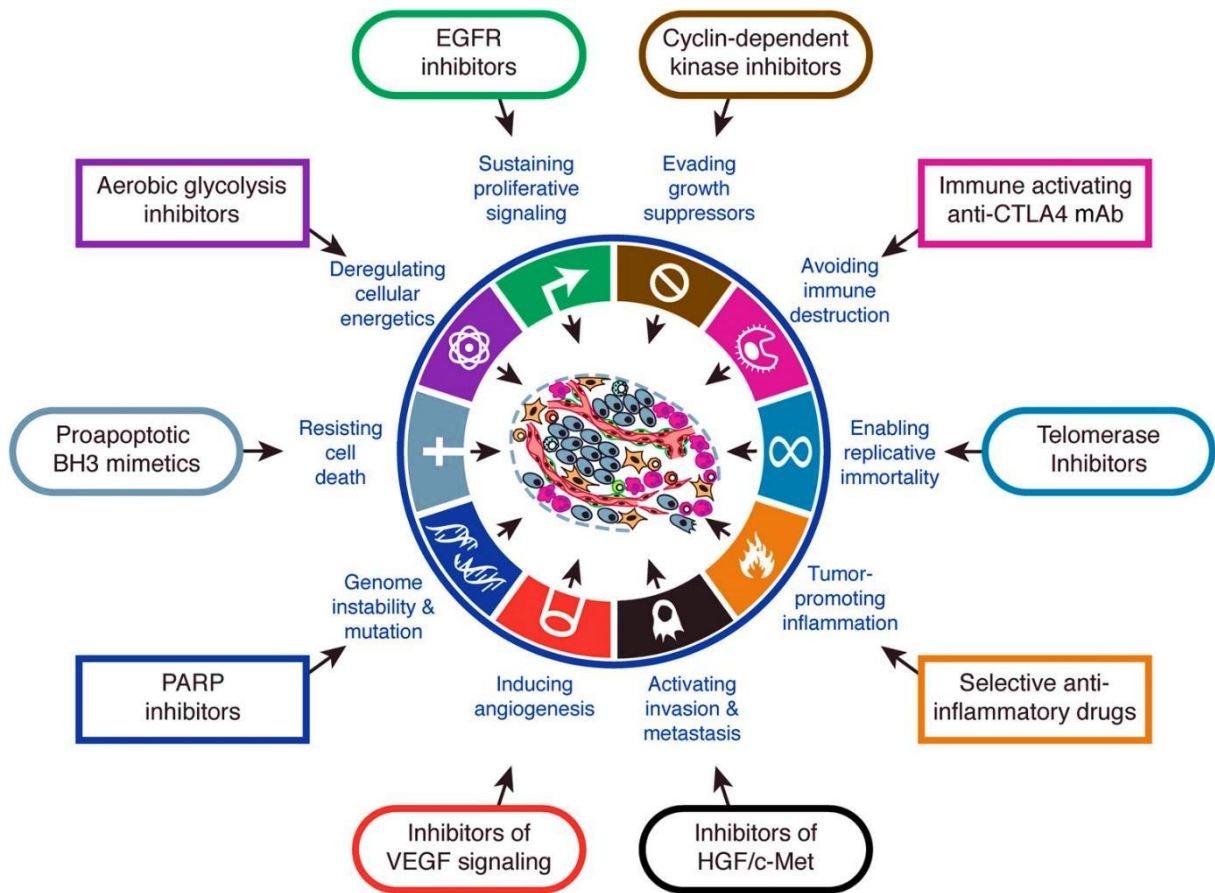


Figure 33: Therapeutic targeting of cancer hallmarks (Hanahan & Weinberg, 2011).

## **Chapter 4**      **Therapeutic targeting of cancer and its limits**

In applying the hallmark concept in cancer medicine, targeted therapies could be categorized according to their respective effects on inhibition of one or several hallmarks in the carcinogenic process. The therapeutic success, defined as inhibition of tumor growth and progression, will depend on the importance of the targeted hallmark in tumor formation. A myriad of drugs targeting each hallmark during cancer progression have been discovered and (pre-) clinically tested or are currently under development (Figure 33). Due to the considerable number of existing therapies, we will not list all of them. Instead, the following paragraph will focus on anti-angiogenic and anti-inflammatory therapies as well as their limits. Furthermore, the emerging link between cancer and the control of mRNA stability will be presented by focusing on expression and functional implication of TIS11 proteins in tumorigenesis and cancer-associated inflammation, emphasizing their potential role as tumor suppressors.



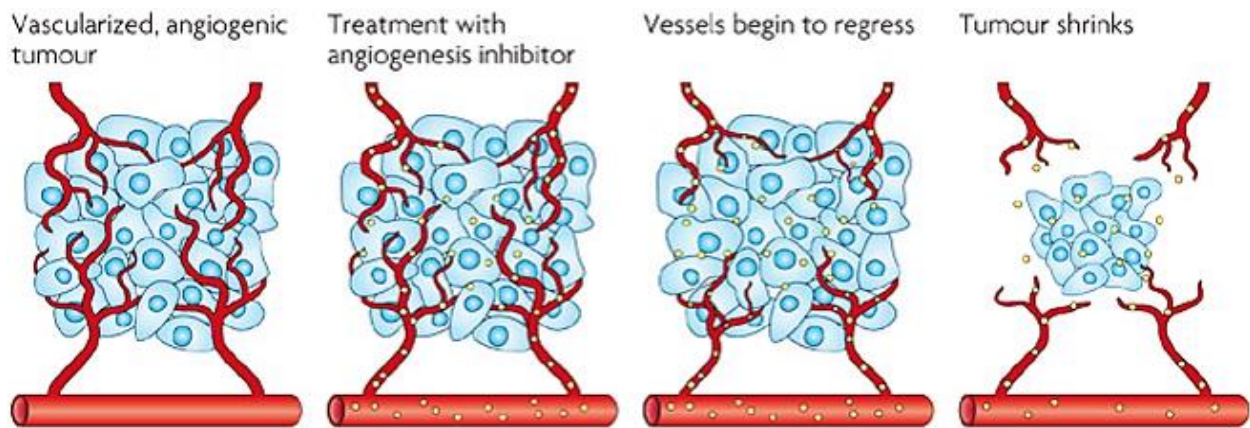


Figure 34: The basic concept of anti-angiogenic cancer therapies (Zetter, 2008).

Anti-angiogenic spectrum	Agent	Target	Cancer type	Phase of development
Single-target	Bevacizumab (avastin)	VEGF-A	Metastatic colorectal cancer, non-squamous non-small cell lung cancer, glioblastoma, metastatic renal cell carcinoma	Approved (U.S./E.U.)
	Cetuximab (erbitux)	EGFR	Squamous cell carcinoma of the head and neck, colorectal cancer	Approved (U.S./E.U.)
	Panitumumab (vectibix)	EGFR	Metastatic colorectal cancer	Approved (U.S./E.U.)
	Trastuzumab (herceptin)	HER-2	HER2-overexpressing breast and gastric cancer	Approved (U.S./E.U.)
Multi-targeted	Aflibercept (VEGF-trap; AVE0005; zaltrap)	VEGF-A, VEGF-B, PlGF	Metastatic colorectal cancer	Approved (U.S.), pending (E.U.)
	Axitinib (AGO13736; inlyta)	VEGFR-1, -2, -3, PDGFR, KIT	Advanced renal cell carcinoma	Approved (U.S./E.U.)
	Cediranib (AZD2171; recentin)	VEGFR-1, -2, -3, PDGFR, KIT	Metastatic colorectal cancer, recurrent glioblastoma, ovarian cancer, lung cancer	Phase III
	Indetanib (BIBF1120; vargatef)	VEGFR, PDGFR, FGFR	Non-small cell lung cancer and ovarian cancer	Phase III
	Pazopanib (GW786034; vortrient)	VEGFR-1, -2, -3, PDGFR, KIT	Advanced renal cell carcinoma, advanced soft tissue sarcoma	Approved (U.S./E.U.)
	Sorafenib (BAY439006; nexavar)	VEGFR-2, -3, PDGFR, Raf, KIT	Hepatocellular carcinoma, advanced renal cell carcinoma	Approved (U.S./E.U.)
	Sunitinib (SU11248; sutent)	VEGFR-1, -2, -3, PDGFR, KIT, FLT3, CSF-1R, RET	Gastrointestinal stromal tumor (GIST), advanced renal cell carcinoma, advanced or metastatic pancreatic neuroendocrine tumors	Approved (U.S./E.U.)
	Vandetanib (ZD6474; caprelsa)	VEGFR-2, EGFR, KIT, RET	Medullary thyroid cancer	Approved (U.S./E.U.)
Broad-spectrum	Vatalanib (PTK787; ZK222584)	VEGFR-1, -2, -3, PDGFR, KIT	Colorectal cancer	Phase III
	ABT-510	CD36	Head and neck cancer, melanoma, lymphoma, renal cell carcinoma, soft tissue sarcoma, non-small cell carcinoma	Phase II
	ATN-161	$\alpha\beta3$ and $\alpha\beta1$ integrins	Brain and central nervous system tumors, renal cell carcinoma	Phase II
	Angiostatin	ATP synthase, NG2, angiomin, $\alpha\beta3$ integrin, c-met, annexin II	Non-small cell lung cancer	Phase II
	Cilengitide	$\alpha\beta3$ and $\alpha\beta5$ integrins	Glioma, glioblastoma	Orphan designation (E.U.), Phase III
	Endostatin (endostar)	$\alpha5\beta1$ and $\alpha\beta3$ integrins, surface nucleolin, caveolin-1, glypicans-1 and -4, MMP-2, VEGFR-2	Non-small cell lung cancer, breast cancer	Approved (China), phase III/IV
	Lenalidomide (CC-5013; revlimid)	CRBN	Multiple myeloma	Approved (U.S./E.U.)
	pomalidomide (CC-4047)	CRBN	Multiple myeloma	Orphan designation (E.U.), Phase III
	Thalidomide (thalomid)	CRBN	Multiple myeloma	Approved (U.S./E.U.)
	TNP-470	MetAp2	Pancreatic cancer	Phase II

Table 7: Anti-angiogenic agents, their molecular targets and current indications for cancer therapy (Limaverde-Sousa et al., 2014).

#### 4.1. Fight against cancer: Focus on anti-angiogenic and anti-inflammatory cancer therapies

##### 4.1.1. Targeting tumor vasculature: Anti-angiogenic therapies

Nutrients and oxygen are essential for the survival of each cell, also for metabolically active tumor cells. *Judah Folkman* assumed in 1971 that starving tumors by inhibiting selectively intratumoral angiogenic processes could induce their death or render them “dormant” (Figure 34) (*Folkman, 1971*). With this idea, *Folkman* opened an intensive research on anti-angiogenic cancer therapies. Since then, numerous therapy approaches were investigated in pre- and clinical trials. These strategies are briefly summarized below.

Table 7 presents anti-angiogenic agents, their molecular targets and current indications for cancer therapy at a glance (*Limaverde-Sousa et al., 2014*). In general, anti-angiogenic therapies could be categorized in (1) single-target strategies, where monoclonal antibodies inhibit specifically one pro-angiogenic factor or receptor, (2) multi-target approaches including tyrosine kinase inhibitors (TKIs) and (3) broad-spectrum agents, which interfere not only endothelial cells (ECs) but also with tumor cells as well as components and cells of the tumor microenvironment such as integrins, leading to the inhibition of the downstream angiogenic phenotype.

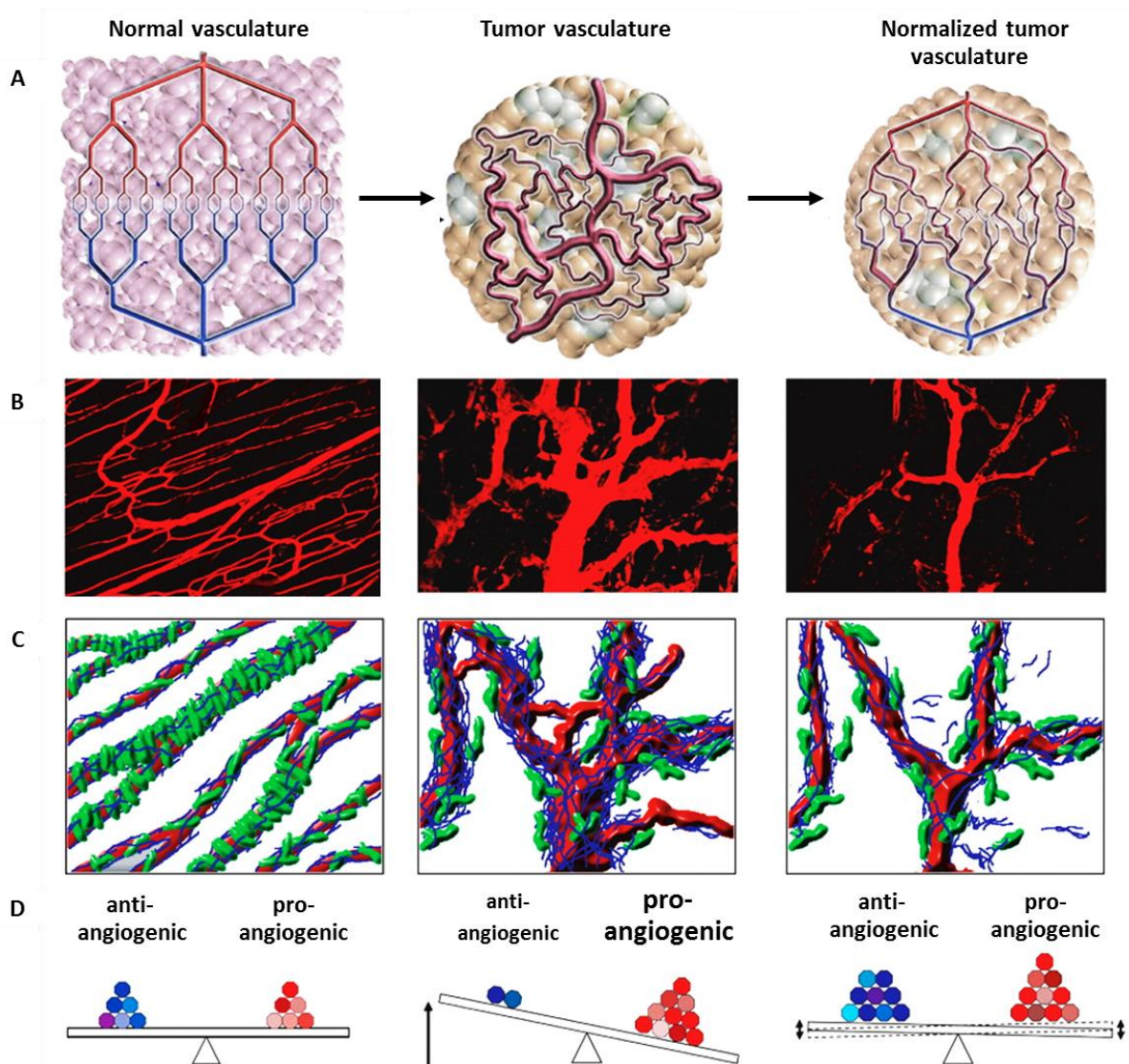


Figure 35: The hypothesis of vessel normalization (*Jain, 2005; Goel et al., 2011*). (A) Abnormal tumor vasculature, which could be initially normalized by anti-angiogenic therapies. (B) Dynamic vessel normalization after VEGFR2 blockade in a pre-clinical model of human colon cancer (day 0 in the middle, day 3 on the right). Normal vascularization of skeletal muscle is shown on the left as reference. (C) Schematic illustration of changing pericyte (green) and basement membrane (blue) coverage during vascular normalization. (D) Phenotypic alteration of the balance between pro- and anti-angiogenic factors through vessel normalization.

#### 4.1.1.1. Single-target angiogenesis inhibitors: the first generation

As VEGF is the key player in tumor angiogenesis and is produced by several cell types in the tumor tissue (tumor cells, fibroblasts, macrophages, ECs), anti-VEGF therapies were developed and indeed, delayed tumor growth after injection of an anti-VEGF monoclonal antibody was initially observed in glioblastoma and human colon carcinoma (*Kim et al., 1993; Warren et al., 1995*). Encouraged by these results, Genentech developed the humanized monoclonal antibody bevacizumab (Avastin), which is intensively tested in numerous clinical

trials.

However, the survival benefits of anti-VEGF monotherapy in phase III trials compromised the exciting results of the initial pre-clinical studies (*Jain et al., 2006; Carmeliet & Jain, 2011*). Interesting results were obtained in phase III clinical trials with bevacizumab combined with systemic chemotherapy. An improvement of patient outcome with this combinatorial approach instead of chemotherapy alone was observed (*Hurwitz et al., 2004*). Significant benefits for patients with metastatic colorectal cancer led the FDA to approve bevacizumab combined to chemotherapy as first-line treatment for this type of cancer in 2004.

Knowing that vessel regression is induced by anti-VEGF therapies and that efficacy of chemotherapy is dependent on efficient tumor blood flow, the observed effects for the combinatorial treatment were intriguing. To elucidate this paradox, the hypothesis of “vascular normalization” was proposed, which assume a normalization of the aberrant tumor vasculature and consequently of the tumor microenvironment induced by anti-angiogenic therapies (Figure 35) (*Jain, 2001; Jain, 2005*). Re-organized blood vessel network leads to a decrease of intratumoral hypoxia and acidosis as well as to a better sensitivity for radiotherapy and cytotoxic treatments, due to a uniform drug delivery. In addition, the improved organization of ECs and pericyte coverage of vessels reduce vessel permeability and consequently the interstitial fluid pressure in tumor tissue as well as the metastatic potential of the tumor. However, “vessel normalization” is transient and limited to a “time window” after the initiation of the therapy. Afterwards, normalization is lost maybe due to prolonged anti-angiogenic therapy, which causes resistance.

Additionally, other monoclonal antibodies inhibiting further single targets of pro-angiogenic signalling pathways, such as EGFR, PLGF or the angiopoietin-TIE2 axis, another key signalling pathway in angiogenesis, were designed and successfully tested (*Petit et al., 1997; Falcon et al., 2009*). For example, EGFR-positive A431 human epidermoid carcinoma cells are dependent on the pro-angiogenic factor VEGF *in vivo*. *Petit et al.* treated these cells with the C225 anti-EGFR neutralizing antibody *in vitro* and demonstrated a dose-dependent inhibition of VEGF protein expression. The treatment of pre-established A431 tumors led to a significant reduction of VEGF expression and tumor vascularization *in vivo*.

Unfortunately, bevacizumab monotherapy induced rapidly a resistance to the treatment (*Bergers & Hanahan, 2008*). It is thought that inhibition of tumor angiogenesis leads to increasing hypoxia and further activation of alternative pro-angiogenic pathways. To overcome this inconvenient, a second generation of anti-angiogenic molecules was developed.

#### **4.1.1.2. Second generation multi-targeted angiogenesis inhibitors: VEGF and related targets**

Within this second generation, the VEGF-trap or “Aflibertcept”, need to be mentioned. Aflibertcept functions as a “decoy” receptor for VEGF as it has a VEGF-binding domain. VEGF-trap prevents the interaction of VEGF-A, VEGF-B and PLGF with their respective receptor by sequestering these factors.

Tyrosine kinase inhibitors (TKIs), such as sunitinib or sorafenib, are of great importance among the second generation angiogenesis inhibitors (*Zhang et al., 2009*). Due to their small molecular weight and hydrophobicity, TKIs are able to cross the membrane and bind directly to the receptors or signalling molecules. These substances compete with ATP at the catalytic binding site of the VEGF receptor instead of interfering with the VEGF binding domain. TKIs are potential multi-target inhibitors and thereby would add benefit to therapy as they are also inhibiting other receptors, like EGFR and PDGFR. Beside differences in the mechanism of action, TKIs induce the same structural changes of tumor vasculature compared to first generation antibodies and expose to a risk of rapid development of resistance to therapy. In addition, monoclonal anti-VEGF antibodies and TKIs cause side-effects maybe due to their impact on the normal vasculature.

#### **4.1.1.3. Broad-spectrum of angiogenesis inhibitors: the next generation**

In contrast to the first and second generation of angiogenesis inhibitors, broad-spectrum agents are counteracting several angiogenic processes by targeting downstream components of the signalling pathways independently of the stimuli.

Angiostatin and Endostatin are two endogenous peptidic anti-angiogenic agents that inhibit efficiently tumor angiogenesis as well as tumor growth and metastasis (*O'Reilly et al., 1997; Lee et al., 2009*). Angiostatin is the 38 kDa C-terminal fragment of plasminogen which could interact with cell membrane proteins such as ATP synthase,  $\alpha v \beta 3$  integrin, c-met, annexin II, angiomin and the chondroitin sulfate proteoglycan NG2. These molecules mediate the anti-angiogenic activity of Angiostatin *in vitro*, including induction of ECs apoptosis, proliferation, migration and tube formation, and the *in vivo* inhibition of tumor growth and metastasis. However, the anti-tumoral mechanism of Angiostatin is poorly understood. *Lee et al.* demonstrated that Angiostatin targets mitochondria as it interacts with the ATP synthase. This enzyme is also present on the cell plasma membrane and seems to be involved in the internalization of Angiostatin *in vitro*. In the same study decreased expression of the pro-apoptotic protein Bcl-2 and increased apoptosis were observed when melanoma-bearing mice were treated with Angiostatin. Interestingly, in

these tumors a strong increase in the expression of the anti-angiogenic factor TSP-1 and a reduced infiltration of pro-inflammatory macrophages were observed (*Lee et al., 2009*).

Endostatin is the 20 kDa amino-terminal fragment of collagen XVIII, a proteoglycan mainly found in the basement membrane around blood vessels. The mechanism of action of Endostatin is still under debate (*Folkman, 2006*). However, Endostatin is known to inhibit VEGF- and FGF-induced proliferation, survival and migration of ECs. Murine models demonstrated an interaction of Endostatin with  $\alpha 5 \nu \beta 1$  and  $\alpha \nu \beta 3$  integrin and Calveolin-1 in ECs. Endostatin was also shown to associate with VEGFR2 and MMP2 (*Limaverde-Sousa et al., 2014*).

Cilengitide, derived from fibronectin, mimics the RDG motif, which is recognized by  $\alpha \nu \beta 3$  and  $\alpha \nu \beta 5$  integrins. Both integrins are present on the endothelium and on tumor cells. Cilengitide induces cell detachment and apoptosis of ECs and glioma cells (*Oliveira-Ferrer et al., 2008*).

These endogenous peptidic molecules interfere with ECs as they inhibit endothelial cell migration, tube formation and induce EC apoptosis, but also directly with tumor cells and cells of the tumor microenvironment. Thanks to their low toxicity, these compounds entered rapidly clinical trials.

#### **4.1.1.4. Indirect pharmacological inhibitors of angiogenesis**

Unlike the agents described so far, certain pharmacological inhibitors are indirectly inhibiting angiogenesis as they are targeting mainly oncogenes upstream of pro-angiogenic factors instead of directly affecting endothelial cells or pericytes in the tumor tissue. Therefore, it is not surprising that these molecules were originally designed as anti-proliferative drugs.

Trastuzumab, a monoclonal antibody against the epidermal growth factor HER2, displays anti-proliferative and anti-angiogenic effects (*Izumi et al., 2002*). Another important signalling pathway in tumor angiogenesis is the PI3K/AKT/mTOR-axis and its upstream regulator Ras. The inhibition of Ras or PI3K and mTOR simultaneously leads to vessel normalization (*Schnell et al., 2008; Qayum et al., 2009*). By inhibiting EGFR, another oncogene, using gefitinib (small TKI) for example, normalized vasculature was observed (*Qayum et al., 2009*).

As already mentioned, anti-angiogenic therapy could be beneficial in combination with chemotherapy. Cytotoxic chemotherapy aims to kill tumor cells and is normally applied in high doses at three-week intervals. One can anticipate that chemotherapeutics could also damage proliferating ECs of tumor vessels. Recent data showed a more efficient anti-angiogenic effect, when metronomic chemotherapy (short-term low-dose chemotherapy) was performed (*Maiti, 2014*). Indeed, low-dose treatment with chemotherapeutics increased intratumoral expression of the anti-angiogenic factor TSP-1 and promoted a subsequent shift to a normal vessel phenotype

(Jain, 2005). One could assume that this effect is even more pronounced in the presence of anti-angiogenic treatment as these molecules cause vessel normalization and therefore improve drug diffusion.

#### **4.1.2. Targeting the wound that never heals: Anti-inflammatory therapies**

The importance of cancer-related inflammation in tumor initiation and progression is well established. Inflammatory cytokines and chemokines produced by tumor cells and their environment promote cell proliferation, survival, differentiation of recruited ECs progenitors and immune cells, angiogenesis as well as invasion and metastasis. Therefore chronic inflammation is accepted as enabling hallmark during cancer progression (Hanahan & Weinberg, 2011).

Due to the constitutive production of pro-inflammatory factors, cancer patients develop a tumor-driven systemic inflammation reaction, which causes symptoms like progressive loss of weight and reduces the quality of life for these patients (Roxburgh & McMillan, 2014). Therefore, a novel tendency to use non-selective cheap drugs, like corticosteroids, non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin (inhibitors of COX-2 and subsequent prostaglandin synthesis) and statins (HMG-CoA reductase inhibitors with cholesterol-reducing and anti-inflammatory activities) to target systemic inflammation and related symptoms came up during the last years.

Beside this non-specific therapy approach, enormous efforts were spent on the development of anti-cancer drugs targeting specific chemokines or cytokines and their respective receptors. The next paragraph will highlight just a few of these therapeutic strategies. Antibodies against TNF $\alpha$  (Infliximab), the prototypical pro-inflammatory cytokine, and antagonists of its receptor (Etanercept) demonstrated therapeutic benefit (Harrison *et al.*, 2007). Siltuximab, a monoclonal anti-IL-6 antibody, is currently tested in clinical trials with first encouraging results (Mantovani *et al.*, 2008).

Depending on their phenotype, which is determined by chemokines in the tumor microenvironment, macrophages either act as tumor-promoting or tumor-inhibiting cells. “Re-educating” pro-tumoral tumor-associated macrophages (TAMs) to reject malignant cells could be a therapeutic approach. Hagemann *et al.* showed that the inhibition of NF- $\kappa$ B in TAMs activated their anti-tumoral phenotype and that those TAMs became cytotoxic for cancer cells (Hagemann *et al.*, 2008). The activation of the TNF receptor family member CD40 is a critical event in the development of tumor specific T cell immunity and may reverse the immunosuppressive tumor microenvironment. Recently, Beatty *et al.* tested an agonist CD40 antibody in combination with gemcitabine chemotherapy in a Phase II clinical trial and observed

tumor regression in pancreatic ductal adenocarcinoma patients (*Beatty et al., 2011*). Interestingly, a corresponding mice model revealed that this treatment caused the rapid infiltration of CD40-activated TAMs, resulting in tumor shrinkage. Antibodies against CCL2 (MCP-1), which regulates macrophages recruitment and tumor angiogenesis via its receptor CCR2, have shown promising effects in mice (*Popivanova et al., 2009*). In addition, the inhibition of the tumor angiogenesis-promoting chemokine CXCL8 by specific antibodies led to reduced angiogenesis and tumor growth in mice (*Huang et al., 2002*). Interestingly, Trabectedin, a chemotherapeutic agent, downregulates CCL2 and CXCL8 production in addition to its anti-proliferative effect (*Allavena et al., 2005*). Blocking the most frequently overexpressed chemokine receptor in cancer, the CXCR4 receptor using, small antagonists including bicyclam AMD3100, led to a reduction of primary tumor size and to anti-metastatic effects (*Richert et al., 2009*).

Tumor angiogenesis and cancer-associated inflammation are related processes during tumor progression as both of them are regulated by pro-angiogenic and pro-inflammatory factors like VEGF and interleukins (*Scaldaferri et al., 2009*). Therefore, it is not surprising that anti-angiogenic and anti-inflammatory therapies could positively interfere with each other. However, they are also sharing the same limits, which will be discussed in the next section.



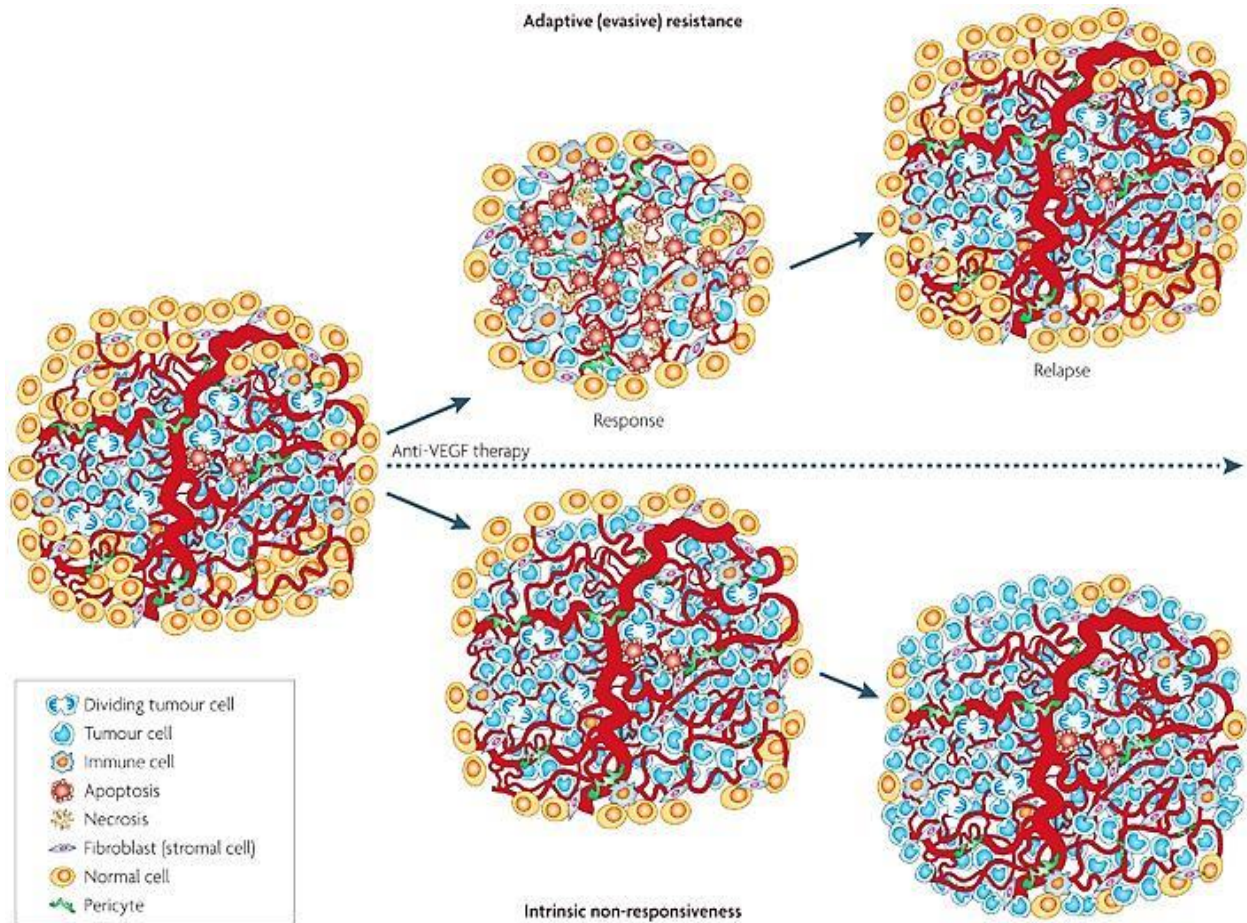


Figure 36: Adaptive (evasive) resistance and intrinsic non-responsiveness are the two modes of tumor resistance to anti-angiogenic cancer therapies (Bergers & Hanahan, 2008).

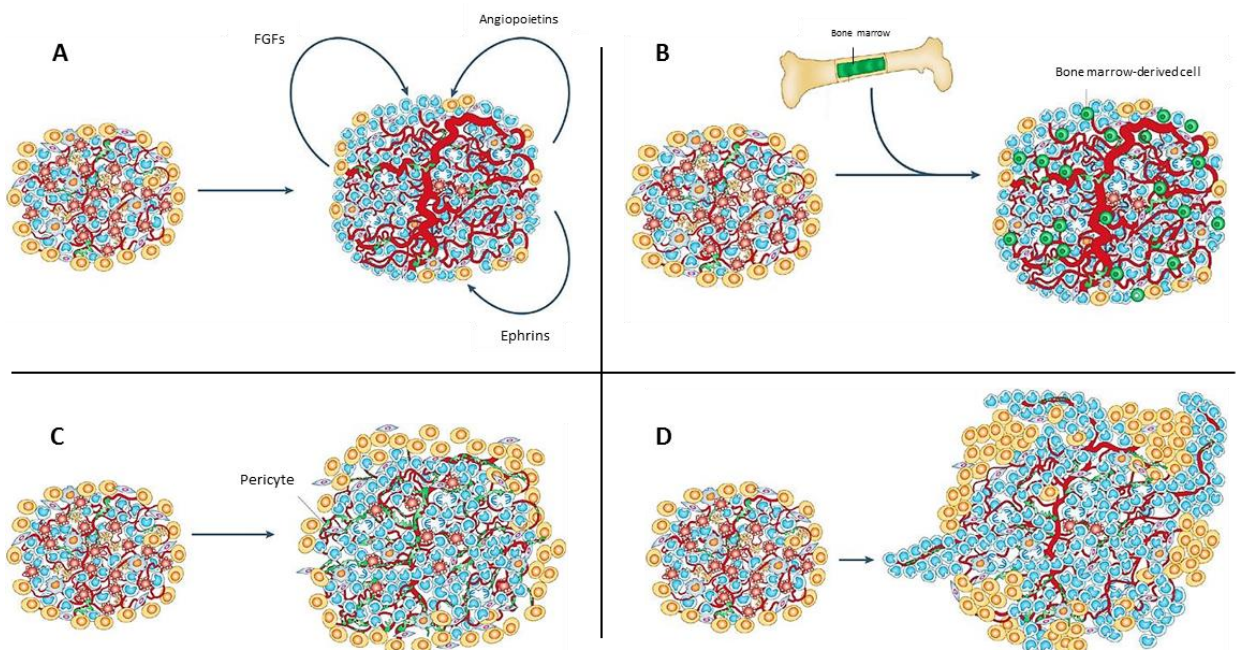


Figure 37: Mechanisms of adaptive resistance (Bergers & Hanahan, 2008). (A) Adaptive resistance by activation of alternative pro-angiogenic pathways. (B) Recruitment of bone marrow-derived cells to restore angiogenesis. (C) Protection of tumor vessels by increased pericytes coverage. (D) Increased invasiveness of tumor cells without angiogenesis.

## 4.2. Limits of targeted cancer therapies

Until now, most of the established hallmark-targeting cancer therapies are directed against specific molecular targets that are involved in one hallmark during tumor development (Figure 33) (Hanahan & Weinberg, 2011). In principle, this specific action has been considered to be beneficial as it is associated with a few off-target effects and low non-specific toxicity of the treatment. For example, it was assumed that anti-VEGF therapies would induce low toxicity as angiogenesis is almost quiescent in adults. However, clinical studies documented a number of side-effects and transitory responses, which means that tumors are initially responding to the treatment and then a relapse follows due to resistance mechanisms. Consequently, multi-target treatments are necessary.

In general, resistance to therapy could be categorized in adaptive (acquired/evasive) resistance (initial response to treatment followed by tumor re-growth in the presence of therapy) and intrinsic resistance (non-responsiveness of the tumor to the treatment) (Figure 36) (Bergers & Hanahan, 2008). Four mechanisms of adaptive resistance are described until now.

First, evasive resistance occurs when signalling pathways grouped in one hallmark are redundant. Inhibition of one circuit could be overcome by activation of an alternative one, causing tumor progression and relapse. *In vitro* studies revealed the important role of numerous other pro-angiogenic factors than VEGF, such as FGF, PDGF, angiopoietins and many more (Figure 37A) (Erber *et al.*, 2004; Casanovas *et al.*, 2005; Crawford *et al.*, 2009; Welte *et al.*, 2011). In cancer therapy, TKIs already address this problem even though they are not a cure-all. Second, tumor vascularization could be re-initiated by hypoxia-driven infiltration of bone-marrow derived cells (BMDCs) and pro-angiogenic monocytic cells (Figure 37B) (Ebos *et al.*, 2009a). BMDCs differentiate into ECs or pericytes and promote angiogenesis by physically incorporating into tumor vessels. Pro-angiogenic monocytes promote neovascularization by secreting cytokines, chemokines and growth factors (Lyden *et al.*, 2001; Shaked *et al.*, 2006). Third, increased pericyte coverage of vessels in the course of vascular normalization protects them from anti-angiogenic therapeutics, which are designed to target tumor-derived survival signals (Figure 37C) (Bergers & Hanahan, 2008). A fourth mechanism of adaptive resistance is that cancer cells could change their dependence on one hallmark towards another one during tumor progression (Figure 37D). This unexpected phenomenon was observed as consequence of anti-angiogenic treatment (Bergers & Hanahan, 2008; Ebos *et al.*, 2009b; Azam *et al.*, 2010). The inhibition of intratumoral vascularization heightened invasiveness and metastasis in pre-clinical models. Similar observations with an increased number of local metastasis were made after anti-angiogenic treatment of human glioblastoma (Norden *et al.*, 2008; Ellis & Reardon,

2009). The mechanisms that underlie the enhanced invasiveness of cancer cells in the presence of anti-VEGF treatment are still under investigation. However, it was shown that TKIs cause changes in a number of circulating factors such as G-CSF, SDF-1 and osteopontin, which are implicated in tumor progression (*Ebos et al., 2007*). Vessel integrity is crucial for the control of metastasis. Assuming that damage of the vasculature facilitates tumor cell extravasation from the primary tumor and intravasation at the metastatic site it appears plausible that anti-angiogenic therapy could enhance metastasis. Indeed, *Cooke et al.* observed in a murine breast cancer model that sunitinib (targets ECs and pericytes) increases the number of lung metastasis due to the upregulation of Twist and Snail as well as activation of the Met-receptor, all inducers of EMT (*Cooke et al., 2012*).

To overcome the inconvenience of acquired resistance, *Hanahan & Weinberg* suggest either to intensify the inhibition of one hallmark, for example by targeting all redundant signalling pathways, or to co-target several hallmarks of cancer progression. An elegant strategy would be the inhibition of common pathways between hallmarks.

A second mode of resistance is the pre-existing non-responsiveness of tumors to therapy. A minority of patients did not even show a transitory clinical benefit, when treated with bevacizumab, sorafenib or sunitinib (*Batchelor et al., 2007*). One could assume that these patients had already developed an evasive mechanism of resistance in response to selective pressure of the tumor microenvironment before starting the therapy. Furthermore, it could be hypothesized that some tumors are hypovascularized, for example pancreatic ductal adenocarcinoma (*Sofuni et al., 2005*). A last possible explanation for intrinsic resistance could be the independence of tumor cells from sprouting angiogenesis as they could use alternative mechanisms, such as vessel co-option and others, which does not require the secretion of pro-angiogenic factors (*Leenders et al., 2004*).

Based on the raising importance of the tumor microenvironment and associated signalling crosstalk during cancer progression, tumor stroma seems to be a suitable target in cancer therapy. *Barcellos-Hoff* and colleagues propose in addition to standard cancer treatment, the therapeutic reversion of the cancer niche to normal tissue microenvironment as a key for long-term therapy success (*Barcellos-Hoff et al., 2013*). This approach implicates the normalization of the tumor extracellular matrix as its altered composition and organization limit drug penetration and intratumoral dissemination (*Bissell & Radisky, 2001; Choi et al., 2013*). This concept remains challenging as anti-stroma therapies failed in clinical trials, maybe due to the dynamic adaptation of the tumor microenvironment during carcinogenesis.

Given the heterogeneous clinical benefit of patients receiving especially anti-angiogenic therapies, there is a need for predictive biomarkers to determine the response to therapy and the outcome. To date, no standardized markers are available. Approaches using circulating angiogenic factors or imaging are emerging but need further validation (*Wehland et al., 2013; Vasudev & Reynolds, 2014*). Circulating IL-6 was proposed to be a potential biomarker for colorectal and lung cancer suggesting the benefit of anti-inflammatory cancer therapy (*Heikkila et al., 2008*). In addition, a recent review of clinical studies present in the literature found that IL-6 is a potential predictive and prognostic biomarker for VEGF-targeted therapy in renal cell carcinoma (*Funakoshi et al., 2014*).

#### **4.3. Emerging link between regulation of mRNA stability and cancer**

Given the above described limitations of existing anti-cancer therapies, it seems worthwhile to develop novel multi-target therapy strategies. Since gene expression is altered in cancer due to somatic mutations as well as to epigenetic and post-transcriptional mechanisms and since the last two mentioned processes are genome-/transcriptome-wide regulatory mechanisms, one can anticipate that they represent relevant targets for the development of novel multi-target anti-cancer therapy approaches. Post-transcriptional control of gene expression implicates, among other mechanisms, the modulation of mRNA stability and/or their translational potential. In the cytoplasm, mRNA stability will determine the translation rate and regulate accordingly the final protein level and subsequent cellular response. As already introduced, AU-rich elements (AREs) are important *cis*-acting elements located in the 3'-untranslated region of mRNAs, which are recognized by *trans*-acting factors (ARE-binding proteins) and implicated in mRNA turnover. ARE-mediated mRNA decay negatively regulates the expression of several key genes in cancer such as cytokines, growth and inflammatory factors or oncogenes. The importance of AREs in cancer is underscored by the fact that the removal of the ARE in the 3'UTR of *c-fos* transforms the proto-oncogene into an oncogene (*Miller et al., 1984*). Most importantly, mRNA stability is often deregulated in cancer and inflammatory diseases, resulting in aberrant stabilization and overexpression of master mediators. Therefore, ARE-binding proteins, such as TIS11 proteins, may be involved in various cellular pathways of tumor development. Once again, most studies addressing the potential role of TIS11 proteins in cancer have been done for TTP, the best known member of the TIS11 protein family. The following section will discuss the role of this protein family in cancer.

#### 4.3.1. Expression of TIS11 proteins in cancer cell lines

*Carrick & Blakeshear* determined the expression of all three TIS11 proteins in a large panel of human cancer cell lines (NCI 60) by RT-QPCR (*Carrick & Blakeshear, 2007*). This screening showed a remarkable heterogeneity concerning the expression levels of the three members, TTP, TIS11b and TIS11d, within one normal organ as well as in different cancer cell lines. TTP mRNA, for example, is less abundant in MDA-MB-231 breast cancer cells compared to TIS11b and TIS11d. On the contrary, all three member transcripts are nearly undetectable in MCF 7 breast cancer cells. By contrast, *Griseri et al.* demonstrated a high TTP and low TIS11b protein level in MCF 7 breast cancer cells (*Griseri et al., 2011*). Nevertheless, from a general point of view, TIS11 proteins seem to be underexpressed in many cancers including colon cancer, breast cancer, malignant gliomas, cervical cancer and hepatocellular carcinoma as well as in tumors of lung, ovary, prostate and thyroid (*Brennan et al., 2009; Sanduja et al., 2009*). Interestingly, within a cohort of 251 breast cancer patients, *Brennan et al.* revealed a negative correlation between TTP mRNA level and tumor grade, indicating that the more advanced tumors showed weak TTP expression (*Brennan et al., 2009*). In addition, low TTP levels were associated with high VEGF mRNA expression in those patients. Regarding the disease outcome, low TTP mRNA levels indicated poor patient survival. Altogether, these correlations revealed TTP as a negative prognostic marker in breast cancer.

So far, few studies have addressed the question of the mechanisms which are responsible for the suppressed expression of TIS11 proteins in cancer.

First, *Sohn et al.* showed that epigenetic mechanisms were able to decrease TTP expression (*Sohn et al., 2010*). The methylation of one TGF $\beta$  response element located in TTP promoter efficiently reduced TTP expression in hepatocellular cancer cells. This epigenetic modification led to the same effect in hepatocellular carcinoma patients. Second, miR-29a, which is overexpressed in metastatic human breast cancer, reduces TTP expression post-transcriptionally (*Gebeshuber et al., 2009*). A third explanation for low TTP expression could be the degradation of TTP mRNA. As already mentioned, TTP mRNA contains AREs in the 3'UTR which could be recognized by TTP itself, thus forming an autoregulatory loop, or by other ARE-binding proteins (*Brooks et al., 2004; Tchen et al., 2004; Pullmann et al., 2007*). Fourth, *Griseri et al.* described a single nucleotide polymorphism in the TTP gene of a triple negative breast cancer cell line which leads to a lower translation rate of the TTP transcript due to a low abundance of TTP mRNA in polysomes, thus resulting in weak TTP protein levels (*Griseri et al., 2011*). In addition, the authors described a positive correlation between this genetic polymorphism and the resistance to Herceptin/Trastuzumab therapy of HER<sup>+</sup> breast cancer patients. Furthermore, TTP expression

and activity is regulated by post-translational modifications, mainly hyperphosphorylation through several signalling pathways. Constitutive activation of ERK results in proteasomal TTP degradation in melanoma cells (*Bourcier et al., 2011*). On the contrary, phosphorylation of TTP by p38MAPK-activated protein kinase 2 (MK2) led to protein stabilization. However, this post-translational modification reduced TTP affinity for ARE-containing mRNAs in macrophages (*Hitti et al., 2006*). In addition, suppression of TTP in head and neck cancer or its p38MAPK-mediated phosphorylation increases the levels of invasion-promoting factors, such as IL-6, MMP2 and MMP9 (*Van Tubergen et al., 2011*).

The suppression of TTP protein expression in many tumors led to the hypothesis that this protein family could be involved in tumorigenic phenotypes.

#### 4.3.2. The impact of TIS11 proteins on cancer hallmarks

Recent studies revealed an increasing number of TIS11 protein targets which are important factors in the different steps of tumor progression, supporting the idea that underexpression of the TIS11 protein family may lead to overexpression of tumor-promoting factors (Figure 38) (*Ross et al., 2012*).

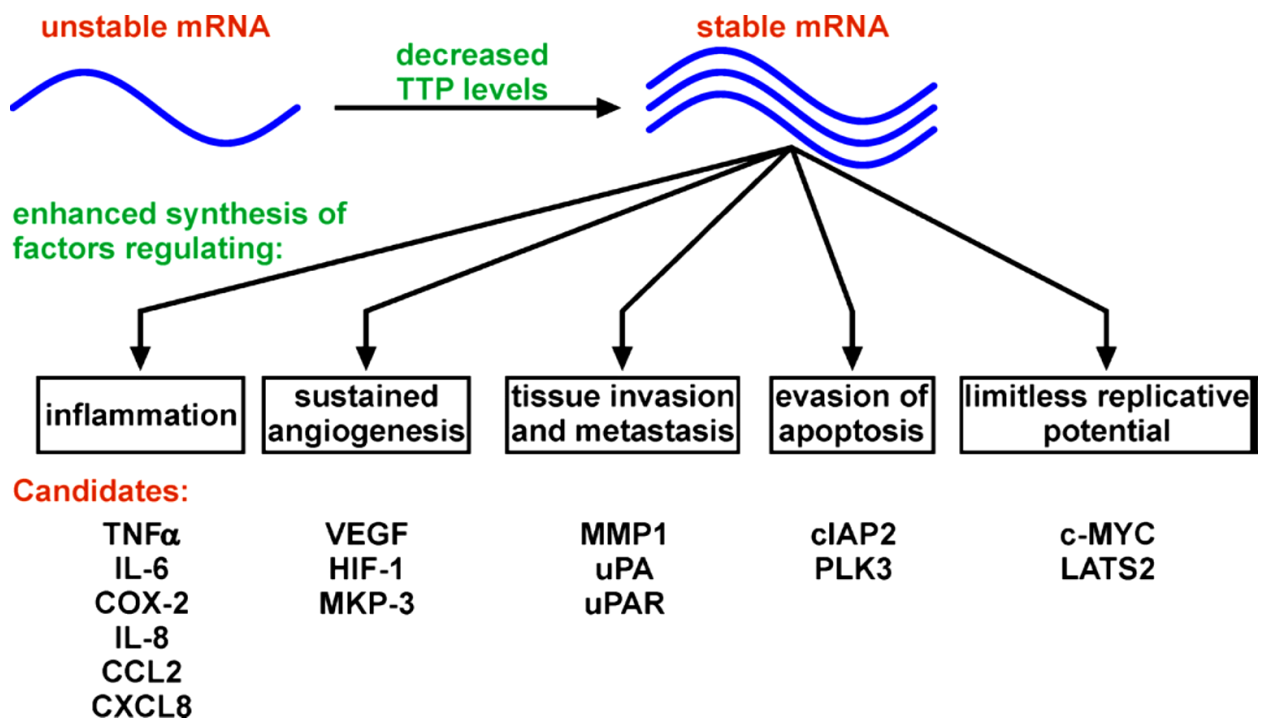


Figure 38: Network of TTP-mediated post-transcriptional control of mRNAs encoding oncogenic and tumor-promoting factors (*Ross et al., 2012*).

A negative correlation between TTP and tumor grade was found in breast cancer patients, suggesting that TIS11 proteins could be tumor suppressors (*Brennan et al., 2009; Al-Souhibani et al., 2010; Griseri et al., 2011*). In addition, low TTP expression is associated with monocytes/macrophages infiltration and correlated with poor outcome (*Milke et al., 2013*).

### Cancer-associated inflammation

**TNF $\alpha$** , the prototypic inflammatory cytokine and main mediator of cancer-associated inflammation, acts as a tumor-promoting factor and is associated with poor prognosis (*Leek et al., 1998; Wu & Zhou, 2010*). TNF $\alpha$  expression is tightly regulated, through ARE-mediated control of mRNA stability. *In vitro*, TTP binds to TNF $\alpha$  mRNA and promotes its degradation (*Carballo et al., 1998; Lai et al., 1999*). TTP-KO mice exhibit a systemic inflammatory syndrome due to abnormal stabilization of TNF $\alpha$  mRNA leading to elevated TNF $\alpha$  circulating levels (*Taylor et al., 1996; Carballo et al., 1998*). The cyclooxygenase 2 (**COX-2**), an enzyme involved in the synthesis of inflammation-regulating prostaglandins, is overexpressed in cancer (*Simopoulos, 2002*). Recent data suggested that loss of TTP in colon cancer promotes COX-2 expression and could contribute to colon tumorigenesis (*Young et al., 2009; Cha et al., 2011*). Ectopic expression of TTP antagonized high COX-2 levels by targeting AREs in the 3'UTR of COX-2 mRNA, thus attenuating cell proliferation of colon cancer cells (*Cha et al., 2011*).

**IL-6** as well as other cytokines are survival and growth factors supporting cancer progression and tumor-related inflammation. Interestingly, *Zhao et al.* brought evidence for TTP-mediated IL-6 mRNA destabilization in TTP-deficient mice-derived embryonic fibroblasts (*Zhao et al., 2011*).

### Sustained angiogenesis

As already mentioned, sustained angiogenesis is a key event in tumor progression which is mainly induced by hypoxia-driven VEGF production. HIF-1 $\alpha$  protein is stabilized by intratumoral hypoxia or oncogenes and is frequently overexpressed in human cancer (*Semenza, 2002*). Our group has shown recently that TTP could also destabilize HIF-1 $\alpha$  mRNA in endothelial cells in response to hypoxia, suggesting that HIF-1 $\alpha$  mRNA levels are regulated by a negative feed-back loop aiming at preventing excessive HIF-1 $\alpha$  protein accumulation (*Chamboredon et al., 2011*). High expression of HIF-1 $\alpha$  mRNA was described in colorectal and gastric carcinomas and was associated with induced angiogenesis due to increased VEGF levels (*Furlan et al., 2007; Ma et al., 2007*). HIF-1 $\alpha$  was therefore proposed as marker for poor disease outcome. In colon cancer, a negative correlation between TTP and VEGF expression was

observed (*Cha et al., 2011*). *In vivo* studies performed by *Essafi-Benkhadir et al.* showed decreased vascularization in tumors derived from TTP-expressing breast cancer cells (*Essafi-Benkhadir et al., 2007; Lee et al., 2010*).

In malignant glioma cells, overexpression of TTP limited tumor growth due to TTP-induced destabilization of VEGF and IL-8 (*Suswam et al., 2008*). Interestingly, TTP expression was detected in both normal and malignant brain tissue, whereas hyperphosphorylated TTP was predominant in gliomas, suggesting that this post-translational modification via p38/MAPK attenuates TTP activity in these tumors. *Bourcier et al.* injected melanoma cells in IL-8-immunized mice (*Bourcier et al., 2011*). The authors observed a delay of tumor appearance and a nearly 50 % reduction of tumor incidence indicating the importance of IL-8 in melanoma development. Additional experiments studying the molecular mechanism of this *in vivo* observation revealed that restored TTP expression in melanoma cells led to the TTP-mediated degradation of IL-8 mRNA *in vitro*. Furthermore, constitutive TTP expression inhibited proliferation, enhanced apoptosis and prevented autophagy in these cells. Interestingly, sorafenib increased TTP expression in melanoma cells. As expected a concomitant decrease of CXCL8 mRNA was detected. These results might explain the anti-angiogenic effect of sorafenib cancer treatment.

### Tissue invasion and metastasis

Closely connected to angiogenesis, invasion and metastasis are essential steps during cancer progression. TTP suppression seems to be associated with higher invasiveness of breast cancer cells (*Gebeshuber et al., 2009*). In this study, low TTP expression was associated with miR-29a/Ras-induced repression of TTP mRNA in human breast cancer. Ectopic TTP expression could reduce invasion capacities of MD-MB-231 cells. Another study showed a lower expression of TTP in MDA-MB-231 cells, a highly metastatic breast cancer cell line, compared to MCF10, a non-invasive one (*Al-Souhibani et al., 2010*). In addition, the authors demonstrated that the restoration of TTP expression in MDA-MB-231 cells reduces the mRNA stability of MMP1, uPA and uPAR, key factors involved in invasion and metastasis, by directly interacting with the 3'UTR of these mRNAs. Results from *Al-Souhibani et al.* further provided that the inhibition of miR-29a restored TTP expression and the balance between TTP and HuR in breast cancer cells. This led to the normalization of CXCR4 mRNA turnover, the most frequently overexpressed chemokine receptor in cancer (*Al-Souhibani et al., 2014*). Low CXCR4 expression was associated with reduced invasiveness of highly metastatic MDA-MB-231 cells.



### Evasion of apoptosis

Less data are available concerning TTP-mediated regulation of apoptosis. Based on several potential targets of TTP that were found by transcriptome-wide studies, one could speculate that TTP may influence apoptotic signalling (*Stoecklin et al., 2008; Al-Souhibani et al., 2010*). TTP expression is lost in human cervical cancer (*Sanduja et al., 2009*). Cervical cancer is induced by the infection with high-risk human papillomavirus (HPV) type 16 or 18. The expression of the early viral genes E6 and E7 neutralizes the tumor suppressors Rb protein and p53 protein and activates the telomerase enzyme via the action of the ubiquitin ligase E6-associated protein (E6-AP). Overexpression of TTP in HPV18-transformed HeLa cervical cancer cells led to the TTP-induced degradation of E6-AP and the further stabilization of p53 as well as inactivation of the telomerase. Finally, cell proliferation was inhibited due to the activation of cellular senescence (*Sanduja et al., 2009*). In a normal cellular context, *Johnson et al.* demonstrated that TTP induces apoptosis in 3T3 fibroblasts (*Johnson & Blackwell, 2002*).

### Limitless replicative potential

Impact of TIS11 proteins on proliferation of cancer cells is sparsely described. Recently, *Lee et al.* showed that TTP regulates mRNA stability of E2F1, a transcription factor involved in cell cycle and often highly expressed in cancer (*Lee et al., 2014*). Ectopic expression of TTP inhibited significantly the proliferation rate of prostate cancer cells by destabilizing E2F1. Restored TTP levels in HeLa cells reduced cell proliferation rate by 50% (*Brennan et al., 2009*). The same observations were made for MDA-MB-231 breast cancer cells (*Griseri et al., 2011*).

### First attempts using ARE-binding proteins in anti-cancer therapy

Based on the fact that ARE-BPs are potential tumor suppressors which regulate various short-lived mRNAs implicated in different hallmarks of cancer, some anti-cancer therapy approaches were pre-clinically investigated.

Patients with non-small cell lung carcinoma develop an immune response caused by the abnormally high expression of the mRNA-stabilizing protein HuD (another member of HuR protein family). Elevated levels of anti-HuD antibodies were associated with a better prognosis for these patients. Interestingly, immunized mice against HuD showed a significant inhibition of HuD-overexpressing neuroblastoma growth and an increased intratumoral infiltration of CD3+ lymphocytes compared to control animals (*Carpentier et al., 1998*). This study provided the first example of treating deregulated expression of ARE-BPs by a neutralizing antibody.

*Stoecklin et al.* proposed another strategy in 2003 (*Stoecklin et al., 2003*). Knowing that IL-3, a frequently overexpressed cytokine in cancer, is a target of TTP, they hypothesized that TTP could downregulate IL-3 mRNA and impairs tumor growth. Indeed, restored TTP expression in v-H-ras-dependent mast cells led to a significant delay in tumor growth *in vivo*. Very interestingly, tumor escape after four weeks was due to loss of TTP expression.

Ras or Raf overexpression maintains permanent activation of the ERK pathway, thus inducing uncontrolled proliferation of cancer cells. *In vitro* activation of ERK in Raf1-ER transformed fibroblasts induced an upregulation of TTP expression and led to VEGF mRNA destabilization (*Essafi-Benkhadir et al., 2007*). In line with *Stoecklin et al.* studies, implantation of these cells and activation of TTP expression by Doxycycline treatment inhibited tumor growth and tumor angiogenesis as measured by low VEGF levels and decreased microvessel density.

Finally, our own group recently developed an anti-tumoral therapy based on the intratumoral injections of a cell-permeable TIS11b fusion protein (*Planel et al., 2010*). As VEGF is an important target of TIS11b, it was hypothesized that TIS11b fused to a small cell-penetrating peptide (9 N-terminal arginine residues (R9)) would restore low TIS11b abundance in tumor cells and would inhibit tumoral angiogenesis. Indeed, treatment of pre-established subcutaneous Lewis lung carcinoma (LLC) tumors decreased significantly tumor growth, tumor vascular density and VEGF expression. Antibody array analyses of tumor protein extracts revealed an additional inhibition of FGF-1, EGF, IL-1 $\alpha$ , IL-6, IL-12 and TNF $\alpha$  in these tumors. This study proposes for the first time a novel multi-target cancer therapy based on an ARE-binding protein targeting key factors of tumor angiogenesis and cancer-associated inflammation.



**In conclusion,** during the last decades, a myriad of targeted cancer therapies were developed and clinically tested or are under current investigation. The seminal paper of *Judah Folkman*, showing the dependence of tumors on angiogenesis, and the work of *Rudolf Virchow*, demonstrating infiltration of tumor tissue by inflammatory cells, initiated this intensive research (*Folkman, 1971; Balkwill & Mantovani, 2001*). The entry of angiogenesis and inflammation inhibitors in clinical trials was a milestone in cancer therapeutics and some of these agents became standard-of-care therapies for certain cancer types during the last years. However, unexpectedly, adaptive or intrinsic therapy resistances attenuated the beneficial effects of these therapeutic strategies. Therefore, *Hanahan & Weinberg* advise combinatorial therapies targeting several crucial hallmarks in cancer progression, in addition to exploring novel strategies. During the last years, an emerging link between mRNA stability and cancer has appeared. The alteration of this tightly controlled post-transcriptional mechanism, mainly due to unusual ratios between stabilizing and destabilizing RNA-binding proteins, leads to the aberrant expression of tumorigenic factors. As described in this chapter, expression of mRNA destabilizing proteins, like members of the TIS11 family, is negatively correlated with aggressiveness of several human tumors. Therefore, their role as tumor suppressors makes them attractive targets in cancer therapy. As TIS11 proteins are involved in nearly every step of the tumorigenic process, a multi-target effect of such a treatment could be anticipated.



## Chapter 5 Breaking through the other side: Cell penetrating peptides

Efficient internalization of therapeutic agents into target cells is critical for successful cancer treatment. However, since the plasma membrane of the cell functions as a physiological barrier, the application of information-rich macromolecules, such as DNA and proteins, to therapies has been restricted. A second restriction is the specific recognition of cancer cells for the delivery of therapeutic macromolecules *in vivo*, without affecting surrounding healthy cells. Various pharmaceutical carriers, like nanocapsules, nanospheres, liposomes, micelles, lipoproteins, cell ghosts and polymers have been generated for the delivery of diagnostic and therapeutic agents. These carriers remain in the circulation long enough to accumulate passively in tumors more than in normal tissue. This phenomenon is called Enhanced Permeability and Retention effect (EPR). Tumors secrete high concentrations of VEGF (also Vascular permeability factor) to induce angiogenesis. The new formed blood vessels are abnormal in their structure and function leading to a leaky vasculature allowing circulating pharmaceutical carriers to enter tumor tissue (*Maeda et al., 2000*). Unfortunately, these carriers do not deliver their cargo specifically to target cells or subcellular compartments.

Another approach in drug delivery is the use of vector molecules, sugar moieties and peptides. Based on their active transport into target cells, these carriers could overcome the cell membrane barrier more efficiently resulting in intracellular delivery of their cargo. However, endocytotic internalization of these constructs is often restricted by insufficient endosome release, limited diffusion or degradation.

The discovery of cell penetrating peptides (CPPs) or protein transduction domains (PTDs) in the 70s of the last century was a milestone in drug delivery and paved the way for the development of biologically active macromolecular carriers with enhanced cellular entry properties to target tumor tissue and intracellular compartments. This chapter aims at briefly introducing the application of CPPs in cancer therapy.

## 5.1. Classes of CPPs

In 1988, *Frankel & Pabo* discovered the first CPP, based on the observation of spontaneous internalization and nuclear translocation of the transcription-transactivating (TAT) protein of HIV-1 (*Frankel & Pabo, 1988*). Shortly after, the cellular uptake of the *Drosophila melanogaster*-derived peptide penetratin (pAntp) was demonstrated (*Derossi et al., 1994*). *Futaki et al.* described in 2001 the cellular uptake of proteins due to polyarginine sequences. Efficiency of internalization was correlated with number of arginine residues. Six to eight arginines were optimal. Proteins containing this CPP entered rapidly into treated cells without causing cytotoxic effects (*Futaki et al., 2001*). Since then, more than 100 peptidic sequences capable to internalize into cells were discovered in various species, including Transportan (chimeric peptide derived from the N-terminal fragment of the galenin neuropeptide fused to the mastoparan peptide), pVEC (peptide derived from the murine Vascular endothelial cadherin), amphipathic peptide (MAP), signal sequence-based peptides and synthetic arginine-enriched sequences (Table 8).

Peptides	Origin	Structure	Proposed mechanism
TAT <sub>(43-58)</sub>	HIV-transcriptional activator	Random coil/PPII helix	Direct penetration, pore formation
Penetratin (pAntp) <sub>(43-58)</sub>	Antennapedia <i>Drosophila melanogaster</i>	Amphipathic, $\alpha$ -helical/ $\beta$ -sheet (higher concentrations)	Direct penetration, endocytosis
Polyarginines	Model peptide (chimeric)	Random coil, $\alpha$ -helical	Direct penetration, endocytosis
pVEC	Murine vascular endothelial cadherin	Amphipathic, $\beta$ -sheet	Direct penetration, transporter-mediated
Pep-1	Chimeric	Amphipathic, $\alpha$ -helical	Direct penetration, pore formation
Transportan	Galanin-mastoparan (chimeric)	Amphipathic, $\alpha$ -helical	Endocytosis, direct penetration
MAP	Model amphipathic peptides (chimeric)	Amphipathic, $\alpha$ -helical	Multiple mechanisms

Table 8: Examples of CPPs, their origin, structure and proposed mechanism of cellular uptake (*Koren & Torchilin, 2012*).

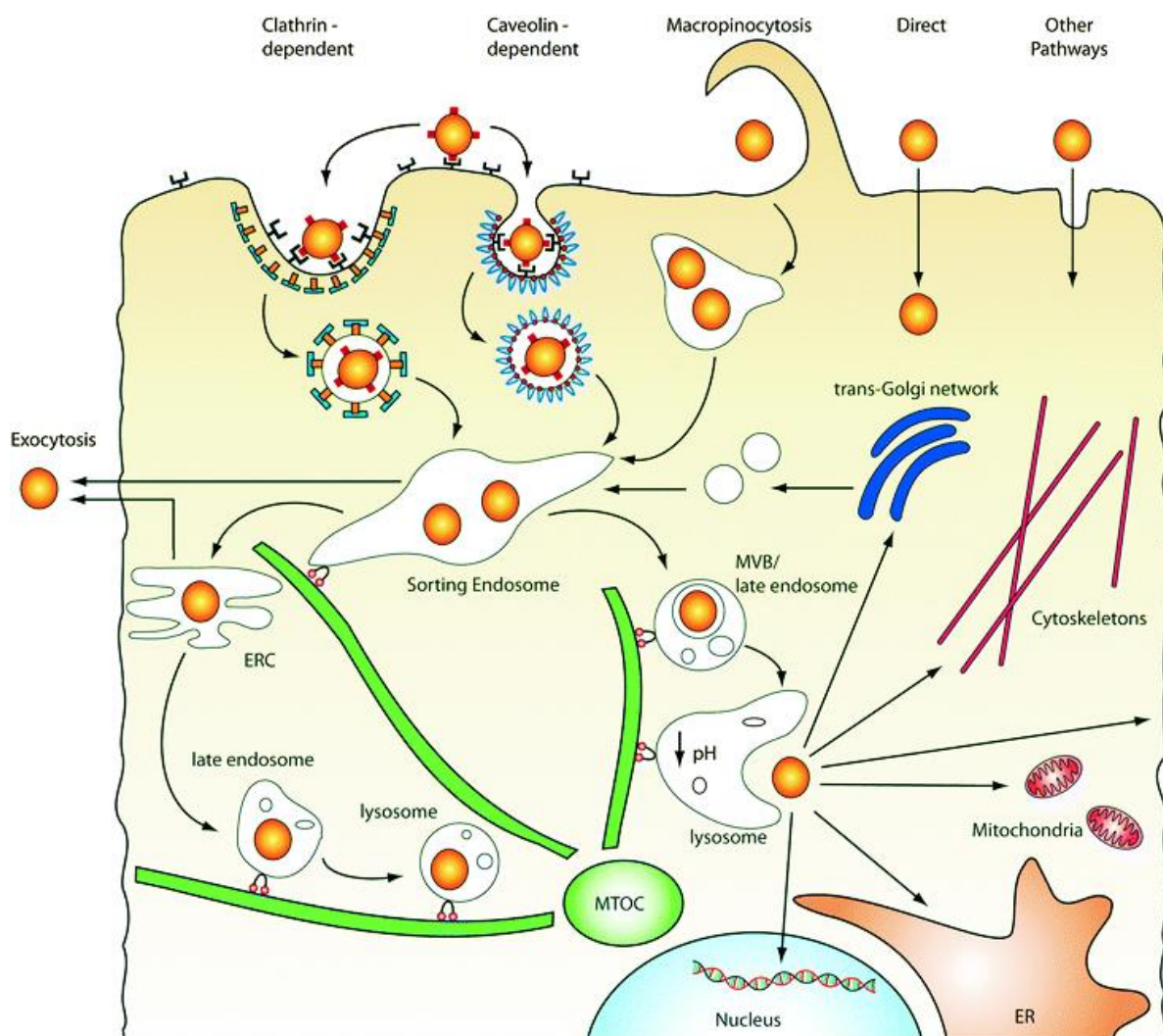
CPPs are in general peptides of around 30 amino acids, derived from synthetic or natural proteins or chimeric sequences. Interestingly, peptides containing unnatural amino acids offer enhanced carrier stability and/or efficiency (*Farrera-Sinfreu et al., 2007*). Due to the obvious heterogeneity, a unique classification of CPPs is difficult. However, based on their origin, CPPs could be classified in two categories, one which requires a chemical link with the cargo and the second forming non-covalent, stable complexes with the cargo. While covalent conjugates with CPPs are recommended because of several advantages for *in vivo* studies, they present the risk of changing the biological activity of the cargo. Non-covalent complexes are based on CPPs

harbouring amphipathic sequences. This type of strategy, mainly developed for oligonucleotide delivery, was described for CPPs, such as polyarginines (*Kim et al., 2006*). Furthermore, structure characteristics of CPPs such as polycationic (clusters of polyarginine) or amphipathic structures serve for their classification.

## 5.2. Different cellular uptake mechanisms of CPPs

The mechanisms of CPP internalization are not completely understood. As sequence homology of proteins in which CPPs were discovered is weak, except for the CPP motif, and CPP uptake is not cell- or tissue-specific, one exclusive internalization pathway is not applicable. Historically, direct translocation of CPPs was described as a major mechanism of internalization. Indeed, CPPs fused to small cargos (peptides fewer than 30-40 amino acids) can enter cells rapidly by transduction. However, the direct translocation process cannot explain the uptake of CPP-cargo complexes of large sizes. Currently, CPPs are considered to be mainly associated with endocytosis-mediated cellular uptake, but the underlying mechanisms remain unclear. Most studies addressing this question used fluorescein-labelled CPPs. In addition, mechanisms independent of the endosome pathway involving the transmembrane potential were described (*Thoren et al., 2003*). However, the first interaction between the CPP via electrostatic interactions with the cell surface proteoglycans platform and the consequent actin network remodelling following the activation of GTPase Rho A or Rac 1 is common to all internalization processes. For example, the guanidine head group of polyarginines can form hydrogen bonds with negatively charged sulphates and phosphates on the surface of the cell membrane. This interaction allows also the accumulation of CPP-cargo complexes on the cell membrane. Further cellular uptake depends on several parameters, such as the cell type, the membrane composition, the nature of CPP structure as well as the type and concentration of the cargo, the nature of the interaction between CPP and cell membrane which is specific for each CPP. Endocytosis could be further distinguished in phagocytosis and pinocytosis. Whereas the first mentioned process is restricted to specialized cells, such as macrophages, pinocytosis occurs in each mammalian cell. Depending on CPP sequence and cargo, four mechanisms of endocytotic internalization were described so far: clathrin-dependent endocytosis, caveolae/lipid raft-mediated endocytosis or a mix of both of them and macropinocytosis as well as clathrin/caveolae-independent endocytosis (Figure 39). Cellular uptake mechanism of polyarginines remains a matter of debate. When conjugated to cargos, they were found in endosomes as well as free in the cytoplasm and nucleus indicating an endocytosis-independent translocation into the cells (*Melikov & Chernomordik, 2005; Ter-Avetisyan et al., 2009*). However, polyarginines need to pass a





Abbreviations: ERC, endocytic recycling compartment; ER, endoplasmic reticulum; MTOC, microtubule-organizing centre; MVB, multivesicular bodies;

Figure 39: Cellular uptake mechanisms and possible intracellular trafficking of CPPs (Chou et al., 2011).

lipophilic bilayer. Recently, a water-pore-assisted translocation mechanism has been proposed to further explain energy-independent internalization of polyarginines (Huang & Garcia, 2013). Polyarginines seem to nucleate a pore into the lipid membrane and translocate along this structure. Very little is known about the intracellular trafficking of CPPs. Clearly, the endosome escape of CPPs is a rate-limiting step before trafficking back to the cell membrane or fusing with lysosomes and regulates mainly bioavailability and activity of their cargos.

### 5.3. The potential of CPPs in anti-cancer drug delivery

*Bolhassani* said: “Cancer could be cured if we knew how to deliver a drug intact to the cytosol of every cancer cell, sparing healthy cells.” (*Bolhassani, 2011*).

CPPs offer several properties, such as their short sequence, low cytotoxicity and ability to carry cargos bigger than 500 Da, cell type-independent internalization and low immune response depending on the CPP. However, the major drawback of CPPs is their lack of cell specificity, which complicates their *in vivo* use. *Schwarze et al.* injected the TAT- $\beta$ -galactosidase fusion protein intraperitoneally *in vivo* and observed the delivery of this protein in almost all organs except the brain (*Schwarze et al., 1999*). It is known that CPPs can cross also the blood-brain-barrier, thus enlarging their spectrum of applications. Since then, CPP-based drug delivery has been successfully used pre-clinical and clinical studies to target different diseases, including ischemia, apoptosis, and stimulation of cytotoxic immunity, cancer as well as other disorders (*Fecke et al., 2009*). To circumvent the “spreading” of CPP-cargo-complexes, they could be either locally applied, as shown in a study by our laboratory in which a R9-fusion protein inhibited tumor growth when intratumorally injected, or fused to molecular systems to improve targeting (*Planel et al., 2010*). In the latter study, one could take advantage of physiological and biological features of the targeted cell/organ, such as its enzymatic activity or microenvironment. In these constructs, the CPP would be first hidden, to avoid unspecific interactions while circulating in the organism. Once the target is reached, the CPP is fully exposed and could efficiently internalize its cargo. *Jiang et al.* provided in 2004 such a targeting system (*Jiang et al., 2004*). The authors fused a polyarginine-based CPP with an inhibitory domain of negatively charged residues linked together via a cleavable metalloprotease (MMP) sequence (Figure 40). This construct leads to the formation of a hairpin structure due to charge-based interaction of polyarginines and the inhibitory domain. Because levels of circulating MMPs are low, no cleavage of the construct occurs in the blood stream avoiding non-specific interactions of the CPP. Tumor cells are secreting actively MMPs in their microenvironment. In tumor tissue, MMP concentration is high enough to cleave the linker, followed by the dissociation of the anionic counterpart of the polyarginine sequence. The CPP can bind to target cells and internalize its covalently-bound cargo.

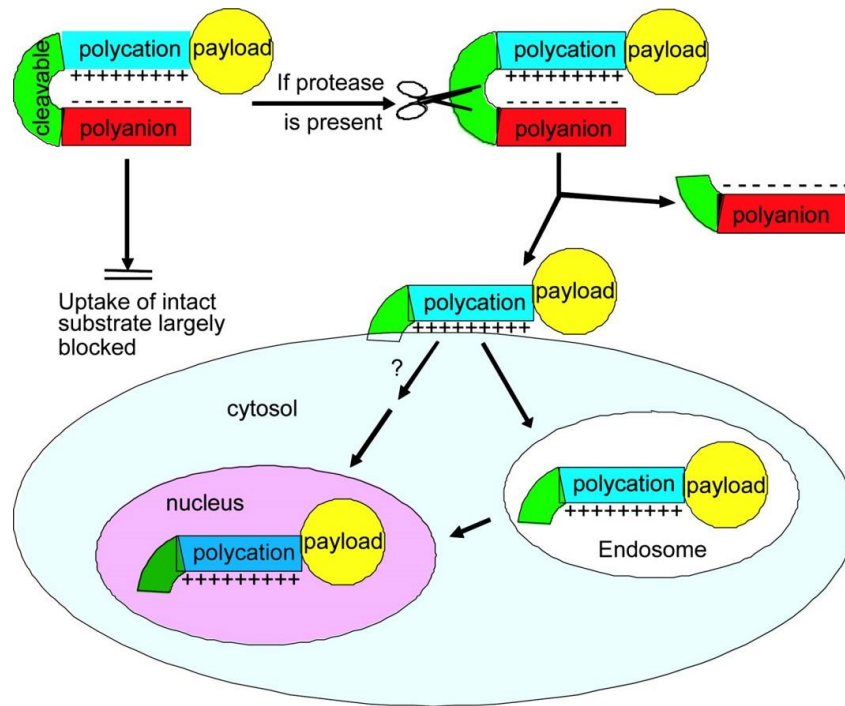


Figure 40: Activatable CPP, which is fused to an anionic inhibitory sequence via MMP-cleavable linker (Jiang *et al.*, 2004).

Sethuraman *et al.* propose a biodegradable pH-sensitive micelle delivery system (Sethuraman *et al.*, 2008). This system consist of hydrophobic core into which drugs can be incorporated, coated with poly-ethylene glycol conjugated to the TAT CPP and a pH-sensitive polymer (PSD, poly sulfonamide) (Figure 41). In the blood system under normal pH, PSD is negatively charged and interacts with TAT. Due to pH decrease, for example near tumor cells, PSD loses its charge and dissociates from the TAT sequence, allowing the CPP to interact with target cells.

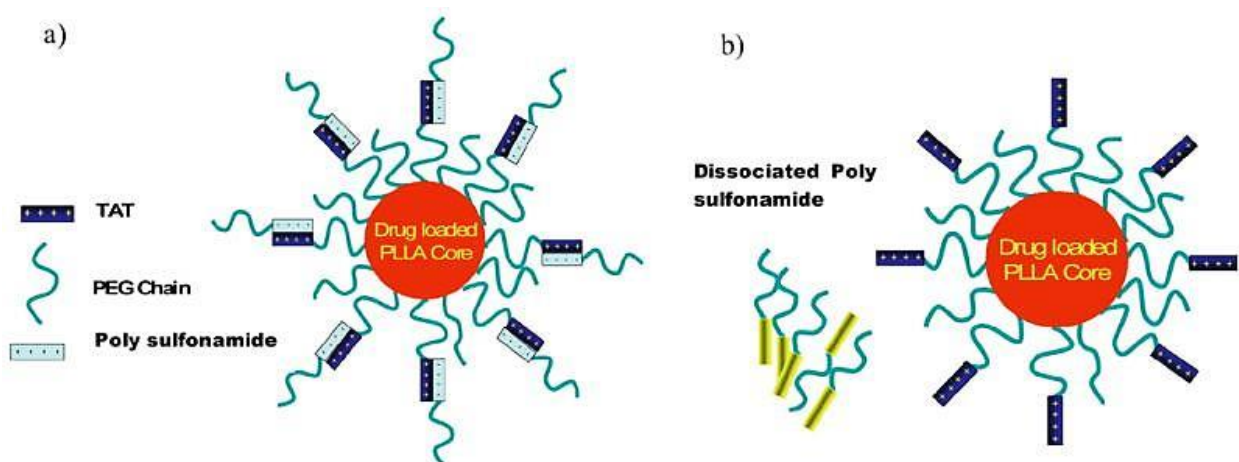


Figure 41: Biodegradable pH-sensitive micelle delivery system (Sethuraman *et al.*, 2008). See text for further explanation.

#### 5.4. CTPs

In addition to CPPs, another group of peptides, cell-targeting peptides (CTP), are described. These peptides recognize and bind with high affinity to receptors exclusively overexpressed by target cells. The best studied CTP is the RGD peptide (*Gehlsen et al., 1988*). The Arg-Gly-Asp (RGD) sequence is present in several circulating proteins and can bind to the  $\alpha\beta3$  integrin receptor, a receptor which plays an important role in angiogenesis, cell migration, invasion and formation of metastases. Therefore,  $\alpha\beta3$  integrin receptor has aroused interest as a target in cancer treatment. To further enhance the specific interaction of the RGD peptide and its target receptor, cyclization and multimerization of the peptide were investigated. *Garanger et al.* developed a cyclic decapeptide, named RAFT, on which they grafted four RGD peptides on the upper face for recognition of the integrin and two potential sites to link drugs on the bottom face (*Garanger et al., 2005*). RAFT(cRGD)4 was designed for the optimal binding to  $\alpha\beta3$  integrin receptor. Indeed, *in vivo* experiments within this study confirmed the tumor-specific delivery of anti-cancer drugs and imaging reagents. Therefore the RAFT(cRGD)4 is a useful tool for cancer treatment and imaging.

Taking together the advantages and drawbacks of CPPs and CTPs, the ideal strategy to improve therapy efficiency would be the combination of both of them. While the CTP would guarantee specific targeting of tumor cells/tissue, the CPP would ensure cellular uptake of the drug. However, this approach remains challenging.



**In conclusion,** efficient and specific treatment of cancer is still a tough issue. The discovery of CPPs and CTPs were milestones in this challenge. Subtle utilization of both of them would allow the specific targeting of cancer cells or their environment and efficient drug delivery. The choice of which CPP/CTP will be used highly depends on the cargo, preferred cellular uptake mechanism and subcellular localization. Taking advantage of these useful tools, numerous pre-clinical trials of CPP-delivery approaches are ongoing (*Bolhassani, 2011; Regberg et al., 2012*).



## Objectives

Our laboratory is mainly interested in the study of physiological and tumor angiogenesis. The expression of vascular endothelial growth factor (VEGF), a key mediator of angiogenesis, is tightly controlled at transcriptional and post-transcriptional levels. Post-transcriptionally, VEGF mRNA levels are regulated by stabilizing/destabilizing trans-acting factors, which bind to AU-rich elements located in the 3'-untranslated region of VEGF mRNA. In 2004, our laboratory identified the zinc finger protein TIS11b as a negative regulator of VEGF mRNA stability. Therefore, TIS11b appeared as a new potential target in anti-angiogenic cancer therapies.

Following this idea, our laboratory published in 2010 a novel concept in anti-angiogenic and anti-tumoral therapy based on the multi-target destabilization of short-lived mRNAs by TIS11b. Indeed, injections of purified cell-penetrating R9-TIS11b in pre-established tumors in mice inhibited significantly tumor growth and expression of angiogenic and inflammatory cytokines, with a consequent decrease in tumor vascularization. Unfortunately, the recombinant protein R9-TIS11b was highly unstable, thus making a further characterization of the experimental therapy more difficult.

In this context, the main task of my thesis was the development of a novel experimental anti-cancer therapy based on a new generation of TIS11b proteins. Therefore, I pursued two complementary objectives which were:

- (1) The study of the impact of TIS11b phosphorylation on its function in ARE-mediated mRNA decay. My work focused on two phosphorylatable serines located in the distal N- and C-terminal domains of TIS11b, respectively.
  
- (2) The evaluation of the anti-tumoral activity of newly generated versions of TIS11b *in vitro* and *in vivo*. In this second part, I aimed to define whether these therapeutic proteins could impair major hallmarks of cancer and to identify their target transcripts in tumor tissue. The most promising candidate was a truncated form of TIS11b lacking the N-terminal domain of the full-length protein and harbouring a substitution of a C-terminal phosphorylatable serine by an aspartate.





# RESULTS



# Article 1

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**“A novel phosphorylation-dependent regulation of TIS11b stability and activity by cAMP-dependent protein kinase (PKA) reveals the important role of two conserved N- and C-terminal serines (S54 and S334)”**



## Introduction

Paracrine acting VEGF is the key driver of physiological and pathological angiogenesis (*Ferrara & Davis-Smyth, 1997*). In addition, autocrine acting VEGF protects adult, quiescent endothelial cells (ECs) in concert with other angiogenic factors to maintain the existing vascular network (*Lee et al., 2007*). *In vivo* mouse models demonstrated that deletion of only one allele as well as a modest overexpression of VEGF lead to embryonic lethality due to defective vascularization (*Carmeliet et al., 1996; Ferrara et al., 1996; Miquerol et al., 2000*). Due to the biological importance of this pro-angiogenic factor, its expression is tightly controlled at the transcriptional and post-transcriptional level. Several response elements located in the promoter region of the *VEGF* gene regulate its transcription (*Pages & Pouyssegur, 2005*). Among other environmental stimuli such as hypoxia or growth factors, *VEGF* gene expression is induced by several hormones such as estrogens or progesterone. Indeed, transcriptional regulation of VEGF expression has been described for endocrine organs such as ovaries or endometrium. Beside its known action on adrenocortical steroidogenesis, the adrenocorticotrophic hormone ACTH plays an instrumental role in the development and maintenance of the vascularization of the adrenal gland at least through its action on VEGF expression (*Keramidas et al., 2004*). In adrenocortical cells, ACTH acts via the melanocortin receptor 2 (MCR2). The stimulation of this seven-transmembrane domain G-protein coupled receptor activates the adenylyl cyclase leading to increased intracellular cAMP levels and activation of the cAMP-dependent protein kinase A (PKA). Our team has previously shown that ACTH stimulates a rapid and transient increase of VEGF mRNA in primary bovine adrenocortical cells (BAC) which peaks around 3 h post-stimulation (*Chinn et al., 2002*). It was shown that Forskolin, an activator of the adenylyl cyclase, was as potent as ACTH in eliciting an increase in VEGF mRNA levels (*Gaillard et al., 2000; Chinn et al., 2002*). Interestingly, this mechanism is transcription-independent (*Gaillard et al., 2000*) and involves post-transcriptional regulations of VEGF mRNA stability.

Several mechanisms control VEGF expression at the post-transcriptional level. Beside alternative splicing, alternative polyadenylation and translational control, VEGF mRNA stability is regulated by several RNA-binding proteins (RNA-BP). These mechanisms involve stabilizing RNA-BP such as HuR and destabilizing RNA-BP like TIS11b which bind to the 3'UTR of the VEGF transcript (*Levy et al., 1998; Cherradi et al., 2006*). In adrenocortical cells, ACTH induces the expression of TIS11b during the decay phase of VEGF mRNA (*Chinn et al., 2002*). This observation led our team to hypothesise that TIS11b is involved in the control of VEGF mRNA turnover. Using RNA interference strategy, it was found that indeed, TIS11b is a negative regulator of basal and ACTH-stimulated VEGF mRNA expression in BAC cells.

Furthermore, it was demonstrated that ACTH modulates the subcellular localization of HuR in BAC cells (*Cherradi et al., 2006*). HuR shuttles from the nucleus into the cytoplasm to stabilize VEGF mRNA. HuR and TIS11b bind to distinct but very close response elements in the 3'UTR of the VEGF transcript. Interestingly, co-overexpression of both proteins abrogated HuR-induced VEGF mRNA stabilization suggesting a predominant role of TIS11b in the regulation of VEGF mRNA stability. Altogether, these studies led our team to propose a model in which ACTH first triggers VEGF mRNA stabilization through the nuclear export of HuR then subsequently promotes VEGF mRNA destabilization through the induction of TIS11b expression, thus allowing for a transient expression of VEGF in adrenocortical cells.

TIS11 family proteins are highly phosphorylated following the activation of several signalling pathways. These post-translational modifications regulate expression, activity, stability and subcellular localization of the TIS11 proteins. In general, their phosphorylation is correlated with an inhibition of their mRNA-destabilizing activity. The p38 MAPK-activated Protein Kinase-2 (MK2) phosphorylates TTP, the prototypical member of the family, thus allowing TTP interaction with 14-3-3 proteins (*Chrestensen et al., 2004; Stoecklin et al., 2004*). This interaction inhibits TTP-mediated degradation of AU-rich elements (ARE)-containing mRNA because phosphorylated TTP is unable to recruit components of the mRNA decay machinery. In addition, binding to the 14-3-3 proteins protected TTP from proteasomal degradation in the cytosol. The impact of TTP phosphorylation on its binding to ARE is still controversial (*Schmidlin et al., 2004; Stoecklin et al., 2004*). MK2-induced phosphorylation of TTP is counterbalanced by the phosphatase PP2A, thus re-activating TTP-mediated mRNA decay (*Sun et al., 2007; Sandler & Stoecklin, 2008*). MK2 phosphorylates TIS11b at Ser<sup>54</sup>, Ser<sup>92</sup> and Ser<sup>203</sup> leading to the stabilization of TIS11b protein and the loss of its mRNA decay activity, but phosphorylated TIS11b can still bind ARE-containing mRNA (*Maitra et al., 2008*). The Protein Kinase B (PKB) phosphorylates the Ser<sup>92</sup> and Ser<sup>203</sup> of TIS11b with the same aforementioned effects on its activity and turnover (*Benjamin et al., 2006*). The phosphorylation of TTP by ERK enhanced its proteasomal degradation (*Bourcier et al., 2011*). *In vitro* studies by *Cao & Lin* showed that PKA phosphorylates TTP (*Cao & Lin, 2008*). In addition, it was demonstrated that PKA induces TIS11b expression in human osteoblast-like cells in response to parathyroid hormone treatment (*Reppe et al., 2004*). Unfortunately, this study did not analyse potential phosphorylation of TIS11b in response to PKA activation. Increasing evidence suggests that the phosphorylation status of TIS11 proteins also regulate their interaction with the mRNA decay machinery. Indeed, MK2-mediated phosphorylation of TTP impairs its activity as it prevents the recruitment of the Caf1 deadenylase in HeLa cells (*Marchese et al., 2010; Clement et al., 2011*).

To our knowledge, no data concerning the impact of TIS11b phosphorylation on its interaction with mRNA decay factors has been reported so far.

In this context, we were interested in investigating whether TIS11b is a target of PKA in response to ACTH and how the phosphorylation of TIS11b could influence its mRNA-destabilizing activity, protein stability, subcellular localization and interaction with components of the mRNA decay machinery.

As this study has been started before I joined the team as a PhD student, I contributed partially but significantly to this work. All the results concerning the hormonal regulation of TIS11b expression were acquired before my arrival in the laboratory. To study the effect of TIS11b phosphorylation at Serine 54 or Serine 334 on the subcellular localization of TIS11b, I generated stable HeLa tet-off cell lines, which express TIS11b phospho-mutants in response to tetracycline. I also studied the effect of hypoxia on endogenous TIS11b expression and phosphorylation in lung cancer cells. Using different approaches, I aimed to identify the kinase(s) and phosphatase(s) which regulate the phosphorylation of TIS11b under hypoxic conditions. Furthermore, I performed co-immunoprecipitation experiments to determine the effect of TIS11b phosphorylation on its interaction with components of the mRNA decay machinery.





**A novel phosphorylation-dependent regulation of TIS11b/BRF1 stability and activity by cAMP-dependent protein kinase (PKA) reveals the important role of two conserved N- and C-terminal serines (S54 and S334)**

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## Abstract

TIS11b is a zinc finger protein which binds to mRNAs containing AU-rich elements (ARE) in their 3'-untranslated region (3'UTR) and promotes their deadenylation and rapid degradation. We have previously shown that VEGF mRNA expression is regulated through antagonistic binding of the mRNA-destabilizing TIS11b and the mRNA-stabilizing HuR to VEGF 3'UTR. The decay of ARE-containing mRNA is regulated by signalling pathways that have been shown to directly target ARE-binding proteins. In the present study, we observed that TIS11b is phosphorylated by the cAMP-dependent protein kinase A (PKA) *in vitro* and *in vivo*. *In vitro* kinase assays using two synthetic peptides spanning a N-terminal or a C-terminal regions of TIS11b as well as recombinant full-length protein suggest that PKA phosphorylates TIS11b at least at two distinct sites, the serine 54 (S54) and the serine 334 (S334). Western blot analyses using anti-phospho-specific antibodies showed that S54 and S334 are indeed phosphorylated *in vivo*, not only in a hormone-regulated cell context but also during the cellular response to hypoxic stress. Mutation of S54 to alanine decreased TIS11b half-life while it increased its mRNA-decay promoting activity. Surprisingly, mutation of S334 to alanine modestly affected TIS11b half-life while it impaired its function in mRNA decay. Moreover, mutation of S334 to aspartate strongly increased TIS11b half-life while it potentiated TIS11b mRNA-destabilizing activity. Co-immunoprecipitations experiments revealed that S54 is involved in the interaction of TIS11b with 14-3-3 proteins while S334 is involved with the interaction of TIS11b with the subunit of the Ccr4-Not complex CNOT1. Altogether, these results suggest that phosphorylation of TIS11b at S54 and S334 exert an opposite action on TIS11b function in ARE-dependent mRNA decay.



## Introduction

Besides transcription, there is now increasing evidence that post-transcriptional mechanisms contribute to a second level of regulation of gene expression. In particular, mRNA stability is a key step that has been understudied but which progressively appears as a highly regulated step. Importantly, this mechanism is responsive to modifications of the cellular environment (hypoxia, hypoglycemia, hormonal variations,...) and regulates the expression of subsets of proteins whose levels need to be rapidly adjusted. The regulation of mRNA stability involves *cis*-sequences located mainly in the 3'-untranslated region (3'UTR) of the target mRNA which are bound by *trans*-acting factors. The most studied *cis*-element is the AU-rich element (ARE) located in the 3'-UTR of short-lived mRNAs. It has been estimated that at least 8% of the human genes code for ARE-containing mRNAs (*Bakheet et al., 2003*). These genes encode proteins such as cytokines, growth factors or metabolic regulators that require to be rapidly turned on or off in numerous transient biological processes (*Chen & Shyu, 1994*). A bioinformatic analysis of the genes involved in angiogenesis reveals that 25% of them contain AREs in their 3'-UTR (personal observations), thus demonstrating the importance of this type of regulatory sequences in such a finely tuned and rapidly-responding biological process.

A great effort has been devoted over the last two decades to the identification of ARE-binding proteins and analysis of their contribution to the control of mRNA stability (*Wilusz et al., 2001; Barreau et al., 2005*). Several mRNA-stabilizing proteins have been identified such the members of the Hu family (HuR/HuA, HuC and HuD), as well as several mRNA-destabilizing proteins, including members of the TIS11 family of double zinc finger nucleo-cytoplasmic shuttling proteins or KSRP (*Ma et al., 1996; Briata et al., 2013; Brooks & Blackshear, 2013*). TIS11 protein family is composed of four known members: TTP (Tristetraprolin/TIS11/ZFP36), TIS11b (ZFP36L1/BRF1), TIS11d (ZFP36L2/BRF2) and ZFP36L3 which is expressed exclusively in mouse placental tissue (*Lai et al., 1999; Blackshear et al., 2005*). The four members exert a destabilizing activity on short-lived mRNA by interacting with ARE sequences via highly conserved tandem zinc finger motives (*Lai et al., 2000*). *In vitro*, the three main members of TIS11 family (TTP, TIS11b and TIS11d) have been shown to interact with several ARE-containing mRNAs and to trigger deadenylation and degradation of the target mRNA. However, *in vivo*, TIS11 family members might have specific mRNA targets. Indeed, knock-out of the different TIS11 proteins in mice leads to different phenotypes (*Taylor et al., 1996; Ramos et al., 2004; Stumpo et al., 2004; Bell et al., 2006; Stumpo et al., 2009; Hodson et al., 2010*).

TTP, the most studied member of TIS11 family, has been shown to trigger degradation of several transcripts including TNF- $\alpha$ , GM-CSF, IL-2, and IL-10 mRNA (*Carballo et al., 1998; Carballo*

*et al.*, 2000; Ogilvie *et al.*, 2005; Stoecklin *et al.*, 2008). TTP is able to recruit the components of the mRNA decay machinery (Johnson & Blackwell, 2002; Fenger-Gron *et al.*, 2005; Lykke-Andersen & Wagner, 2005). We have previously shown that TIS11b destabilizes the mRNA of the angiogenic cytokine Vascular Endothelial Growth Factor (VEGF) (Ciais *et al.*, 2004), via its interaction with two consensus ARE motives composed by a nonamer (UUAUUUAUU) and a pentamer (AUUUA) in VEGF mRNA 3'UTR. In endocrine cells, hormone-induced expression of VEGF is regulated through interplay between the mRNA-stabilizing protein HuR and the mRNA decay-promoting protein TIS11b (Cherradi *et al.*, 2006). More recently, we have developed a preclinical anti-angiogenic and anti-tumoral therapy using the mRNA-destabilizing function of TIS11b (Planel *et al.*, 2010).

ARE-binding proteins are distal targets for signalling pathways in conveying external stimuli to the mRNA decay machinery. The p38 MAP Kinase (MAPK) and its downstream kinase MAPK-activated protein kinase 2 (MK2) appear to play a pivotal role in ARE-mediated mRNA decay. Activation of p38 MAPK has been shown to impair the deadenylation of ARE-containing mRNA *in vivo*, leading to mRNA stabilization ((Winzen *et al.*, 1999; Winzen *et al.*, 2007). Macrophages from MK2<sup>-/-</sup> mice show severely reduced levels of TNF $\alpha$ , IL-1, IL-6 and IFN $\gamma$  due to decreased cytokine mRNA stability (Kotlyarov *et al.*, 1999; Neininger *et al.*, 2002). A major target of MK2 is TIS11/TTP protein which is directly phosphorylated at Serine 52 (S52) and Serine 178 (S178) allowing binding of 14-3-3 adaptor proteins. This interaction reduces the destabilizing activity of TTP (Johnson *et al.*, 2002; Stoecklin *et al.*, 2004). In addition, phosphorylation of TTP on S52 and S178 by MK2 stabilizes TTP protein by preventing TTP degradation by the proteasome and favours its cytoplasmic localization (Brooks *et al.*, 2002). It has been suggested that MK2 is counterbalanced by protein phosphatase 2A (PP2A) which directly competes with 14-3-3 protein for binding to TTP. PP2A then dephosphorylates TTP at Ser178 (and possibly at other serine residues) and thereby activates mRNA decay. More recently, it has been shown that TTP phosphorylation by MK2 prevents the recruitment of the deadenylation machinery to the target mRNA (Clement *et al.*, 2011).

By contrast to the accumulating data on the impact of TTP phosphorylation on its function, few studies addressed the role of TIS11b phosphorylation in ARE-mediated mRNA decay. Phosphorylation of TIS11b by the Protein kinase B (PKB) at S92 and S203 abrogated mRNA decay of an IL-3 ARE-containing probe and led to TIS11b binding to 14-3-3 proteins as well as to TIS11b protein stabilization (Schmidlin *et al.*, 2004; Benjamin *et al.*, 2006). More recently, using *in vitro* kinase assays, Maitra *et al.* reported that phosphorylation of TIS11b by MK2 at

S54, together with S92 and S203 did not affect its ability to bind to ARE or to recruit mRNA degradation enzymes but did nonetheless inhibit its ability to destabilize ARE-containing mRNA (Maitra *et al.*, 2008). The striking number of potential phosphorylation sites in TIS11b sequence suggests that the protein has to integrate multiple signals to provide the appropriate cellular response. In this study, we identified new phosphorylation sites in TIS11b and investigated the role of these phosphosites in the control of TIS11b activity and protein stability. We show that two putative Protein Kinase A (PKA) phosphorylation sites, the S54 and S334 regulate not only the mRNA-destabilizing activity of TIS11b but also TIS11b protein stability. Our data indicate that in addition to the previously identified serines, S54 and S334 play a critical role in the control of TIS11b function.

## Material and Methods

### *Cell culture*

Bovine adrenal glands were obtained from a local slaughterhouse. *Zona fasciculata-reticularis* cells (BAC) were prepared by enzymatic dispersion with trypsin and primary cultures were established as previously described in detail elsewhere (Duperray & Chambaz, 1980). BAC cells were cultured in Ham's F12 medium supplemented with 10 % horse serum, 2.5 % fetal calf serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 20 µg/mL gentamicin (Invitrogen, Saint Aubin, France). On day 4,  $3 \times 10^6$  cells/10 cm-petri dish were stimulated with 10 nM ACTH for the indicated periods of time in the presence or in the absence of the PKA inhibitor H89 (5µM) (Sigma-Aldrich, Saint-Quentin Fallavier, France). A549 cells were purchased from ATCC and cultured in DMEM GlutaMAX High Glucose medium (Invitrogen) containing 10 % fetal bovine serum (GE Healthcare, Velizy-Villacoublay, France) and 100 U/mL of penicillin, 100 µg/mL of streptomycin (Invitrogen, Saint Aubin, France) and 30 µg/mL of gentamicin (Invitrogen).  $7.5 \times 10^5$  A549 cells were seeded in 35 mm-petri dishes. The day after, they were exposed to hypoxia (1.5 % O<sub>2</sub>) and harvested at the time point indicated for Western blot analyses. Alternatively, A549 cells were treated with 100 nM okadaic acid or DMSO (Sigma-Aldrich) then incubated under hypoxia. COS7 cells and Hela cells were cultured as described previously (Planel *et al.*, 2010). All cell types were grown at 37 °C in a 5 % CO<sub>2</sub>-95 % air atmosphere.

### *Plasmids*

The plasmids pTarget-TIS11b-S54A, pTarget-TIS11b-S54D, pTarget-TIS11b-S334A, pTarget-TIS11b-S334D, and pTarget-TIS11b-S54A-S334A were generated from pTarget-TIS11b wild



type (*Ciais et al., 2004*) by site directed mutagenesis (QuickChange XL site-directed mutagenesis kit, Agilent Technologies, Massy, France), using the primers indicated in Supplemental Table 1. pTarget-TIS11b-S54A served as a template to construct pTarget-TIS11b-S54A-S334A. pTarget-TIS11b plasmid was also used to amplify the human TIS11b truncated forms by PCR using the primers indicated in Table 1. Following their amplification, the NZn (amino acids 1-195) and ZnC (amino acids 109-338) fragments were inserted into the pTarget plasmid using the T-overhangs to generate pTarget-NZn and pTarget-ZnC. For recombinant TIS11b, the constructs used were pET15b-Flag-TIS11b-S54A, pET15b-Flag-TIS11b-S334A, pET15b-Flag-NZn and pET15b-Flag-ZnC, which were constructed in the same way as pET15b-Flag-TIS11b (*Planel et al., 2010*), using their respective pTarget plasmid. Recombinant proteins were purified as previously described (*Planel et al., 2010*). To derive a tetracycline-regulated expression vector for wild type and mutant TIS11b, pTarget-TIS11b or pTarget-TIS11b mutants were digested with *Bam*H1-*Not*I then the fragments were inserted into the *Bam*H1-*Not*I sites of pTRE-Tight vector (Clontech, Saint-Germain-en-Laye, France) which harbours a tetracycline-responsive element.

#### *Transient Transfections and Dual Luciferase Activity Assay*

$1.5 \times 10^5$  COS7 cells were seeded in triplicate into 12 well-plates and transfected the day after using Lipofectamine (Invitrogen) according to the manufacturer's recommendations. Ten ng of pTarget-TIS11 plasmids were transfected in the presence of 500 ng of pLuc-3'-UTR, and 25 ng of pRL-TK (Promega, Charbonnières Les Bains, France) to compensate for variations in transfection efficiency. Renilla and Firefly luciferase activities were measured sequentially 24 h after transfection using the Dual-Luciferase reporter assay system (Promega) on a LUMAT LB 9507 luminometer (EGG-Berthold, Bad, Wildbad, Germany). Results are expressed as relative light units of Firefly luciferase activity over relative light units of renilla luciferase activity, and are represented as a percentage of the luciferase activity in control cells. Each transfection condition was performed in triplicate.

#### *Metabolic labelling*

BAC cells in primary culture ( $5 \times 10^6$  cells/10 cm-petri dish) were pre-labeled for 60 min in phosphate-free Ham's F12 medium containing 200  $\mu$ Ci/mL [ $^{32}$ P]-orthophosphate, before exposure to 10 nM of ACTH in the presence or in the absence of 10  $\mu$ M H89 for the time indicated, at 37 °C. At the end of the stimulation period, cells were washed twice with ice-cold PBS and lysed in 0.5 mL ice-cold RIPA lysis buffer containing a protease inhibitor cocktail, 5

mM sodium fluoride, 100 nM okadaic acid, and 200  $\mu$ M sodium orthovanadate (Sigma-Aldrich). Samples were briefly centrifuged at  $10,000 \times g$  for 15 min. Total cell lysates were pre-cleared with 20  $\mu$ l protein A/G agarose mixture and incubated with 1  $\mu$ g/mL of rabbit polyclonal anti-TIS11b antibody at 4 °C for 12 h. The immune complex was isolated by adding 30  $\mu$ l protein A/G mixture for 2 h at 4 °C and then centrifuging at 2 000 rpm for 5 min. The pellet was washed four times with RIPA buffer and analyzed by SDS PAGE and autoradiography.

#### *In vitro phosphorylation*

Recombinant Flag-TIS11b, Flag-TIS11b-S54A, Flag-TIS11b-S334A, Flag-NZn, Flag-ZnC or synthetic peptides were incubated with the catalytic subunit of the protein kinase A (PKA, 1  $\mu$ g) in the presence of [ $\gamma^{32}$ -P]-ATP (10  $\mu$ Ci), 0.2 mM of cold ATP and 15 mM of MgCl<sub>2</sub> in Tris-HCl 50 mM pH 7.4 for 20 min, at 30 °C in a final volume of 50  $\mu$ L. Phosphorylation was stopped by the addition of 10 mM EDTA. The samples were analyzed by SDS-PAGE and phospho-proteins or phospho-peptides visualized by autoradiography.

#### *Generation of TIS11b/TIS11d phospho-specific antibodies*

Polyclonal, phospho-specific antibodies against TIS11b-phospho-S54 and TIS11b-phospho-S334 were generated by injection of the synthetic KLH-conjugated peptides CAGGGFPRRH(Sp)VTL or RRLPIFSRL(Sp)ISD, respectively, into rabbits (CovalAb, Lyon, France). Sera were affinity-purified using the same peptide, and non-phospho-specific antibodies were depleted by affinity purification using peptides containing unmodified serines. Due to the conservation of these sequences between TIS11b and TIS11d, the anti-phospho-serines antibodies recognize both phospho-proteins.

#### *Northern blot*

RNA extraction and Northern blot analysis have been described previously (Planel *et al.*, 2010).

#### *Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-qPCR)*

Total RNA was extracted using the Nucleospin RNA kit according to the manufacturer's recommendations (Macherey-Nagel, Hoerd, France). 1  $\mu$ g of total RNA was reverse-transcribed with the iScript System (BioRad, Marnes-la-Coquette, France) according to the manufacturer's guidelines and random primers from Invitrogen. cDNAs were diluted in a 50  $\mu$ L final volume. Quantitative PCR was performed using the GoTaq qPCR Master Mix (Promega) and 2  $\mu$ L aliquots of the RT reaction. Amplification of VEGF was carried out in a final volume of 20  $\mu$ L

using a CFX96 Real-Time System thermocycler (Bio-Rad, Marnes-la-Coquette, France) with the following program: Initial denaturation at 95 °C for 5 min followed by 40 cycles of denaturation at 95 °C for 10 sec and annealing at 60 °C for 30 sec. Amplification of human VEGF mRNA was performed using the forward 5'-AAG GAG GAG GGC AGA ATC AT-3' and reverse 5'-ATC TGC ATG GTG ATG TTG GA-3' primers. The size of the amplified fragment was 226-bp for the VEGF transcript. The primers for HPRT amplification were as follows: 5'-ATG GAC AGG ACT GAA CGT CTT GCT-3' and 5'-TTG AGC ACA CAG AGG GCT ACA ATG-3'. This primer pair sequence amplifies a 80-bp fragment.

#### *Western Blot*

Western blotting was performed as described previously (*Planel et al., 2010*). In addition to the anti-phospho-TIS11b antibodies, the following antibodies were used: rabbit anti-TIS11b/TIS11d (BRF1/BRF2) and anti-TIS11b (BRF1) (Dr C Moroni, University of Basel), rabbit polyclonal anti-human VEGF-A (Santa Cruz Biotechnology, Heidelberg, Germany), mouse monoclonal anti-Actin (Sigma-Aldrich), rabbit polyclonal anti-pan-14-3-3 (K-19, Santa Cruz Biotechnology, Heidelberg, Germany), rabbit polyclonal anti-human CNOT1 (Proteintech, Manchester, UK).

#### *Measurement of the half-life of wild type and mutant TIS11b*

1.5 x 10<sup>5</sup> COS7 cells were seeded into 12 well-plates and transfected the day after with 10 ng of pTarget-TIS11b plasmids (pTarget-WT, pTarget-TIS11b-S54A, pTarget-TIS11b-S54D, pTarget-TIS11b-S334A, pTarget-TIS11b-S334D, and pTarget-TIS11b-S54A-S334A) using Lipofectamine (Invitrogen, Saint Aubin, France) according to the manufacturer's recommendations. 48 hours post-transfection, cycloheximide (10 µg/mL) was added to each well for various periods of time. Cells were washed with cold PBS then lysed in RIPA buffer on ice as described above. Protein extracts were centrifuged at 10 000 x g for 15 min at 4 °C then protein concentration was determined using Micro BCA Protein Assay Kit (Thermo Fisher, Illkirch, France) according to the manufacturers' instructions. Samples were subsequently analyzed by western blot.

#### *Generation of stable HeLa Tet-off cells expressing wild type TIS11b and TIS11b mutants*

Human HeLa tet-off cells (2 x 10<sup>6</sup> cells) (Clontech) in DMEM/10 % fetal bovine serum (Tet System approved FBS, Clontech) at 50 % confluency in 100-mm diameter plate were transfected in the presence of 100 ng/mL doxycycline, using Lipofectamine 2000 (Invitrogen), with 2 µg of pTRE-Tight-TIS11b, pTRE-Tight-TIS11b-S54A, pTRE-Tight-TIS11b-S54D, pTRE-Tight-

TIS11b-S334A or pTRE-Tight-TIS11b-S334D, and Linear Hygromycin Marker (100 ng, Clontech). Clones stably expressing TIS11b constructs were selected in the presence of 400 µg/mL G418 (Invitrogen) and 100 µg/mL Hygromycin (Clontech) according to the manufacturer's recommendations. A Transcriptional pulse from TIS11b or TIS11b mutant plasmids was initiated by washing the cells twice with phosphate buffer saline and feeding with DMEM/10 % serum containing no doxycycline.

### *Immunofluorescence*

$2.0 \times 10^4$  stably transfected Hela tet-off cells were plated on an eight-chamber Lab-Tek Coverglass plate (Thermo Fisher, Illkirch, France) and cultured overnight in growth medium containing doxycycline (1 µg/mL). The day after, medium was removed and growth medium without doxycycline was added to the cells to induce TIS11b expression. Forty eight hours after induction, cell culture medium was removed and cells were washed three times (5 min) with PBS. Cells were fixed for 20 min using 4 % Paraformaldehyde (PFA) and washed twice (5 min) with PBS then permeabilized during a 5 min-incubation with 0.2 % Triton X 100 in PBS. After three 5 min-washes with PBS, cells were then sequentially incubated for 1 hour in PBS buffer containing 0.5 % BSA and for 2 h with of rabbit anti-human TIS11b/BRF1 antiserum (1:200 dilution; generous gift of Dr. C Moroni, University of Basel). After three washes of 5 min in PBS buffer containing 0.5 % BSA, cells were incubated for 1 h with 3 µg/mL donkey anti-rabbit Alexa 488 (Jackson Immuno Research, Marseille, France). Then, cells were washed three times (5 min) with PBS buffer containing 0.5 % BSA prior to the addition of 1 ng/mL Hoechst 33258 (Invitrogen) for 3 min to stain nuclei. Intracellular localization of TIS11b was assessed by laser confocal microscopy (Leica, TCS-SP2).

### *Immunoprecipitation assays*

COS7 cells cultured in 6-well plates were transfected with 50 ng of pTarget-TIS11b or pTarget-TIS11b mutants. HEK 293 cells cultured in 10-cm petri dishes ( $4.5 \times 10^6$  cells / dish) were transfected with 250 ng of pTarget-TIS11b or pTarget-TIS11b mutants. Total cell lysates of each cell line was cleared by centrifugation for 15 min at 12,000 x g at 4° C. A mixture of rabbit anti-N-terminal and anti-C-terminal peptide fragments of TIS11b (1 µg/mL) (Planel *et al.*, 2010) as well as anti-TIS11b/TIS11d (BRF1/BRF2) polyclonal antibody (1/200 dilution; Ozyme, Montigny-le-Bretonneux, France) was added to whole supernatants, which were then gently rocked overnight at 4° C, before being incubated for 2 h with Rabbit IgG TrueBlot® beads (Tebu-Bio, Le Perray En Yvelines, France). Immunoprecipitates were pelleted, washed four

times with RIPA buffer, analyzed by SDS-PAGE and transferred to PVDF membranes. Blots were probed with anti-14-3-3 (Santa Cruz Biotechnologies, Heidelberg, Germany) or anti-CNOT1 (Proteintech, Manchester, UK) antibodies. The membranes were thoroughly washed with TBS containing 0.1 % Tween20 then incubated for 1 h with HRP-conjugated IgG fraction monoclonal mouse anti-rabbit IgG, light chain specific antibodies (Jackson Immuno Research, Marseille, France).

## Results

### *PKA signalling pathway regulates TIS11b expression and phosphorylation*

We have previously shown that the cAMP-mobilizing hormone adrenocorticotropin (ACTH) increases TIS11b protein expression in adrenocortical cells and that TIS11b-mRNA destabilizing activity is involved in the decay phase of ACTH-increased VEGF mRNA levels (*Chinn et al., 2002; Cherradi et al., 2006*). In addition, activation of the cAMP signalling pathway induced a broad series of 38-50 kDa TIS11b bands which collapsed into a single band after  $\lambda$ -phosphatase treatment, indicating that the slower mobility species arose because of phosphorylation (*Duan et al., 2009*). To investigate the potential role of the protein kinase A (PKA) in hormone-elicited increase in TIS11b protein levels, bovine adrenocortical (BAC) cells in primary culture were stimulated by ACTH for various periods of time in the absence or in the presence of H89, a PKA specific inhibitor. The hormone induced a marked time-dependent increase in TIS11b protein levels (Fig. 1A and 1B) which was accompanied by a shift towards high molecular weight species (Fig. 1A). In contrast, VEGF mRNA and protein expression peaked at 3 h and 2 h, respectively, then decreased towards basal levels (Fig. 1A and 1B). Interestingly, the high induction of TIS11b at 6 h after the addition of ACTH was correlated to low VEGF mRNA and protein levels. In the presence of H89, the stimulation of TIS11b expression by ACTH was significantly reduced with changes in TIS11b electrophoretic mobility (Fig. 1A and 1B), while VEGF mRNA and protein induction was completely prevented (Fig. 1A and 1B). Note that the remaining TIS11b protein was still phosphorylated, suggesting an additional role for kinases other than PKA.

To examine whether ACTH affects indeed TIS11b phosphorylation, TIS11b was immunoprecipitated from  $^{32}\text{P}$ -labeled adrenocortical cells. A basal phosphorylation level of TIS11b was detected in control cells while ACTH induced a robust and time-dependent increase of  $^{32}\text{P}$  incorporation into TIS11b which was markedly impaired in the presence of the H89 (Fig.

1C). We next performed *in vitro* phosphorylation experiments to determine whether TIS11b is a direct substrate of PKA. Purified recombinant GST-TIS11b was incubated in the presence of the catalytic subunit of PKA and <sup>32</sup>P-labeled orthophosphate. As shown in Fig. 1D, TIS11b proved to be efficiently phosphorylated by PKA *in vitro*. In addition, the protein was detected as a doublet, probably corresponding to different phosphorylation states. Altogether, these results suggest that TIS11b expression and phosphorylation are positively regulated by PKA in endocrine adrenocortical cells.

*Identification of PKA target sites within TIS11b sequence: conserved S54 and S334 are phosphorylated in vitro*

TTP, TIS11b and TIS11d each consist of an RNA-binding zinc finger domain (Zn) flanked by N-terminal (NTD) and C-terminal (CTD) domains which can activate mRNA decay (Lykke-Andersen & Wagner, 2005). Inspection of the coding sequence of TIS11b (SwissProt, accession number Q07352) using the Phosphorylation Site Predictor DISPHOS 1.3 (<http://www.dabi.temple.edu/disphos/>) revealed that the CTD of TIS11b harbours a majority of putative phosphorylatable serine residues as compared to the NTD (Supplementary Fig. 1). By contrast, analysis of TTP sequence showed that putative phosphorylatable serines were almost equally distributed between the NTD and the CTD of TTP. Interestingly, TIS11d displayed a similar profile of putative phosphorylatable serines in its distal CTD to that of TIS11b, while an additional phosphorylation hotspot was predicted close to TIS11d zinc finger domain (Supplementary Fig. 1). We next wished to test which regions of TIS11b were responsible for the observed PKA-mediated phosphorylation. Because of the high instability of GST-TIS11b fusion proteins, we generated novel Flag-tagged constructs encoding full-length TIS11b, NZn (NTD + Zn) or ZnC (CTD + Zn) domains (Fig. 2A). Each construct was expressed in *E. coli* and purified using anti-Flag affinity chromatography. Recombinant proteins were tested as substrates for PKA *in vitro*. As shown in Fig. 2B (left panel), the ZnC domain (25 kD) was heavily phosphorylated as compared to the NZn (21 kD). However, it is worth mentioning that the apparent low phosphorylation of the NZn was due to the low purification yield of this fragment (Fig. 2B, silver stained gel). Indeed, quantification of independent experiments taking into account the purification yield revealed that the NZn domain was potently phosphorylated by PKA *in vitro* (Figure 2B, right panel).

In order to identify putative PKA phosphorylation sites within TIS11b, we used NetPhosK (<http://www.cbs.dtu.dk/services/NetPhos/>) software. Four motives corresponding to PKA

consensus phosphorylation sites (RXS or RRXS), including Serine 54 (S54), Serine 92 (S92), serine192 (S192) and serine 334 (S334) were found. As S54 and S334 presented the highest predictive score and as they were highly conserved not only between the three TTP family members TTP, TIS11b and TIS11d, but also between species (Fig. 2C), we focused on both serines. Interestingly, S54 has been reported previously as a target of MAPK-activated kinase kinase-2 (MK2) *in vitro* (Maitra *et al.*, 2008). This could be due to the similarity between MK2 and PKA consensus phosphorylation sites (RXXS and RRXS, respectively). We first tested whether TIS11b peptides spanning either S54 (amino acids 50 to 60) or S334 (amino acids 330 to 338) were phosphorylated by PKA *in vitro*. SDS-PAGE analysis and autoradiography revealed a heavy dose-dependent phosphorylation of the S54-bearing peptide while PKA also phosphorylated the S334-bearing peptide by but to a lesser extent (Fig. 2D). To determine whether S54 and S334 were directly targeted by PKA, we generated Flag-tagged TIS11b mutants in which S54 or S334 were replaced by an alanine to prevent phosphorylation (S54A or S334A). Purified wild-type (WT) or mutant proteins were incubated *in vitro* with PKA. As shown in Fig 2E, phosphorylation of TIS11b S54A mutant was markedly impaired when compared to that of WT TIS11b, suggesting that S54 is a major target of PKA. By contrast, phosphorylation of TIS11b S334A mutant was modestly although significantly affected, suggesting that S334 is a minor PKA phosphorylation site.

To determine whether S54 and S334 were phosphorylated *in vivo*, we generated phospho-S54- and phospho-S334-specific antibodies in rabbits immunized with a phosphopeptide spanning S54 (CAGGGFPRRH(Sp)VTL) or a phosphopeptide spanning S334 (RRLPIFSRL(Sp)ISD). The specificity of the sera was characterized using the non-phosphorylatable TIS11b mutants (S54A or S334A). Wild-type or TIS11b mutants were overexpressed in COS7 cells then cells extracts were probed for phospho-S54- or phospho-S334-specific signals. A strong signal was obtained with the WT protein but no phosphorylation could be detected with the mutants (Fig. 2F and G), thus demonstrating the specificity of the newly raised antibodies.

#### *TIS11b is phosphorylated at S54 and S334 in vivo*

To assess whether TIS11b is phosphorylated at S54 and S334 *in vivo*, BAC cells were challenged with ACTH for various periods of time (Fig. 3A). Cell lysates were probed either with anti-total TIS11b/TIS11d or with anti-phospho-S54 or anti-phospho-S334 antibodies. It is worth mentioning that TIS11d expression was variable between primary cultures of BAC cells. In this experiment, TIS11d was detected at the basal level and slightly decreased at 6 hours post-

stimulation while TIS11b which was nearly undetectable at (t=0 h) and markedly increased by ACTH treatment. ACTH induced an increase in phospho-S54 signal which paralleled the hormone-induced increase in total TIS11b protein levels. Interestingly, TIS11d was heavily labelled with the anti-phospho-S54 due to the conservation of the RRHS motif (S57 in TIS11d) between TIS11b and TIS11d. No anti-phospho-S334 signal was detected for TIS11b before 6 h of stimulation by the hormone, suggesting a delayed phosphorylation of this residue by ACTH (Fig. 3A). The single band detected belongs to the high molecular weight species of TIS11b. As observed with the anti-phospho-S54, TIS11d was markedly labelled with the anti-phospho-S334 antibodies (S490 in TIS11d). Again, this result is likely due to the perfect conservation of the antigenic C-Terminal peptide between TIS11b and TIS11d. Importantly, co-treatment of BAC with ACTH and the PKA inhibitor H89 abrogated TIS11b induction as well as TIS11b phosphorylation at S54 and S334.

In parallel with these experiments, we sought to evaluate whether phosphorylation of S54 and S334 could be observed in another cellular context. Besides hormones, hypoxia is another regulator of TTP protein family expression (*Sinha et al., 2009; Kim et al., 2010*). In particular, TIS11b expression was shown to be increased in VHL-expressing renal cell carcinoma in response to hypoxia (*Sinha et al., 2009*). We choose the A549 lung carcinoma cell line where low levels of TIS11b mRNA have been reported (*Carrick & Blackshear, 2007*). To examine whether hypoxia could modulate TIS11b expression and/or phosphorylation at the S54 and S334 residues, A549 cells were exposed to normoxia or hypoxia (1.5 % O<sub>2</sub>) for 2 to 8 h then total cell extracts were analyzed by western blot. A transient but robust increase in TIS11b levels was observed in A549 cells under hypoxia, peaking between 2 h and 4 h of exposure and declining at 8 h (Fig. 3B). This increase in total protein level was accompanied by an increase in the quantity of phosphorylated TIS11b at the S54 and S334 residues. Interestingly, as observed for TIS11b, TIS11d was heavily labelled with anti-phospho-S54-antibodies in response to hypoxia. By contrast, TIS11d was labelled with anti-phospho-S334-antibodies (S490 in TIS11d) in both normoxic and hypoxic conditions.

As the phosphorylation status of TIS11b protein family depends on dynamic equilibrium of kinase and phosphatase activities (*Benjamin et al., 2006; Brook et al., 2006; Sun et al., 2007*), we sought to determine whether inhibition of Serine/Threonine phosphatases could impact the level of hypoxia-induced phosphorylation of S54 and S334 within TIS11b. Therefore, A549 cells were exposed to normoxia or hypoxia for 8 h in the absence or in the presence of okadaic acid (OA), a potent inhibitor of protein phosphatases PP1 and PP2A. As shown in Fig. 3C, treatment of normoxic A549 cells by OA led to a dramatic increase in total TIS11b protein levels in



normoxia and this upregulation was even more pronounced in response to hypoxia. In addition, OA caused accumulation of low mobility bands of TIS11b that were shifted upward under hypoxia (Fig. 3C). OA-induced TIS11b protein levels in normoxia were highly phosphorylated at S54 (Fig. 3C). Under hypoxia, OA led to a disappearance of TIS11b high-mobility bands, presumably corresponding to hypophosphorylated forms of the protein. Importantly, OA-induced TIS11b under hypoxia was heavily phosphorylated at S334 with appearance of low mobility-forms of the protein (Fig. 3C).

Altogether, these observations demonstrate that the S54 and S334 residues of TIS11b as well as their counterparts in TIS11d are phosphorylation target sites. Moreover, these sites are regulated under different physiological conditions and in different cell types, suggesting that they may play a wide biological role in the function of TIS11b.

#### *S54 and S334 regulate TIS11b mRNA-destabilizing activity*

Like TTP, TIS11b is a direct substrate of the kinases PKB (Serines 90/92/203) (*Schmidlin et al., 2004; Benjamin et al., 2006*) and MK2 (Serines 54/92/203) (*Maitra et al., 2008*). PKB- and MK2-induced phosphorylation of TIS11b at Serines 90/92/203 was reported to exert an inhibitory on TIS11b-mediated ARE mRNA decay (*Schmidlin et al., 2004; Stoecklin et al., 2004; Benjamin et al., 2006*). To determine the role of S54 and S334 in TIS11b function, we constructed different mutants by replacing S54 and S334 by either an alanine, to block phosphorylation, or by an aspartate, to mimic phosphorylation. The mRNA-destabilizing activity of wild type TIS11b (WT), TIS11b S54A, TIS11b S54D, TIS11b S334A, TIS11b S334D and TIS11b S54A/S334A mutants was assessed using a Luciferase-VEGF 3'UTR fusion construct (*Ciais et al., 2004*) of which activity is driven by ARE-containing VEGF 3'UTR. As shown in Fig. 4A, overexpression of WT TIS11b decreased luciferase activity to 60 % of controls while substitution of S54 by an alanine (S54A) decreased luciferase activity to 40 %. By contrast, the activity of the mutant S54D was not statistically different from that of the WT. Interestingly, when the S334 was replaced by an alanine (S334A), luciferase activity was modestly decreased (78 % of controls). Replacing S334 by an aspartate (S334D) decreased luciferase activity to ~ 60%, as observed for the WT. The double mutant (S54A/S334A) displayed a luciferase activity to 70 % of control. However, the low activity of this mutant was correlated to its consistent low expression level when compared to the single mutants. We next examined the effect of TIS11b mutant expression on endogenous VEGF mRNA steady state levels. Overexpression of each mutant followed by northern blot analysis revealed that both S54A and S334D mutants were

more efficient in decreasing VEGF mRNA than the WT (Fig. 4B and 4C) whereas the mutant S334A was less active. Interestingly, while the activity of the mutant S334D was not significantly different from that of the WT in reporter gene assays (Fig. 4A), this mutant appeared more active in decreasing endogenous VEGF mRNA. Altogether, these results indicate that the S54A mutant is more efficient in triggering mRNA decay than the WT and therefore that phosphorylation on S54 negatively modulates TIS11b mRNA-destabilizing activity. By contrast, the S334A mutant is less active than the WT suggesting that phosphorylation of the residue S334 potentiates TIS11b function.

#### *TIS11b protein stability is regulated by S54 and S334*

Importantly, TIS11b protein turnover and mRNA decay activity were shown to be regulated by PKB at the same phosphorylation sites (*Benjamin et al., 2006*). We therefore investigated whether S54 and S334 were involved in the regulation of TIS11b protein half-life. COS7 cells were transfected with wild type TIS11b, (S54A), (S54D), (S334A), (S334D) or (S54A/S334A) mutants then translation was arrested by cycloheximide at the time points indicated. Western blot analyses of total cell extracts from independent experiments showed that the half-life of the WT TIS11b was of  $4 \pm 0.6$  h (Fig. 5A). Notably, the slower-migrating bands corresponding to more highly phosphorylated forms of TIS11b appear to decay less rapidly. The mutant (S54A) displayed a shorter half-life of  $2 \pm 0.8$  h while the (S54D) mutant half-life was similar to that of the WT ( $4.1 \pm 0.4$ ). The S334A mutant was less stable ( $3.5 \pm 0.5$  h) than the WT. By contrast, TIS11b protein stability was strikingly increased when the S334 was replaced by an aspartate (S334D), with a half-life around  $7.7 \pm 0.3$  h. Interestingly, the half-life of the double mutant (S54A/S334A) was in between the half-lives of (S54A) and (S334A) mutants ( $3.0 \pm 0.6$  h). These results demonstrate that in addition to their regulatory role in TIS11b-mediated mRNA decay, S54 or S334 are critical residues in the control TIS11b turnover.

#### *S54 and S334 modulate the proteasome-dependent degradation of TIS11b*

TIS11b degradation has been previously shown to proceed through the proteasome (*Benjamin et al., 2006*). Our observation that (S54A) and (S334A) mutants are rapidly degraded suggests that these serines are key residues in the regulation of TIS11b protein stability. To examine the molecular mechanisms of TIS11b and TIS11b mutant degradation, we assessed their stability in

the presence of the proteasome inhibitor MG132 in COS7 cells in overexpression experiments. With the exception of the mutant (S334D), WT TIS11b as well as the other phosphorylation mutants accumulated within the cells, indicating that their degradation is mediated, at least partially, by the proteasome (Fig. 5B). The S334D mutant was less sensitive to MG132-mediated blockade suggesting that this highly stable mutant might be degraded by an alternative degradation pathway.

#### *Mimicking a phosphorylation at S54 changes TIS11b subcellular localization*

TIS11b and related proteins are nucleocytoplasmic shuttling proteins. They all contain a specific nuclear localization sequence (NLS) located between the two zinc fingers. In addition to these import sequences, nuclear export signals (NES) are present in the N-terminus of TTP or in the C-termini of both TIS11b and TIS11d. Importantly, the S334 residue is located in the NES of TIS11b. As the function of TTP family members in mRNA decay is known to occur in the cytoplasm, we hypothesized that changes in the subcellular distribution of TIS11b mutants could explain the differential regulation of VEGF mRNA decay by these mutants (Fig. 4A and 4C), in particular for the mutant (S334A) (Fig. 4). We therefore generated HeLa-tet-off cells stably expressing WT or mutant TIS11b. Transcription of TIS11b was controlled by a tetracycline regulatory promoter, which allows for gene expression when HeLa cells were cultured in the absence of the doxycycline. Immunofluorescence analyses using an antibody recognizing specifically TIS11b (and not TIS11d) revealed that WT TIS11b was distributed throughout the cytoplasm and the nucleus of HeLa cells (Fig. 6). The mutants (54A), (334A) and (334D) displayed a similar pattern of subcellular localization to that of the WT. Of note, (334D) mutant was expressed at higher levels than the WT or the other mutants, possibly due to its enhanced protein stability (Fig. 6). Interestingly, the expression of the mutant (54D) was restricted to the cytoplasm, suggesting that this mutant was not able to translocate to the nuclear compartment or is sequestered in the cytoplasm. Altogether, these observations indicate that the differences that we observed in the mRNA-destabilizing activities of TIS11b mutants compared to the WT are unlikely related to a distinct subcellular localization.

### *Mimicking a phosphorylation at S54 promotes the binding of TIS11b to endogenous 14-3-3 proteins*

Serines S90, S92 and S203 were previously shown to be important for phosphorylation-dependent binding of TIS11b to 14-3-3 proteins (*Benjamin et al., 2006*). Mutation of all these three sites to alanine abolished TIS11b-14-3-3 interaction. In addition, the triple mutant (S54A/S92A/S203A) failed to interact with 14-3-3. We investigated the role of S54 and S334 alone or in conjunction with each other in the interaction of TIS11b with 14-3-3 proteins (Fig.7A). Transfection experiments followed by TIS11b immunoprecipitation and western blot analysis of 14-3-3 proteins showed that a weak basal interaction of WT TIS11b with a 14-3-3 protein doublet (~30 kD) was detected in COS7 cells, suggesting a basal activity of an unidentified kinase. The level of immunoprecipitated 14-3-3 was markedly increased for the S54D mutant, indicating that phosphorylation of this serine is involved in TIS11b/14-3-3 protein interaction. However, the mutant (S54A) also interacts with 14-3-3, thus confirming that other phosphorylated serine residues within TIS11b sequence are implicated in TIS11b/14-3-3 interaction. Both (S334A) and (334D) mutants interact with 14-3-3 with an enhanced binding to the higher molecular weight species. Remarkably, this enhanced selective interaction was impaired in the presence of the double mutant (S54A/334A). These results suggest that S54 regulates the binding of TIS11b to 14-3-3 and that S334 does not play a major role in this process. The preferential localization of the (S54D) mutant in the cytoplasm (Fig. 6) is likely due to its sequestration by 14-3-3 proteins.

### *TIS11b interacts with endogenous CNOT1*

TTP, the prototypical member of TIS family as well as TIS11b were shown to activate mRNA decay by recruiting the deadenylase complex including Ccr4, Caf1a as well as the decapping enzyme Dcp2 and the exonuclease Xrn1 (*Lykke-Andersen & Wagner, 2005; Marchese et al., 2010; Clement et al., 2011; Sandler et al., 2011; Fabian et al., 2013*). However, there is some controversy regarding the location of the domain of interaction within TTP. While some studies concluded that the Ccr4-NOT complex interacts with the N-terminal part of TTP (*Lykke-Andersen & Wagner, 2005*), a more recent data reported interactions with the C-terminal domain of TPP (*Sandler et al., 2011; Fabian et al., 2013*). Recently, a crystal structure of the NOT1 subunit associated with a C-terminal peptide of TTP identified a TTP-Ccr4-NOT interaction motif (TTP-CIM, Fig. 7B). TTP-CIM is highly conserved between TTP family members and

comprises the S334 residue (S323 in TTP, Fig. 7A). To investigate whether TIS11b interacts with CNOT and whether S344 is an important residue in this interaction, we transfected HEK 293 cells with WT TIS11b or with the S334A or S334D mutants and performed co-immunoprecipitation assays. Fig. 7C shows that TIS11b exists in complex with endogenous CNOT1 in cell extracts. In addition, the mutants S334A and S334D also interact with CNOT1. However, we observed in independent experiments that mimicking phosphorylation at S334 caused reduced association with CNOT1. These results indicate that the S334 residue is involved in the interaction of TIS11b with CNOT1, thus confirming the importance of this highly conserved serine among TIS11 family members.

### ***Discussion***

Upon hormone stimulation, adrenocortical cells exhibit a transient induction of VEGF mRNA mainly through post-transcriptional mechanisms. These regulations involve antagonistic roles of the mRNA-stabilizing protein HuR and the mRNA-destabilizing protein TIS11b (BRF1/ZFP36L1) which both, bind to AU-rich elements in the 3'-untranslated region of VEGF mRNA (Ciais *et al.*, 2004; Cherradi *et al.*, 2006). TIS11 proteins are targets of several kinases which modulate their mRNA-decay promoting activity as well as their protein stability (Brooks *et al.*, 2004; Baou *et al.*, 2009; Brooks & Blackshear, 2013). In this report, we provide evidence that hormone-stimulated expression of VEGF mRNA is accompanied by a PKA-dependent phosphorylation of the N-terminal serine 54 in the early phase of stimulation while a phosphorylation of the C-terminal serine 334 occurs in the late phase. We subsequently focused on both serines and demonstrated that mimicking or preventing their phosphorylation impact TIS11b function in mRNA decay as well as TIS11b protein turnover. Unexpectedly, while MAPK-activated protein kinase-2 (MK2)- and protein kinase B (PKB)-mediated phosphorylations of TIS11b at specific serines were reported to inhibit its mRNA-destabilizing activity (Stoecklin *et al.*, 2002; Schmidlin *et al.*, 2004; Benjamin *et al.*, 2006; Maitra *et al.*, 2008), we found that mimicking a phosphorylation at serine 334 potentiates TIS11b function, suggesting that the regulatory role of TIS11b phosphorylation in mRNA decay may be more complex than anticipated.

In adrenocortical endocrine cells, ACTH is a major physiological agonist which mainly activates the cAMP messenger system and the protein kinase A to induce steroid hormone biosynthesis. We have previously shown that ACTH triggers a transient increase in VEGF mRNA levels which involves TIS11b in the decay phase of VEGF mRNA expression (Chinn *et al.*, 2002;

*Cherradi et al., 2006*). Our data *in vivo* show that the hormone also induces a rapid phosphorylation of TIS11b which is significantly impaired in the presence of H89. We subsequently used prediction software to identify putative PKA phosphorylation sites. As previous studies have reported that the N-terminal and the C-terminal domains of TIS11 proteins family might have different functions in the recruitment of the mRNA decay machinery (*Lykke-Andersen & Wagner, 2005; Sandler et al., 2011*), we focused our attention on S54 and S334 which are located in the N-terminal and the C-terminal domains of TIS11b, respectively. Using peptides fragments containing each of S54 and S334 residues, we found that these sites are indeed phosphorylated by PKA *in vitro*, although to a lesser extent at S334. Substitution of S54 or S334 by an alanine in recombinant proteins expressed in *E. coli* prevented PKA-mediated phosphorylation, thus confirming that they are PKA target sites. Recently, mass spectrometric analyses revealed that S54 was phosphorylated by MK2 *in vitro* in conjunction with S92 and S203 (*Maitra et al., 2008*). Interestingly, as the PKA consensus phosphorylation site RXXS is embedded within the MK2 consensus site (HyXRXXSXX, where X is any amino acid and Hy is any hydrophobic amino acid), a phosphorylation of this serine residue by both kinases is not surprising. Nevertheless, the role of S54 alone in the regulation of TIS11b-dependent mRNA decay has not been investigated. Importantly, using anti-phospho-specific antibodies, we demonstrate that S54 and S334 are phosphorylated *in vivo* not only in hormonally-regulated cellular context but also during cancer cell response to hypoxic stress. These observations suggest that both serines may play an important role in the regulation of TIS11b-dependent mRNA decay. Remarkably, phosphorylation at S334 occurs in the late phase of TIS11b induction by ACTH which is associated with decreased levels of VEGF mRNA, suggesting that S334 is a regulatory phosphosite which is required for the destabilization of VEGF mRNA by TIS11b. The kinase(s) involved in the phosphorylation of S54 and S334 in response to hypoxia remains to be identified. Nevertheless, we observed that inhibition of phosphatase PP2A by okadaic acid increased the phosphorylation of both serines. These data are in agreement with previous studies showing that PP2A inhibition increased TTP phosphorylation in macrophages (*Sun et al., 2007*) and indicate that the phosphorylation status of TIS11 proteins depends on dynamic equilibrium of kinase and phosphatase activities. It is worth mentioning that the role of S334 in the regulation of TIS11b function has not been previously addressed. A recent study reporting the mammalian target of rapamycin (mTOR)-regulated phosphoproteome identified S334 as target residue in TIS11b (*Hsu et al., 2011*).

Phosphorylation of TIS11b by the protein kinase B (PKB) at Ser92 and Ser203 abrogated mRNA decay of an IL-3 ARE-containing probe and led to TIS11b binding to 14-3-3 proteins as well as

to TIS11b protein stabilization (*Schmidlin et al., 2004; Benjamin et al., 2006*). More recently, using *in vitro* kinase assays, *Maitra et al* reported that phosphorylation of TIS11b by MK2 together at Serine 54, Serine 92 and Serine 203 did not affect its ability to bind to ARE or to recruit mRNA degradation enzymes but did nonetheless inhibits its ability to destabilize ARE-containing mRNA (*Maitra et al., 2008*). We therefore investigated the role of PKA phosphosites in TIS11b-mediated VEGF mRNA decay by replacing Serine 54 and Serine 334 by an alanine. We found that substitution of S54 potentiates TIS11b activity in VEGF-3'UTR-driven luciferase activity as well as in northern blot analyses of endogenous VEGF mRNA, suggesting that phosphorylation of S54 negatively regulates TIS11b function. This result is in agreement with studies by *Maitra et al* (*Maitra et al., 2008*), reporting that phosphorylation of S54 is likely necessary for the inhibition of TIS11b-dependent mRNA decay. By contrast, we show for the first time that preventing phosphorylation of S334 through its alanine substitution impairs TIS11b function. Intriguingly, the mutant TIS11b-S334D even proved to be more active than wild type TIS11b in triggering VEGF mRNA degradation. Given the striking number of phosphorylatable serines in TIS11b (49 out of 338 amino acids), in particular in the C-terminal domain of the protein (Supplementary figure 1), we hypothesize that TIS11b function in ARE-mediated mRNA decay could be modulated by antagonistic phosphorylation events.

We tested the impact of S54 and S334 mutations on a second aspect of TIS11b biology, namely the phosphorylation-dependent regulation of TIS11b turnover. We determined the half-life of TIS11b which is about 4 h. These results are similar to those obtained by *Benjamin et al.*, even though the half-life of TIS11b in their experiments was slightly shorter (around 3h) (*Benjamin et al., 2006*). Although highly unstable compared to the wild type TIS11b, the mutant TIS11b-S54A displayed the highest mRNA-destabilizing activity, suggesting that its intrinsic activity is probably higher than the one measured in transfection experiments. The substitution of S334 by an alanine did not change significantly TIS11b half-life, indicating that the altered activity of TIS11b-S334A is not due to protein instability. Importantly, the mutant TIS11b-S334D displayed a markedly enhanced stability when compared to TIS11b suggesting that the S334A mutation would favour a long-lasting action of the protein. In agreement with previous studies (*Benjamin et al., 2006*), proteasome blockade with the specific inhibitor MG-132 led to stabilization of pre-existing TIS11b, indicating that TIS11b undergoes degradation through the proteasome pathway. On the other hand, all the mutants bearing an alanine substitution (S54A, S334A and S54A/S334) were highly stabilized when the proteasome was blocked. These data are in line with their short half-lives. By contrast, the mutant S334D was resistant to proteasome

blockade, suggesting that its degradation might occur through alternative mechanisms, such as the lysosomal pathway.

The recruitment of Ccr4-Not deadenylase complexes by TIS11 proteins is emerging as a major mechanism in ARE-mediated mRNA decay. Phosphorylation of TTP by MK2 impairs its activity by preventing deadenylase recruitment (*Marchese et al., 2010; Clement et al., 2011*). In addition, phosphorylated TTP and TIS11b are sequestered by the 14-3-3 adaptor proteins (*Chrestensen et al., 2004; Schmidlin et al., 2004; Stoecklin et al., 2004; Benjamin et al., 2006; Sun et al., 2007*). Phosphorylation of serine 92 and serine 203 has been shown to protect TIS11b from dephosphorylation, promote cytoplasmic localization and protects TIS11b from proteasomal degradation. Interestingly, we observed that mimicking phosphorylation at S54 (TIS11b-S54D) retained TIS11b in the cytoplasm and increased its association with 14-3-3 proteins. These results are in line with previous studies by others and point at S54 as an additional regulatory site in TIS11b/14-3-3 protein interaction. In attempts to identify the role of S334 in TIS11b-mediated mRNA decay, we conducted co-immunoprecipitations experiments of TIS11b and components of the Ccr4-Not complex. Very interestingly, *Fabian et al.* reported recently a high-resolution crystal structure of the TTP-CNOT1 complex in which they identified the serine 323 of TTP (Serine 334 in TIS11b) as a critical serine for the interaction of TIS11b with CNOT1 (*Fabian et al., 2013*). It was postulated that phosphorylation of this serine would probably impair TTP-CNOT1 binding. Our results showing that the association of the mutant TIS11b-S334D with CNOT1 is decreased as compared to that of the wild type TIS11b confirm this hypothesis. However, this perturbed association is not correlated to a decrease in TIS11b-mediated mRNA decay as we observed that TIS11b-S334D mutant is highly active in decreasing VEGF-3'UTR-driven luciferase activity as well as endogenous VEGF mRNA steady state levels. Therefore, we hypothesize that recruitment of other untested mRNA decay enzymes is increased upon phosphorylation of serine 334. Alternatively, unknown additional critical factors may regulate the activity of the mRNA decay machinery.

Altogether, our results led us to propose a model for TIS11b function in cAMP-regulated VEGF mRNA expression (figure 8). Upon activation of cAMP signalling pathway, TIS11b protein is induced and is concomitantly phosphorylated at S54 by PKA, binds to 14-3-3 proteins and is prevented from participating to VEGF mRNA decay, in addition to be protected from degradation. This allows HuR-triggered stabilization of VEGF mRNA (*Cherradi et al., 2006*). At the end of the stabilizing signal, TIS11b undergoes concomitant dephosphorylation at S54 and phosphorylation at S334 and is active in promoting the decay of VEGF mRNA.



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## Legends of the figures

### Figure 1: ACTH induces a cAMP-dependent expression and phosphorylation of TIS11b.

- (A) Primary cultures of BAC cells were treated with 10 nM ACTH in the absence or in the presence of 5  $\mu$ M H89 for the indicated periods of time. TIS11b and VEGF protein levels of whole cell extracts (20  $\mu$ g) were analysed by Western blot. The blot was subsequently probed with an anti- $\beta$ -Actin to assess equal loading of samples.
- (B) Quantification of TIS11b, VEGF mRNA and protein levels from independent experiments (n=3-5, means  $\pm$  s.e.m). Protein level values were normalized to actin values and are expressed as percentage of control values at time 0 (unstimulated cells).
- (C) Time-course of TIS11b phosphorylation in BAC cells stimulated with 10 nM ACTH in the presence of  $^{32}$ P-orthophosphate and in the presence or absence of H89. TIS11b was immunoprecipitated from cell extracts and resolved by SDS-PAGE then visualized by autoradiography. One representative experiment of four is shown.
- (D) Phosphorylation of recombinant TIS11b by the catalytic subunit of PKA. Purified GST-TIS11b fusion protein was produced as described previously (Ciais *et al*, 2004). Increasing doses of GST-TIS11b were subjected to *in vitro* phosphorylation as described in Material and Methods. Vect: 30  $\mu$ g of protein extract from *E. coli* transformed with empty vector (pGEX) were used as control in the phosphorylation assay. \*, degradation product consistently detected in purified GST-TIS11b samples.

### Figure 2: The serines S54 and S334 are PKA-target sites *in vitro*.

- (A) Schematic representation of full length (FL) TIS11b and TIS11b truncated forms used in *in vitro* phosphorylation experiments (NZn and ZnC). The tandem zinc fingers are shown in gray.
- (B) Dose-dependent phosphorylation of FL TIS11b, NZn and ZnC by the catalytic subunit of PKA. Recombinant purified proteins were used in kinase assay and resolved by SDS-PAGE. Protein labelling was visualized by autoradiography (upper panel). The low molecular weight bands migrating in TIS11b samples are degradation products of the protein (Planel *et al.*, 2010). The lower panel is the corresponding silver stained gel showing that the NZn truncated protein is less abundant than the FL and the ZnC. Shown on the right is the quantification of three independent experiments. Relative ratios of the band intensity of phosphorylated protein/total purified protein are reported (n=3, mean  $\pm$  s.e.m).
- (C) Sequence alignment of conserved amino acid within the N-terminus and the C-terminus between TIS11b, TIS11d and TTP showing PKA consensus motives (highlighted in red, RRHS and RLS). These motives are also conserved between species in TIS11b sequence and harbour the serine 54

and the serine 334 (*hs*, *homo sapiens*; *Bt*, *bos taurus*; *Rt*, *rattus norvegicus*; *mm*, *mus musculus*; *xl*, *xenopus laevis*).

- (D) Dose-dependent *in vitro* phosphorylation of synthetic N-terminal and C-terminal peptides of TIS11b by the catalytic subunit of PKA. Both peptides contain the PKA consensus motives RRHS or RLS (S54 and S334 are shown in bold). Phosphorylated peptides were resolved by SDS-PAGE (15 %) and visualized by autoradiography.
- (E) *In vitro* phosphorylation of recombinant WT TIS11b and TIS11b mutants (S54A) and (S334A). Protein extracts from *E. coli* transformed with empty vector (pET15b) served as control (Vect). The lower panel shows a western blot analysis of TIS11b and its mutants in the phosphorylated fractions using an anti-Flag antibody. PKA-mediated phosphorylation of TIS11b was significantly impaired when S54 was replaced by an alanine while it was altered to a lesser extent for the S334A mutant (right panel, n=3, mean  $\pm$  s.e.m, \*\* and \*\*\*, significantly different from the WT with p<0.01 and p<0.001, respectively).
- (F) COS7 cells were transfected with pTarget-TIS11b, pTarget-TIS11b (S54A) or pTarget-TIS11b (S334A) plasmids. To assess the specificity of the anti-phospho-TIS11b antibodies, cell lysates were analyzed by western blot using the anti-phospho-S54- or anti-phospho-S334-TIS11b antibodies.

**Figure 3: TIS11b is phosphorylated at serine 54 and serine 334 *in vivo*.**

- (A) BAC cells were stimulated with ACTH in the presence or in the absence of H89 for the indicated periods of time. Total cell extracts were probed for total TIS11b/TIS11d, phospho-S54- and phospho-S334-TIS11b. Due to the conserved PKA consensus motives (RRHS and RLS, see Fig. 2) within TIS11b and TIS11d sequences, both phosphoproteins were detected. Note the shift of TIS11b towards high molecular weight species. The asterisk \* indicates the phospho-(S334)-TIS11b species appearing at 6 h post-stimulation by ACTH.
- (B) A549 lung carcinoma cells were exposed to normoxia (0h) or hypoxia for 2, 4, and 8 hours. In the left panels, cells extracts were probed by western blot as described in (A). In the right panels, A549 cells were exposed to normoxia or hypoxia for 8 hours in the absence or in the presence of okadaic acid, an inhibitor of the phosphatases PP2A/PP1. Note the phospho-(S334)-TIS11b species appearing under hypoxia in the presence of OA (indicated by \*). Blots were subsequently probed with an anti- $\beta$ -Actin to assess equal loading of samples.

**Figure 4: Serine 54 and Serine 334 regulate TIS11b ARE-mediated mRNA decay.**

- (A) COS7 cells were co-transfected with pLuc-3'UTR and pTarget plasmids encoding wild type TIS11b (WT), TIS11b (S54A), TIS11b (S54D), TIS11b (S334A), TIS11b (S334D) or TIS11b (S54A/S334A) mutants as described in Material and Methods. Firefly/Renilla luciferase activities

of cell lysates were measured as described in Material and Methods. Results are expressed as relative light units of Firefly luciferase activity over relative light units of Renilla luciferase activity, and are represented as a percentage of the luciferase activity in control cells transfected with empty pTarget plasmid. Luciferase activity is inversely proportional to TIS11b mRNA-destabilizing activity. Transfections were performed in triplicate and values are means  $\pm$  s.e.m. from 5 to 7 independent experiments. Values of luciferase measured for TIS11b mutants were compared to the value of WT TIS11b. \*, significantly different from WT with  $p < 0.05$ ; ns: no significant difference when compared to the WT. The lower panel is a representative western blot analysis of overexpressed TIS11b proteins showing that equivalent amounts of TIS11b were recovered except for the double mutant S54A/S334A which is consistently less expressed.

- (B) Northern blot analysis of VEGF mRNA in COS7 cells transfected as in (A).
- (C) Quantification of VEGF mRNA steady state levels in 3 independent experiments. Note that TIS11b VEGF mRNA-destabilizing activity is potentiated when the S54 residue was replaced by an alanine while it was altered when the serine S334 was replaced by an alanine. \*, \*\*, significantly different from WT with  $p < 0.05$  and  $p < 0.01$ , respectively; ns: no significant difference when compared to the WT.

**Figure 5: Serine 54 and Serine 334 regulate TIS11b protein stability.**

- (A) COS7 cells were co-transfected with 10 ng of pTarget plasmids encoding wild type TIS11b (WT), TIS11b (S54A), TIS11b (S54D), TIS11b (S334A), TIS11b (S334D) or TIS11b (S54A/S334A) mutants. The half-life of the different forms of TIS11b was determined in the presence of the translation inhibitor cycloheximide as reported in Material and Methods. The graphs are quantification of the western blots shown on the left. TIS11b protein levels were normalized to Actin levels and plotted as a percentage of the initial value against time. The numbers on the right indicate the half-lives calculated from 4 independent experiments. The most unstable TIS11b protein is the S54A mutant.
- (B) WT TIS11b and TIS11b phosphorylation mutants are differentially stabilized in the presence of the proteasome inhibitor MG-132. COS7 cells were transfected with 10 ng of pTarget plasmids encoding wild type or mutant TIS11b. 24h post-transfection, cells were incubated with the proteasome inhibitor MG-132 (10  $\mu$ M) for 3h and cell lysates were analyzed by western blot.

**Figure 6: Serine 54 but not Serine 334 regulates TIS11b subcellular localization.**

Laser confocal microscopy analysis of HeLa-tet-off cells expressing WT or mutant TIS11b. TIS11b expression was induced in the absence of Doxycycline. Immunofluorescence labelling was performed using antibodies recognizing only TIS11b and not TIS11d as described in Material and Methods. Control cells were transfected with empty pTRE-Tight vector. Note that the S54D mutant localized exclusively to



the cytoplasm. Nuclei were stained with Hoechst. Scale bar, 20  $\mu$ m. Embedded images are of higher magnification.

**Figure 7: Serine 54 is involved in the interaction of TIS11b with 14-3-3 proteins while S334 is involved in the interaction with CNOT1.**

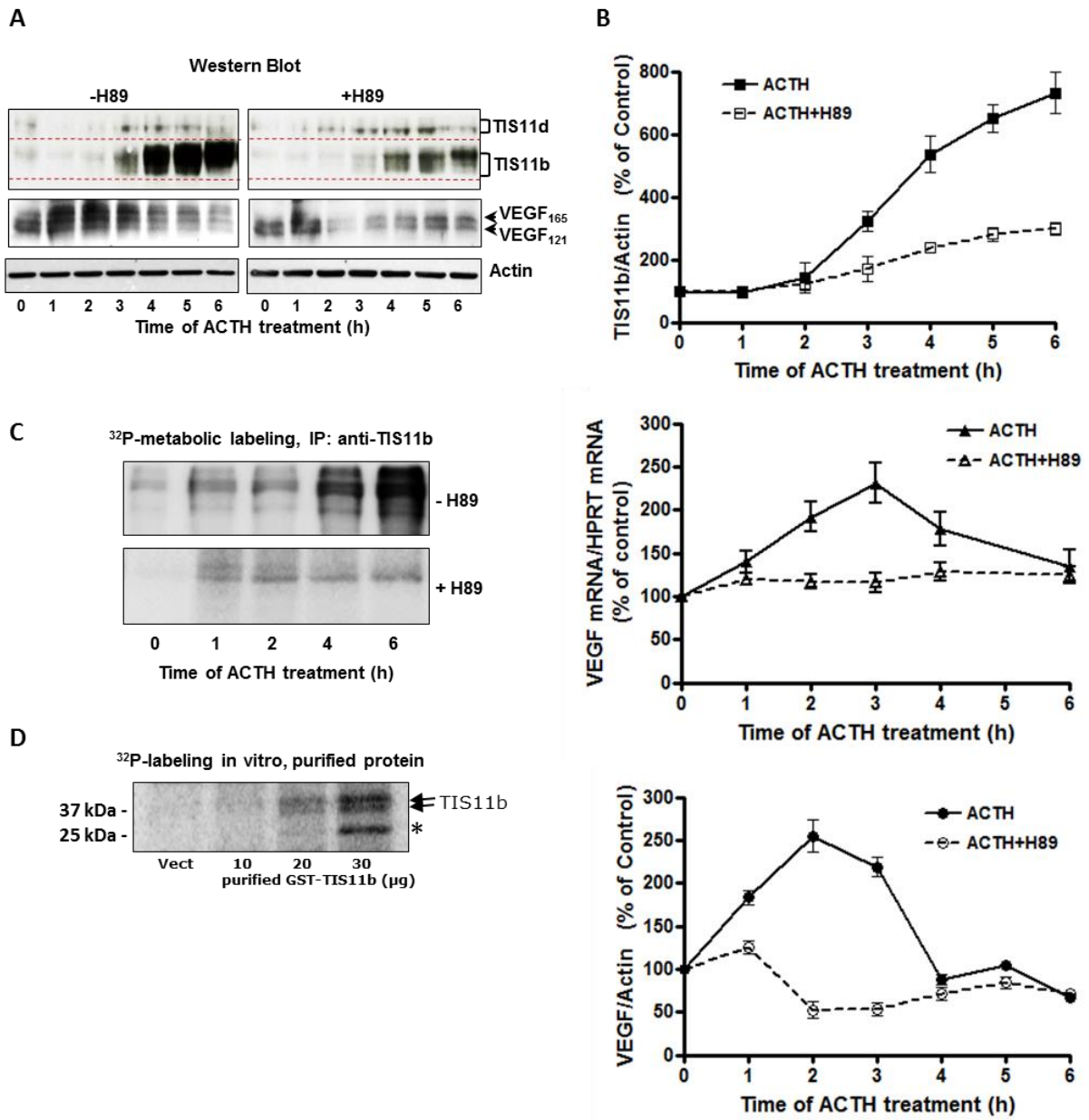
- (A) Western blot showing co-immunoprecipitation assays. Transiently expressed WT or mutant TIS11b were immunoprecipitated from HEK 293 cell extracts and precipitates were probed for the presence of TIS11b and endogenous 14-3-3 proteins. IgG: immunoprecipitation reaction using normal rabbit serum in place of TIS11b antibodies.
- (B) Sequence alignment of the distal C-terminus of TTP family members showing that the TTP-CCR4-NOT interaction motif is highly conserved between TTP family members (*Fabian et al., 2013*).
- (C) Western blot showing co-immunoprecipitation assays performed as described in A. Transiently expressed WT or mutant TIS11b were immunoprecipitated from HEK 293 cell extracts and precipitates were probed for the presence of TIS11b and endogenous CNOT1. Input: 5% total cell extract. The western blot is representative of 3 independent experiments.

**Figure 8: Model for TIS11b function in cAMP-regulated VEGF mRNA decay.**

We identified two putative protein kinase A (PKA) phosphorylation sites, S54 and S334, in TIS11b protein sequence with important roles in protein activity and stability. ACTH stimulation increases intracellular cAMP levels through the action of the G protein Gs and the Adenylyl cyclase (AC). This leads to activation of PKA. (1) Phosphorylation of TIS11b at S54 by PKA does not alter significantly protein stability, but inhibits protein activity. We hypothesize that TIS11b-phospho-S54 is sequestered in the cytoplasm due to enhanced interaction with 14-3-3 proteins. This mechanism would promote VEGF mRNA induction. (2) To turn off VEGF production, phosphorylation of TIS11b at S334 by PKA increases protein stability and activity. Dephosphorylation of both serines by the phosphatase PP2A leads to degradation of TIS11b via the proteasome.

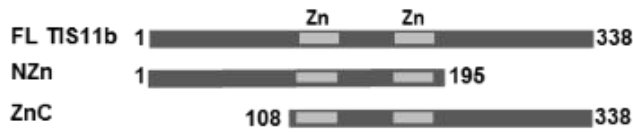
**Supplementary Figure 1:** Phosphorylation Site Predictions in TTP family members according to DISPHOS 1.3 software. S is serine and T threonine. Zn: zinc finger. Note that the N-terminal domain of TTP is enriched in putative phosphorylation sites while the N-terminal domain of TIS11b and Tis11d harbours few putative phosphorylation sites

**FIGURE 1**

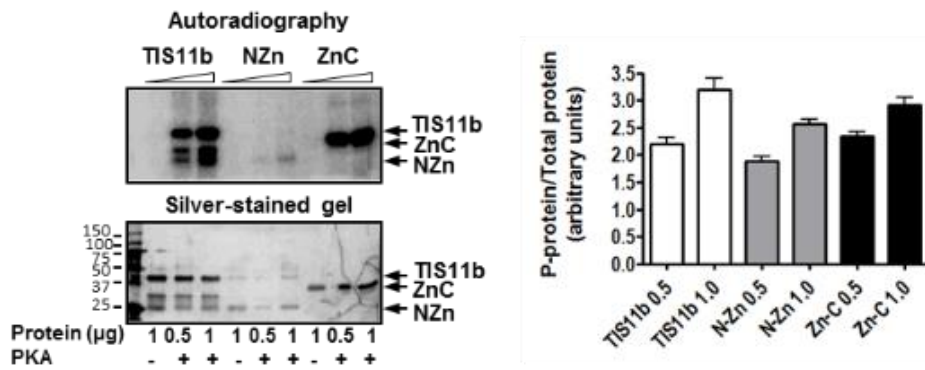


**FIGURE 2**

**A**



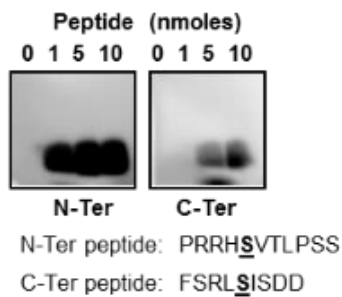
**B**



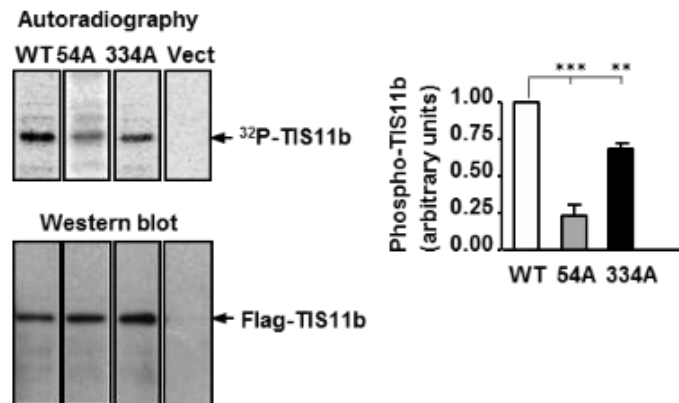
**C**

	N-terminus	C-terminus
<i>Hs</i> Tis11b/BRF-1	48 GFFRRHSVTL 57	321 LDNSRRLPIFSRLSISDD 338
<i>Hs</i> Tis11d/BRF-2	51 GFLRRHSASN 60	477 LDPGRRLPIFSRLSISDD 494
<i>Hs</i> Tis11/TTP	43 --PWSLSPS- 49	316 VAAPRRLPIFNRISVSE- 326
<i>Hs</i> Tis11b/BRF-1	48 GFFRRHSVTL 57	321 LDNSRRLPIFSRLSISDD 338
<i>Bt</i> Tis11b/BRF-1	48 GFFRRHSVTL 57	321 LDNSRRLPIFSRLSISDD 338
<i>Rt</i> Tis11b/BRF-1	48 GFFRRHSVTL 57	321 LDNSRRLPIFSRLSISDD 338
<i>Mm</i> Tis11b/BRF-1	48 GFFRRHSVTL 57	321 LDNSRRLPIFSRLSISDD 338
<i>Xl</i> Tis11b/BRF-1	53 GFFRRHSVTL 62	328 LDTTKRLPIFSRLSISDD 345

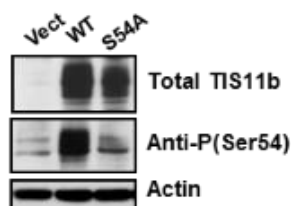
**D**



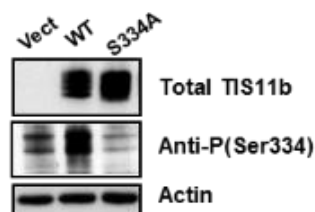
**E**



**F**

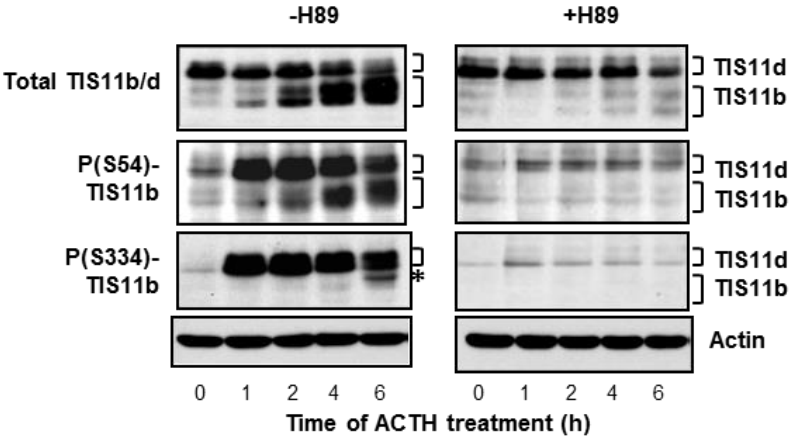


**G**

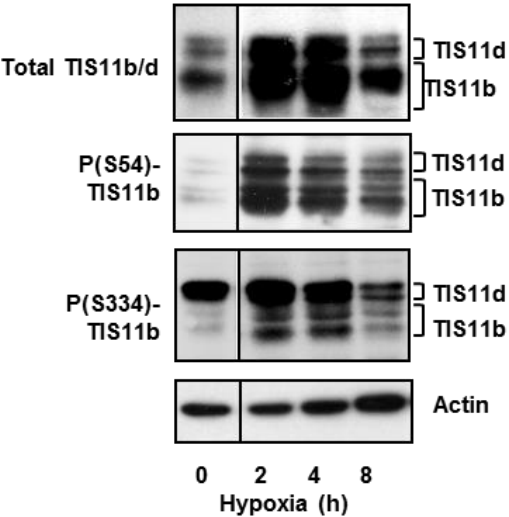


**FIGURE 3**

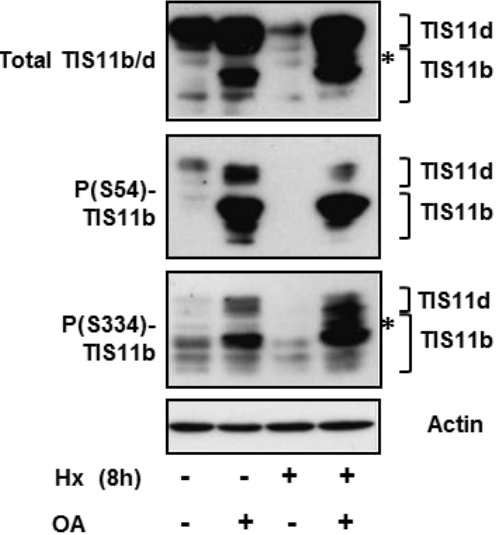
**A**



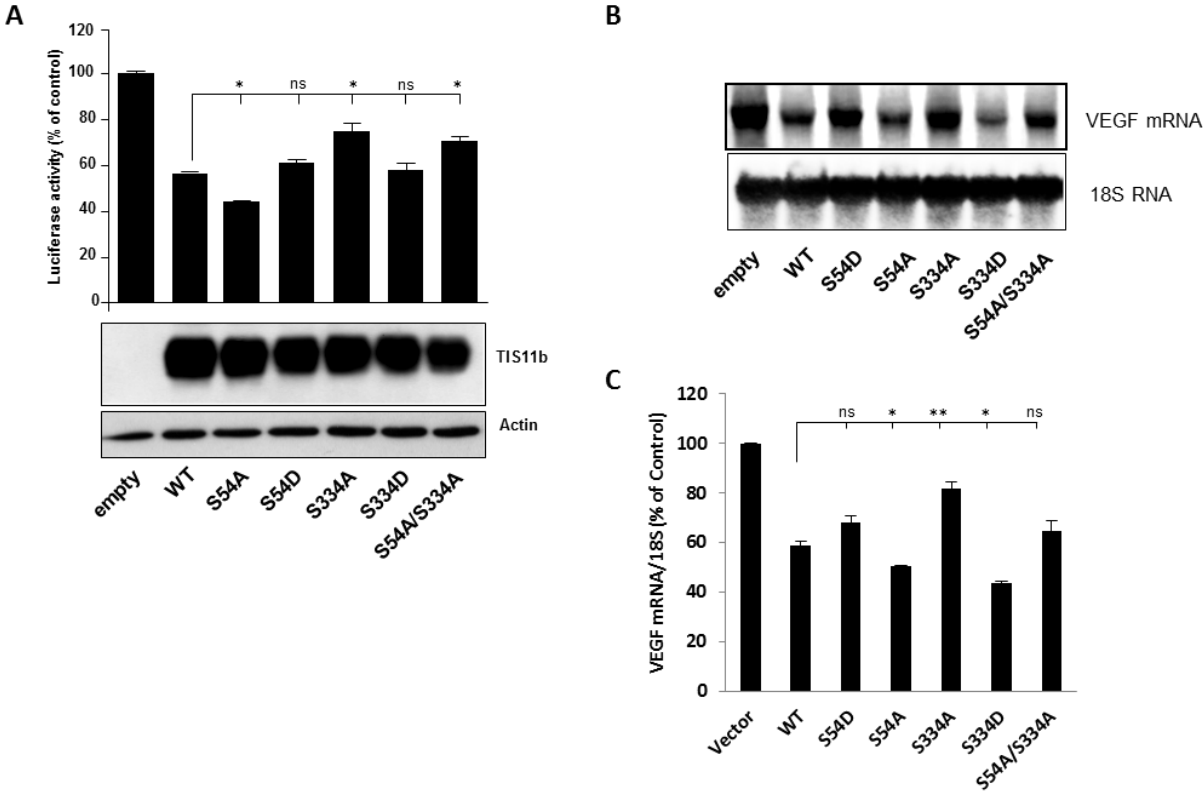
**B**



**C**

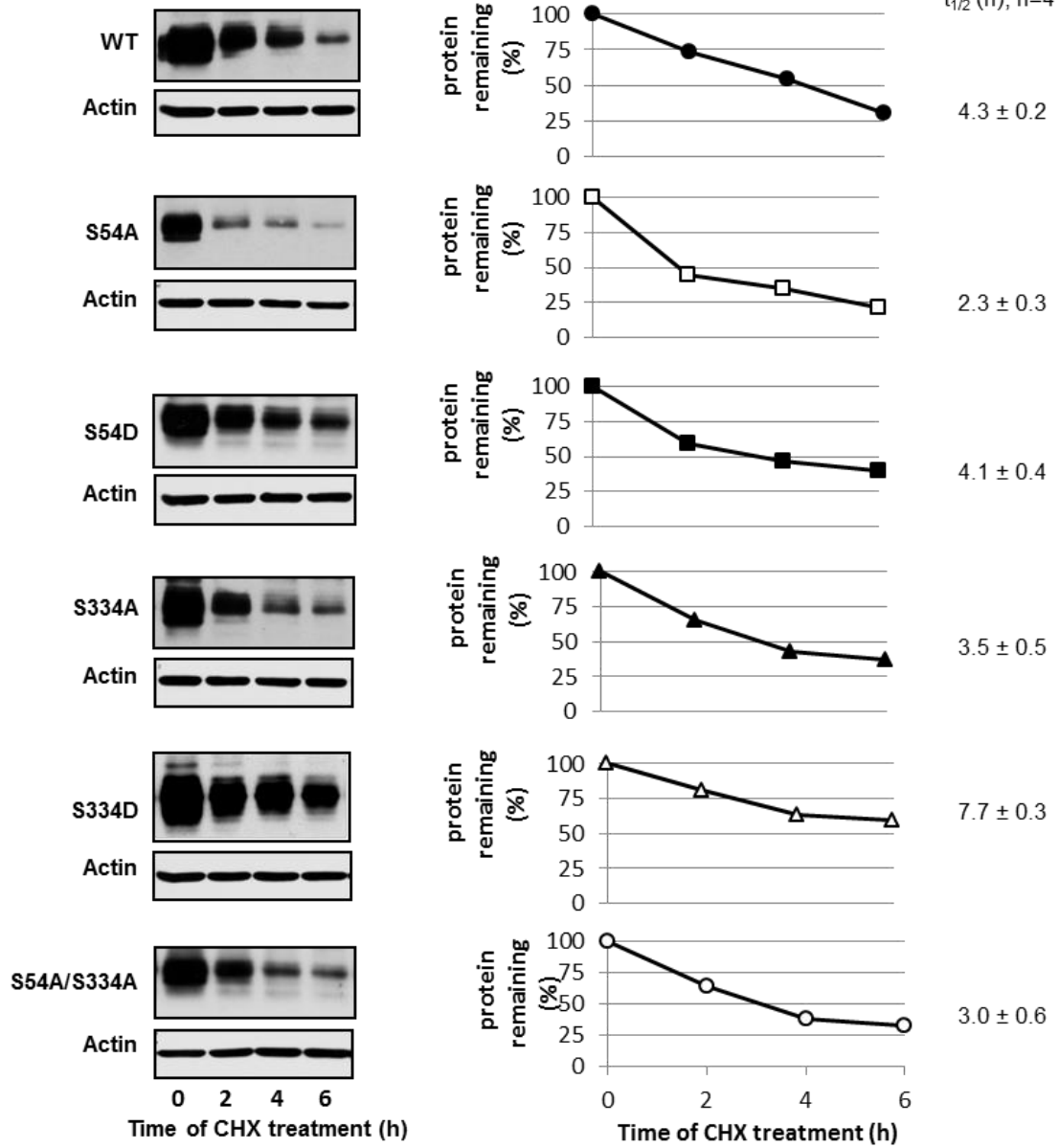


**FIGURE 4**

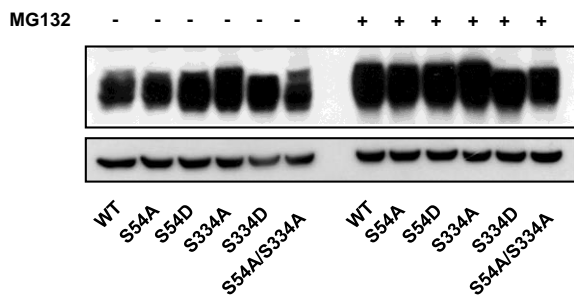


**FIGURE 5**

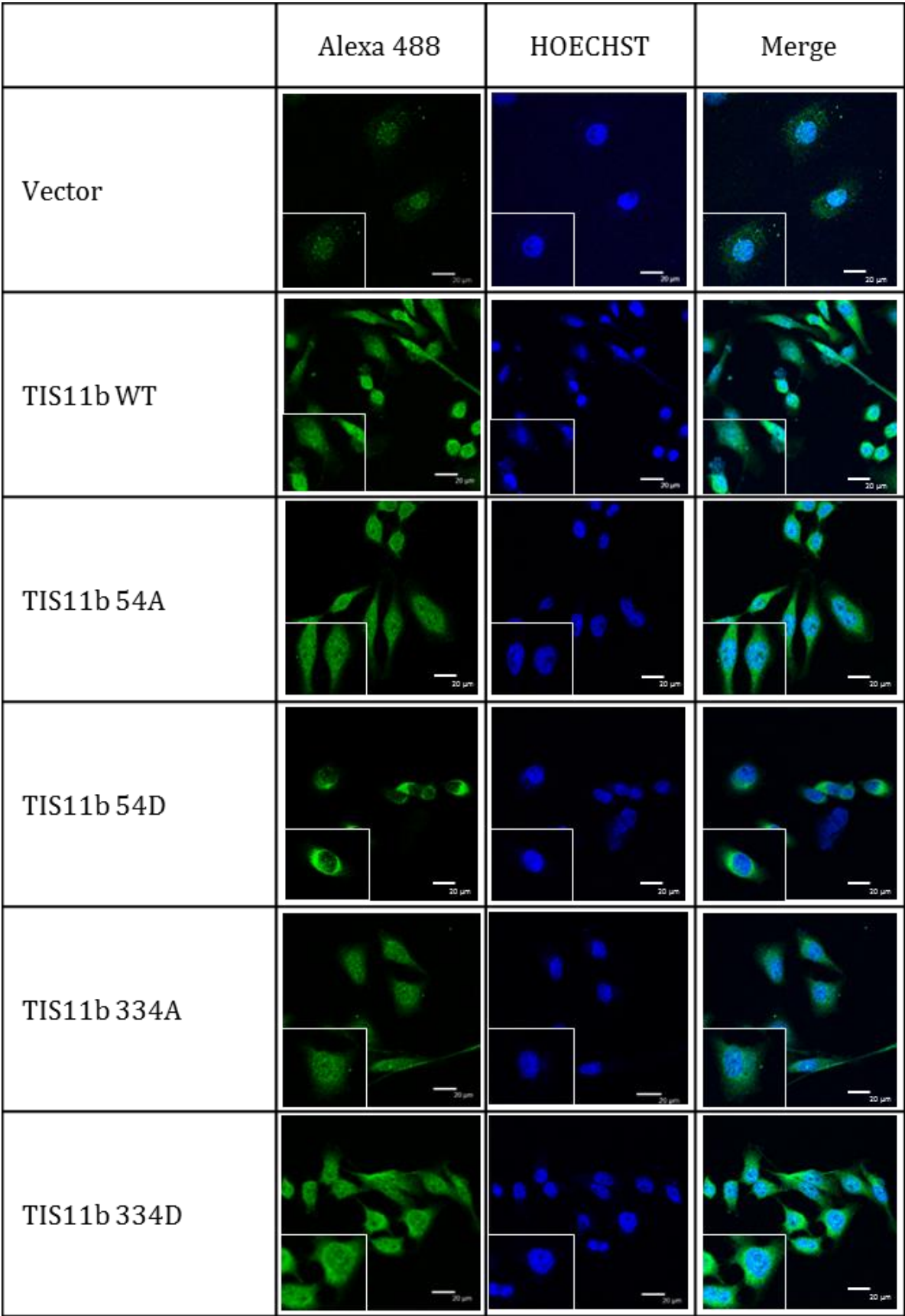
**A**



**B**

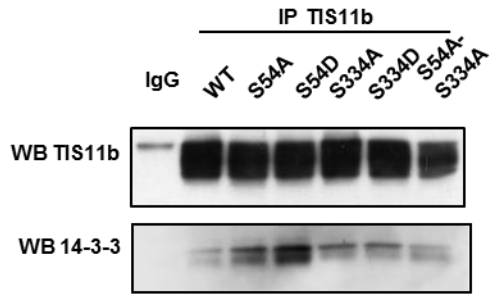


**FIGURE 6**



**FIGURE 7**

**A**



**B**

<i>Hs</i> Tis11b/BRF-1	321	LDNSRRRLPIFS	RLSISDD	338
<i>Hs</i> Tis11c/BRF-2	477	LDPGRRRLPIFS	RLSISDD	494
<i>Hs</i> Tis11/TTP	316	VAAPRRRLPIFN	RLSVSE-	326

**TTP-CIM**  
(TTP-CCR4-NOT interaction motif)

**C**

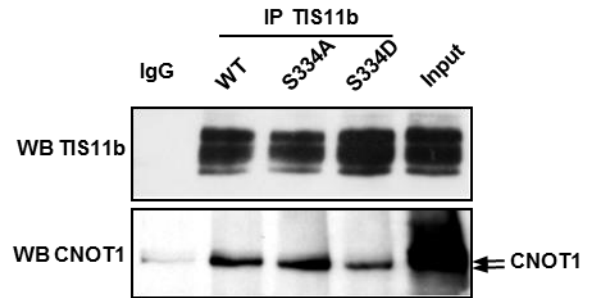
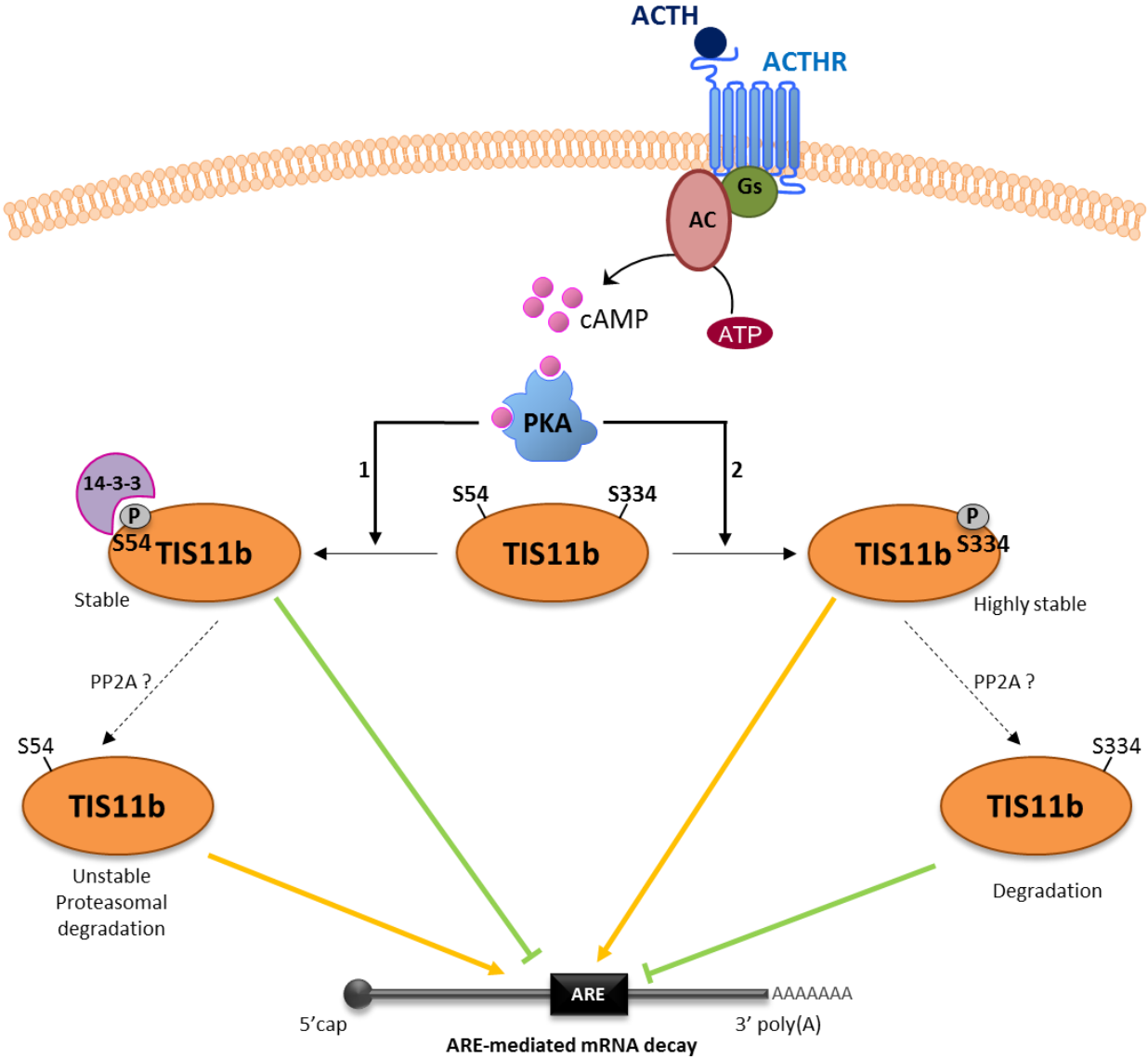
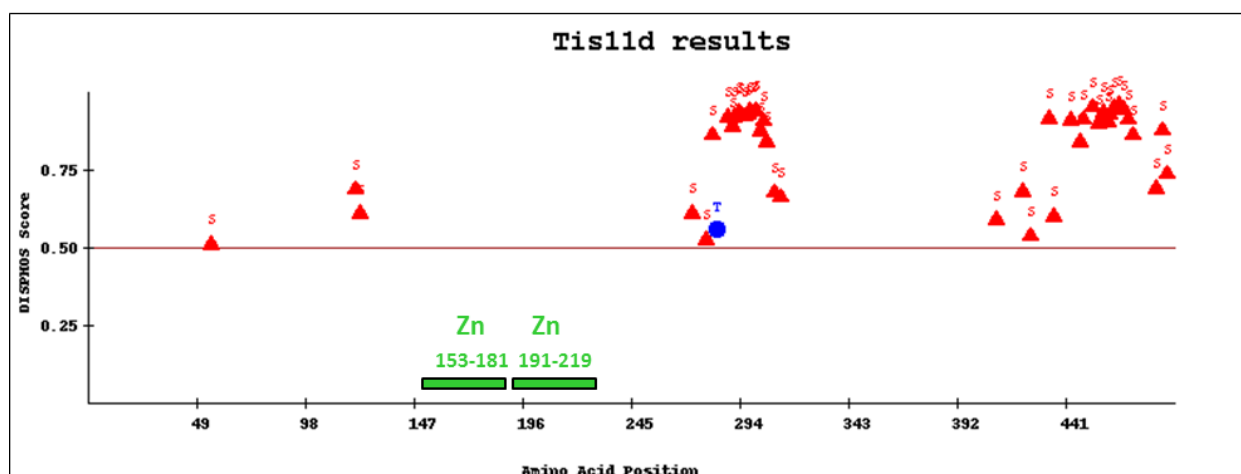
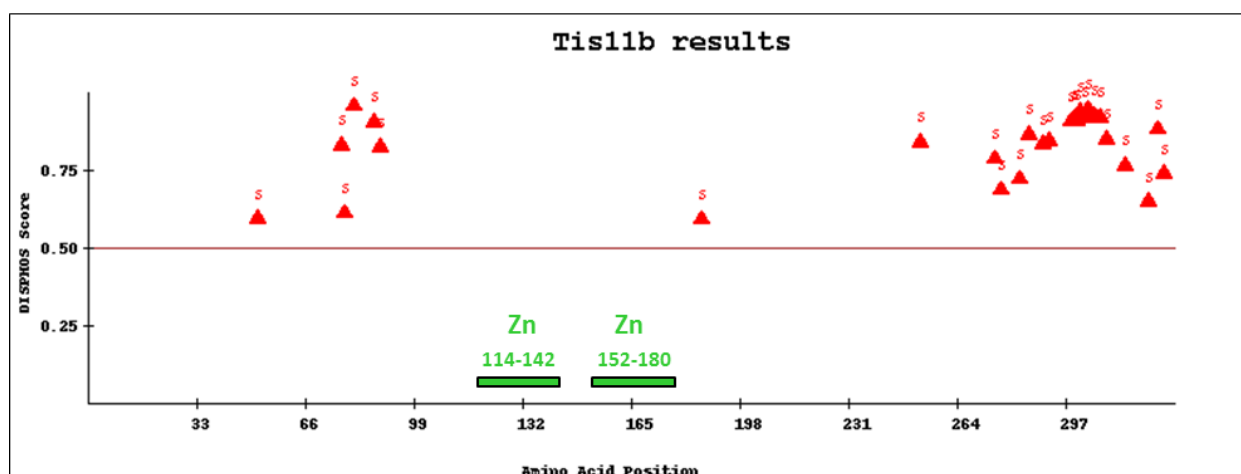
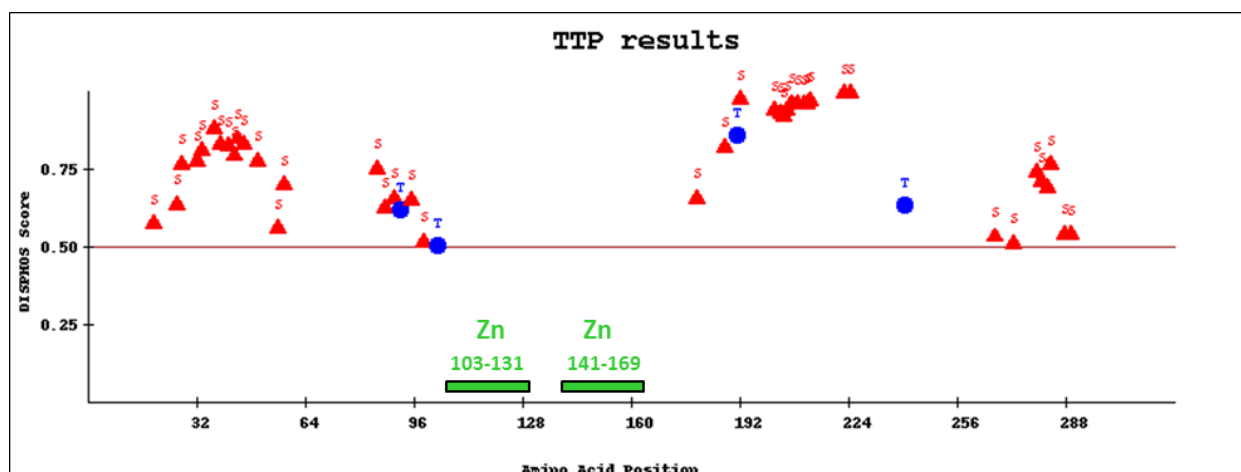




FIGURE 8



# SUPPLEMENTARY FIGURE 1



Disorder-Enhanced Phosphorylation Sites Predictor  
DISPHOS 1.3  
<http://www.dabi.temple.edu/disphos/>

**SUPPLEMENTARY TABLE 1**

<b>Mutant</b>	<b>Forward primer</b>	<b>Reverse primer</b>
TIS11b-S54A	5'-CCCTCGGAGGCACGCAGTCACCCTGCCAGC-3'	5'- GCTGGGCAGGGTGACTGCGTGCCTCCGA GGG-3'
TIS11b-S54D	5'-CCTCGGAGGCACGACGTCACCCTGCCGAGC-3'	5'- CTGGGGAGGGTGACGTCGTGCCTCCGAG GG-3'
TIS11b-S334A	5- 'GCCCATCTTCAGCAGACTTGCCATCTCAGATGAC TAAGCGGC-3'	5'- GCCGCTTAGTCATCTGAGATGGCAAGTCT GCTGAAGATGGGC-3'
TIS11b-S334D	5'- CTGCCATCTTCAGCAGACTTGACATCTCAGATG ACTAAGCG-3'	5'- GCCGCTTAGTCATCTGAGATGTCAAGTCT GCTGAAGATGGGCAG-3'
TIS11b- S54A/S334A	5'-CCCTCGGAGGCACGCAGTCACCCTGCCAGC-3'	5'- GCCGCTTAGTCATCTGAGATGGCAAGTCT GCTGAAGATGGGC-3'

## Article 2

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**“Targeting AU-rich element-mediated mRNA decay using a mutant version of ZFP36L1/TIS11b zinc finger protein impairs major hallmarks of tumorigenesis”**



## Introduction

Gene expression is highly dysregulated in many types of cancer. It has appeared that modifications of mRNA stability are significantly contributing to these deregulations (*Griseri and Pages, 2014; Han et al., 2013; Srikantan et al., 2012; Sanduja et al., 2012; Benjamin & Moroni, 2007*). Messenger RNA stability is regulated in part through the binding of stabilizing or destabilizing proteins to AU-rich elements (AREs) within the 3'-untranslated region (3'UTR) of target mRNAs. Destabilizing ARE-binding proteins enhance the decay of their targets through their association with the mRNA decay machinery. Therefore, mRNA decay-promoting ARE-binding proteins, such as TIS11 proteins, may be involved in various cellular pathways of multi-step tumor development (*Ross et al., 2012*). Indeed, TIS11 proteins are generally underexpressed in many different types of cancer, suggesting their potential role as tumor suppressors (*Brennan et al., 2009; Sanduja et al., 2012*).

The TIS11 (TPA-Inducible Sequence 11) protein family consists of three members, including TTP, TIS11b and TIS11d in mammals. The three members share structural similarities (two CCCH-type zinc fingers) and mechanisms of action. However, these proteins may have specific mRNA targets *in vivo* as suggested by their gene invalidations *in vivo*, which results in dramatically distinct phenotypes (*Taylor et al., 1996; Ramos et al., 2004; Stumpo et al., 2004; Bell et al., 2006; Hodson et al., 2010*). Our team was first to show that TIS11b inhibits VEGF expression by destabilizing its mRNA and to identify the molecular mechanisms which are implicated in this regulation (*Ciais et al., 2004*). Our observations were confirmed by the fact that TIS11b-deficient mouse embryos show deleterious vascular defects related to high VEGF concentrations (*Bell et al., 2006*). These embryos die *in utero*. Furthermore, other pro-inflammatory and pro-angiogenic mRNAs such as those encoding GM-CSF, TNF $\alpha$ , IL-6 and pre-messenger mRNA of Dll4, were described as TIS11b mRNA targets *in vitro* (*Baou et al., 2009; Desroches-Castan et al., 2011*).

Tumor growth is highly dependent on the supply of nutrients and oxygen from blood vessels. Above a size of 2-3 mm<sup>3</sup>, tumors induce their own vascularisation through the “angiogenic switch” (*Folkman, 1971; Bergers & Benjamin, 2003; Carmeliet & Jain, 2011*). Among other pro-angiogenic factors, VEGF is a key player in the initiation of tumor angiogenesis and therefore provides an attractive target for anti-cancer therapies. Promising results using antibodies targeting VEGF (bevacizumab/Avastin, Genentech/Roche) or tyrosine kinase inhibitors affecting VEGF receptors' activity combined with chemotherapy could be obtained in

several types of cancer (*Limaverde-Sousa et al., 2014*). However, during the last years, mounting evidence of resistance to these anti-angiogenic strategies appeared (*Bergers & Hanahan, 2008*).

Resistances seem to occur either through the selection of a hypoxia-resistant subpopulation of tumor cells, or the redundancy of pro-angiogenic factors apart VEGF (e.g. FGF, IL-6, IL-8 etc).

Since TIS11b inhibits VEGF expression differently from bevacizumab and since this protein targets also various inflammatory and pro-angiogenic cytokines *in vitro*, we hypothesized that TIS11b could be an innovative multi-target anti-angiogenic and anti-tumoral molecule. To set up this novel anti-cancer strategy, TIS11b was fused to a cell-penetrating peptide (polyarginine R9). Our team reported recently that purified R9-TIS11b efficiently enters living cells and reduces VEGF mRNA and protein levels *in vitro* (*Planel et al., 2010*). A single-dose injection of R9-TIS11b markedly decreased VEGF expression in the mouse adrenal gland *in vivo*. Furthermore, intratumoral injection of R9-TIS11b protein into pre-established tumors (Lewis Lung carcinoma) in mice reduced tumor growth and dramatically decreased the expression of several angiogenic and inflammatory cytokines including VEGF, TNF $\alpha$ , IL-1 $\beta$  and IL-6, with a concomitant obliteration of tumor vascularization. Unfortunately, this study was limited by the instability of the purified R9-TIS11 protein.

Therefore, the main objective of my PhD thesis was the optimization of the TIS11b protein stability and activity for our experimental therapeutic purposes taking advantage of the results presented in article 1, followed by the evaluation of the multi-target anti-tumoral and anti-angiogenic activity and of the newly generated TIS11b protein constructs *in vitro* and *in vivo*.

The results that we obtained *in vitro* are reported in the following manuscript “Targeting AU-rich element-mediated mRNA decay using a mutant version of ZFP36L1/TIS11b zinc finger protein impairs major hallmarks of tumorigenesis”. Unfortunately, we faced unpredictable difficulties in our *in vivo* studies. Indeed, the weak reproducibility due to the spontaneous regression of tumors in control animals complicated the analysis of our preclinical models. Therefore, the preliminary but promising data that we have obtained *in vivo* were not integrated in the manuscript but are presented as supplementary results after the article. We plan to perform further *in vivo* studies and to include our observations before the submission of the article for publication.

**Targeting AU-rich elements-mediated mRNA decay using a mutant version of ZFP36L1/TIS11b zinc finger protein impairs major hallmarks of tumorigenesis**

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Key words: tumor angiogenesis, inflammation, mRNA stability, TIS11b (ZFP36L1/BRF1), multi-target therapy, protein truncation





## Abstract

Alterations in mRNA stability can lead to various diseases including cancer and chronic inflammation. A considerable number of mRNAs encoding for cytokines, growth factors, transcription factors and oncogenes are abnormally stabilized in tumorigenic processes, including angiogenesis, inflammation and invasion/metastasis. Therefore, therapeutic interventions that aim at controlling aberrant mRNA stability are needed. The RNA-binding protein family TIS11 (TPA-Inducible Sequence 11) mediate mRNA decay through their binding to AU-rich elements in mRNA 3'UTR and subsequent recruitment of the mRNA degradation machinery. We have previously reported that the cell-permeable R9-TIS11b fusion protein displays an anti-angiogenic and anti-tumoral activities *in vivo*. The aim of the present study was to produce a new generation of more stable versions of TIS11b to improve our existing anti-tumoral strategy. We demonstrate that the deletion mutant lacking the N-terminal domain of TIS11b (ZnC) as well as its phosphorylation mutant ZnC<sup>S334D</sup> are more stable and highly active *in vitro*. Fusion of both proteins to the R9 (polyarginine) cell-penetrating peptide induced efficient cellular uptake and destabilizing activity of VEGF mRNA. *In vitro* studies using the murine breast cancer cell line 4T1 revealed that VEGF expression was significantly reduced in the presence of R9-ZnC while it was completely obliterated in the presence of R9-ZnC<sup>S334D</sup>. Analysis of the effect of both recombinant proteins on several hallmarks of tumorigenesis showed that R9-ZnC<sup>S334D</sup> inhibits 4T1 cell proliferation, migration, invasion and anchorage-independent growth while the non-mutated R9-ZnC only impedes 4T1 cell invasion. Furthermore, the mRNA expression of Snail and Twist, two markers of epithelial-mesenchymal transition, was reduced in the presence of R9-ZnC<sup>S334D</sup>. Finally, angiogenesis assays revealed that the ability of HUVEC endothelial cells to form tube-like structures was impaired in the presence of R9-ZnC and R9-ZnC<sup>S334D</sup>. Altogether, our results indicate that R9-ZnC and R9-ZnC<sup>S334D</sup> not only alter angiogenesis but also reduce breast cancer cells aggressiveness, and suggest that these novel proteins are potential innovative multi-target agents in cancer therapy.



## Introduction

Gene expression is a tightly controlled mechanism in eukaryotic cells. Various regulatory mechanisms control the amount of gene products, which results in part from a balance between mRNA synthesis and degradation rates. Besides transcription, post-transcriptional regulatory mechanisms, such as pre-mature mRNA processing, nuclear mRNA export, RNA interference, mRNA sequestration, codon usage, translational repression by miRNAs or proteins and the control of mRNA turnover have emerged as key steps in the regulation of gene expression.

mRNA stability is controlled by both *cis*-acting elements, located in the 3' untranslated region (3'UTR) of the mRNA, and *trans*-acting factors. Among regulatory sequences, AU-rich elements (AREs) are present in 8-10 % of total mammalian mRNAs (Bakheet *et al.*, 2006). AREs were initially discovered as instability determinants (Shaw & Kamen, 1986). Their presence correlates with patterns of rapid mRNA decay for genes encoding cytokines, chemokines or proto-oncogenes (Shaw & Kamen, 1986). AREs are 40- to 150-nt long adenylate- and uridylylate-rich sequences harbouring various copies of an AUUUA motif. *Trans*-acting factors such as ARE-binding proteins induce either stabilization of the transcript or mRNA degradation. The TIS11 protein family is a key player in ARE-mediated mRNA decay. This family of tandem CCCH zinc finger-containing RNA-binding proteins consists of three members in humans: TTP (ZFP36), TIS11b (ZFP36L1) and TIS11d (ZFP36L2) (Lai *et al.*, 1990). All members share common structural characteristics. TIS11 proteins comprise three protein domains: the N-terminal domain, the tandem zinc finger domain and the C-terminal domain. While the tandem zinc finger domain, which is essential for RNA-binding, is highly conserved, the N- and C-terminal parts of the three proteins differ more strongly in their amino acid sequences. TIS11 proteins act by recruiting multiple factors implicated in the mRNA decay machinery via protein-protein interactions. Post-translational modifications, mainly phosphorylations, modulate TIS11 protein activity, stability and subcellular localization (Ciais *et al.*, 2013). *In vitro*, TIS11 proteins bind to ARE-containing mRNA targets and induce their degradation (Baou *et al.*, 2009). By contrast, TIS11 proteins have unique roles *in vivo* as demonstrated by their respective murine knock out models (Taylor *et al.*, 1996; Ramos *et al.*, 2004; Stumpo *et al.*, 2004; Bell *et al.*, 2006; Hodson *et al.*, 2010). Homozygous TIS11b deletion is embryonic lethal because of abnormal placentation and vascular defects related to high VEGF-A levels (Stumpo *et al.*, 2004; Bell *et al.*, 2006). Thymus-specific double knock out of TIS11b/TIS11d in mice led to the development of leukaemia six months after birth (Hodson *et al.*, 2010).

Tumorigenesis is a dynamic, multi-step process comprising several hallmarks such as genome instability and mutation, uncontrolled proliferation, sustained angiogenesis, tumor-promoting inflammation, and activation of migration/invasion as well as the subsequent activation of metastasis (*Hanahan & Weinberg, 2011*). Most tumors are heterogeneous and contain multiple cell types, which communicate through heterotypic signalling. Beside tumor cells, endothelial cells, cancer-associated fibroblasts and tumor-associated immune cells create and maintain the pro-tumoral cancer niche and promote tumor progression (*Barcellos-Hoff et al., 2013*).

Over the last decade, a tight link between mRNA stability and cancer has emerged (*Benjamin & Moroni, 2007*). mRNA stability is often deregulated in cancer and inflammatory diseases leading to the abnormal stabilization of transcripts implicated in these pathologies. TIS11 proteins are underexpressed in many types of cancers and their expression is negatively correlated with tumor aggressiveness (*Carrick & Blackshear, 2007; Brennan et al., 2009; Griseri et al., 2011; Sanduja et al., 2012*). In addition, low TTP expression is associated with monocyte/macrophage infiltration and correlates with poor clinical outcome (*Milke et al., 2013*). Indeed, the repertoire of TIS11 protein family mRNA targets that includes cytokines and chemokines such as VEGF, GM-CSF, TNF $\alpha$  and IL-6, is associated with inflammation, angiogenesis and cancer, thus supporting the potential role of TIS11 proteins as tumor suppressors (*Ciais et al., 2004; Baou et al., 2009; Desroches-Castan et al., 2011; Ross et al., 2012*).

We have previously shown that intratumoral injections of a cell-permeable TIS11b fusion protein significantly decreased tumor growth, tumor vascularization and VEGF expression (*Planel et al., 2010*). However, this study was limited by the instability of the purified recombinant TIS11b protein under storage. In the present work, we generated truncations of the TIS11b protein by deleting either the N-terminal or C-terminal part of the protein. The deletion mutant ZnC, containing the zinc finger and the C-terminal domains proved to be the most stable truncated form of TIS11b. In addition, substitution of the distal C-terminal serine 334 by an aspartate residue increased ZnC protein stability. We demonstrate that ZnC and ZnC<sup>S334D</sup> are still active as they induce ARE-mediated mRNA-decay. Truncated TIS11b proteins fused to the R9 cell-penetrating peptide (R9-ZnC, R9-ZnC<sup>S334D</sup>) were efficiently internalized into murine breast cancer 4T1 cells and induced the degradation of endogenous VEGF mRNA with a concomitant decrease in VEGF protein secretion. Furthermore, purified R9-ZnC<sup>S334D</sup> inhibited proliferation, migration, invasion and anchorage-independent cell growth of 4T1 cells. The expression of different markers implicated in the epithelial-mesenchymal transition such as SNAIL and TWIST was also reduced upon treatment of 4T1 cells with purified R9-ZnC<sup>S334D</sup> protein. Finally,

we observed an inhibition of endothelial cell organization into pseudo-capillaries in the presence of R9-ZnC and R9-ZnC<sup>S334D</sup>, suggesting that our novel protein constructs could target not only tumor cells but also the tumor endothelium.

## Material and Methods

### *Cell culture*

COS7 cells were purchased from ATCC and cultured in DMEM GlutaMAX High Glucose medium (Invitrogen, Saint Aubin, France) containing 10 % fetal bovine serum (GE Healthcare, Velizy-Villacoublay, France) as previously described (*Planel et al., 2010*). Luciferase-expressing 4T1 cells (4T1-luc) were a generous gift from Dr. Jean-Luc Coll (Albert Bonniot Institute, Grenoble, France). They were cultured in RPMI 1640 GlutaMAX medium (Invitrogen, Saint Aubin, France) containing 10 % fetal bovine serum (GE Healthcare, Velizy-Villacoublay, France) and 200 µg/ml of G418 (Invitrogen, Saint Aubin, France). HUVEC cells were purchased from Lonza and cultured in EGM-2MV medium containing 5 % fetal bovine serum (Lonza, Levallois-Perret, France). All cell culture media were supplemented with 100 U/ml of penicillin, 100 µg/ml of streptomycin (Invitrogen, Saint Aubin, France) and 30 µg/ml gentamicin (Invitrogen, Saint Aubin, France).

### *Cloning of TIS11b deletion mutants*

pTarget-NZn, pTarget-Zn and pTarget-ZnC plasmids:

pTarget-TIS11b plasmid (*Ciais et al., 2004*) was used to amplify the human TIS11b truncated forms by PCR using the forward 5'-CCA GAC AGG AGA GGC TGC G-3' and the reverse 5'-G ACG GTC AGC GGA GAG TCA CC-3' primers for NZn (671 bp-fragment including the ATG codon of TIS11b), the forward 5'-ATG CCC ACC CAG AAG CAG CCC-3' and the reverse 5'-G ACG GTC AGC GGA GAG TCA CC-3' primers for Zn (292 bp-primers including the ATG and stop codons), the forward 5'-AT GGT CAA CTC CAG CCG CTA-3' and the reverse 5'-TGC AGG GTA GGG GCT GGA G-3' primers for ZnC (740 bp-fragment including the stop codon of TIS11b). Following their amplification, the NZn-, Zn- or ZnC-fragments were inserted into the pTarget plasmid using the T-overhangs to generate pTarget-NZn, pTarget-Zn and pTarget-ZnC plasmids.

pTarget-Flag-R9, pTarget-Flag-R9-ZnC and pTarget-Flag-R9-ZnC<sup>S334D</sup> plasmids:

The construction of the mammalian expression vector pTarget-Flag-R9-TIS11b was previously described (*Planel et al., 2010*). pTarget-Flag-R9-TIS11b plasmid was digested by *Kpn1* to generate the pTarget-Flag-R9 control plasmid. pTarget-ZnC plasmid was used to amplify ZnC (744 bp-fragment) flanked by *Kpn1* and *Sal1* restriction sites using the forward 5'-TT GGT ACC GTC AAC TCC AGC CGC TAC-3' and the reverse 5'-AA GTC GAC TT GCA GGG TAG GGG CTG-3' primers. ZnC was inserted in pTarget vector between *Kpn1* and *Sal1* restriction site downstream of Flag-R9 to generate pTarget-Flag-R9-ZnC plasmid. The pTarget-Flag-R9-ZnC<sup>S334D</sup> was obtained by a site-directed mutagenesis of the pTarget-Flag-R9-ZnC plasmid using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Massy, France) according to the manufacturer's instructions with the forward 5'-CTG CCC ATC TTC AGC AGA CTT GAC ATC TCA GAT GAC TAA GCG-3' and the reverse 5'-GCC GCT TAG TCA TCT GAG ATG TCA AGT CTG CTG AAG ATG GGC AG-3' primers to replace the serine 334 by an aspartate.

pET15b-Flag-ZnC, pET15b-Flag-ZnC<sup>S334D</sup>, pET15b-Flag-R9-ZnC and pET15b-Flag-R9-ZnC<sup>S334D</sup> plasmids:

The prokaryotic expression vectors pET15b-Flag-TIS11b and pET15b-Flag-R9-TIS11b were previously described (*Planel et al., 2010*). ZnC or ZnC<sup>S334D</sup> fragments were inserted in the bacterial expression vectors pET15b-Flag or pET15b-Flag-R9 between the *KpnI* and *NotI* restriction sites downstream of Flag-R9 to generate the pET15b-Flag-ZnC and pET15b-Flag-R9-ZnC as well as pET15b-Flag-ZnC<sup>S334D</sup> and pET15b-Flag-R9-ZnC<sup>S334D</sup> plasmids.

All constructs were verified by sequencing analyses (GATC, Mulhouse, France).

pLuc-3'UTR reporter gene plasmid and pRL-TK plasmid:

The plasmid pLuc-3'UTR containing the Firefly luciferase cDNA cloned upstream of the rat VEGF 3'-UTR was described previously (*Ciais et al., 2004; Planel et al., 2010*). pRL-TK plasmid encoding Renilla luciferase was obtained from Promega Corp (Charbonnières, France).

#### *Transient Transfections and Dual Luciferase Activity Assay*

COS7 cells were grown in DMEM medium (Invitrogen, Saint Aubin, France) supplemented with 10 % fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin.  $1.0-1.5 \times 10^5$  cells were seeded in duplicates or triplicate into 12-well plates and transfected the day after, using Lipofectamine (Invitrogen, Saint Aubin, France) according to the manufacturer's

recommendations. 1 ng, 10 ng or 25 ng of TIS11b, NZn, Zn or ZnC pTarget plasmids or 100 ng of Flag-R9-empty, Flag-R9-TIS11b, Flag-R9-ZnC, Flag-R9-ZnC<sup>S334D</sup> pTarget plasmids were transfected together with 500 ng of pLuc-3'UTR (Firefly luciferase) and 25 ng of pRL-TK (Renilla luciferase). Activities of Firefly and Renilla luciferase were measured sequentially 24 h post-transfection using the Dual-Luciferase reporter assay system (Promega, Charbonnières, France) on a LUMAT LB 9507 luminometer (EGG-Berthold). Results are presented as relative light units of Firefly luciferase activity over relative light units of Renilla luciferase activity to compensate for variations in transfection efficiency, and are represented as a percentage of the luciferase activity in control cells.

### *Northern blot*

2.0 x 10<sup>5</sup> COS7 cells per well were transfected in 6-well plates with 250 ng Flag-R9-empty, Flag-R9-TIS11b, Flag-R9-ZnC or Flag-R9-ZnC<sup>S334D</sup> pTarget plasmids using Lipofectamine (Invitrogen, Saint Aubin, France) according to the manufacturer's recommendations. Cells were washed with PBS and total RNA was extracted from triplicate samples using the NucleoSpin RNA kit (Macherey-Nagel, Hoerd, France) according to the instructions of the manufacturer. 20 µg of RNA were size-fractionated on a 1 % formaldehyde agarose gel, vacuum-transferred onto Hybond-N+ membranes (GE Healthcare, Velizy-Villacoublay, France) and fixed by UV cross-linking. Northern blots were pre-hybridized in Rapid Hybridization Buffer (GE Healthcare, Velizy-Villacoublay, France) at 65 °C for 30 min. [ $\alpha$ -<sup>32</sup>P]dCTP-labelled VEGF 3'UTR cDNA probe (2 x 10<sup>6</sup> cpm / ng DNA, Rediprime random primer labelling kit, GE Healthcare, Velizy-Villacoublay, France) was then added and the incubation was continued for 2 h at 65 °C. Blots were washed for 5 min and 15 min successively at room temperature in 2 x saline sodium citrate (SSC), 0.1 % SDS, and then for 15 min in 1 x SSC, 0.1 % SDS. The final wash was performed at 65 °C for 15 min in 0.5 x SSC, 0.1 % SDS. RNA-cDNA hybrids were visualized on phosphor screen (Molecular Dynamics) after a 12- to 24-h exposure period. Blots were stripped and reprobed with 18S cDNA probe to assess RNA loading. Quantitation of autoradiograms was performed using phosphorimager and QuantityOne software.

### *SDS-PAGE*

SDS-polyacrylamide gel electrophoresis was performed according to *Laemmli (Laemmli, 1970)*. Total proteins extracts or purified proteins were solubilized in sample buffer (60 mM Tris-HCl, pH 6.8, 2 % SDS, 5 %  $\beta$ -mercaptoethanol, 10 % glycerol, 0.01 % bromophenol blue), boiled for



5 min and loaded onto a 12 % SDS-PAGE minigel (Mini Protean II System, BioRad, Marnes-la-Coquette, France) or NuPAGE 4-12 % Bis-Tris gel (Invitrogen, Saint Aubin, France). Electrophoresis was performed at 100 V for 30 min and afterwards at 150 V for 1-2 h.

### *Western blot*

SDS-PAGE-resolved proteins were electrophoretically transferred onto a polyvinylidene fluoride (PVDF) membrane as previously described (*Towbin et al., 1979*). Following transfer, the membrane was incubated in a blocking buffer (Tris-buffer saline (TBS) containing 0.1 % Tween 20 and 5 % non-fat dry milk) for 1 h at room temperature. The blots were probed with monoclonal mouse anti-Actin (1:5000, Sigma-Aldrich, Saint-Quentin Fallavier, France), rabbit anti-BRF1/2 (1:1000, Ozyme, Montigny-le-Bretonneux, France), mouse anti-E-Cadherin (1:2500, Becton Dickinson, Le Pont de Claix, France), mouse anti-FLAG M2-HRP (1:1000, Sigma-Aldrich, Saint-Quentin Fallavier, France), mouse anti-N-Cadherin (1:2500, Becton Dickinson, Le Pont de Claix, France), mouse anti-Tubulin (1:40000, kindly provided by Dr. A. Andrieux (Grenoble Institute of Neuroscience, Grenoble, France)) or monoclonal mouse IgM isotype anti-Vimentin (1:1000, Sigma-Aldrich, Saint-Quentin Fallavier, France) for 2 h at room temperature or overnight at 4 °C in TBS containing 0.1 % Tween. The membrane was thoroughly washed with the same buffer (3 x 10 min), then incubated for 1 hour with either horseradish peroxidase (HRP)-labelled goat anti-mouse IgG (1:5000, Thermo Fisher, Illkirch, France), HRP-labelled goat anti-mouse IgM (1:5000, Invitrogen, Saint Aubin, France), HRP-labelled goat anti-rabbit IgG (1:3000, Thermo Fisher, Illkirch, France) or HRP-labelled goat anti-rat IgG (1:3000, Jackson Immuno Research, Marseille, France). The PVDF sheet was washed as above and the antigen-antibody complex revealed by Enhanced Chemiluminescence, using the Western blotting detection kit from Perkin Elmer and BioMax Kodak films (Sigma-Aldrich, Saint-Quentin Fallavier, France) or the Chemidoc<sup>TM</sup> MP imaging system (BioRad, Marnes-la-Coquette, France). Quantification of the hybridization signals in pixel density was either performed using ImageJ software for BioMax Kodak films or by ImageLab Version 4.0.1 software (BioRad, Marnes-la-Coquette, France) when the Chemidoc<sup>TM</sup> MP imaging system was used. Protein levels were normalized to Actin or Tubulin to compensate for protein loading variations. Values are presented relative to protein content of control samples.

### *Determination of protein half-life*

COS7 cells were grown in DMEM medium (Invitrogen, Saint Aubin, France) supplemented with 10 % bovine calf serum, 100 U/ml penicillin and 100 mg/ml streptomycin.  $1.0 \times 10^5$  cells were seeded in into 12-well plates and transfected the day after with 100 ng of R9-empty, R9-TIS11b, R9-ZnC or R9-ZnC<sup>S334D</sup> pTarget plasmids, using Lipofectamine (Invitrogen, Saint Aubin, France) according to the manufacturer's recommendations. 24 h post-transfection, cells were treated with the translation inhibitor Cycloheximide (CHX) (10 µg/ml) and harvested at the time-point indicated using RIPA buffer (10 mM Tris HCl, pH 7.4, 150 mM NaCl, 0.1 % SDS, 0.5 % Na deoxycholate, 1 mM EDTA, 1 % Triton X100, and protease inhibitor cocktail (Sigma-Aldrich, Saint-Quentin Fallavier, France)). Total protein extracts were analysed by Western blot. Results are represented as a percentage of the protein level at time 0 h of CHX treatment.

### *Semi-quantitative RT-PCR and Quantitative real-time PCR*

COS7 cell total RNA was extracted using the Qiagen RNeasy Mini kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. For semi-quantitative PCR analysis of full-length or truncated TIS11b or HPRT (hypoxantin phosphoribosyl transferase), 1 µg of total RNA was reverse-transcribed with ImProm II reverse transcriptase (Promega, Charbonnieres, France) and PCR-amplified using Taq-polymerase (MP Biomedicals, Illkirch-Graffenstaden, France). The forward 5'-CGG AGC TGT GCC GCC CCT TTG-3' and reverse 5'-GGG CAC GGC GCT CTT CAG CGT TGT-3' primers were used to amplify a 200 bp-long internal region containing the tandem zinc finger domain of TIS11b. The amplification conditions were as follows: 94 °C for 5 min followed by 28 amplification cycles, each consisting of 94 °C for 1 min, 63 °C for 1 min, 72 °C for 1 min, and 72 °C for 5 min for final extension. The primers for HPRT amplification were as follows: 5'-GCC ATC ACA TTG TAG CCC TCT-3' and 5'-TGC GAC CTT GAC CAT CTT TGG-3'. This primer pair amplifies a 305-bp fragment. The amplification conditions were as follows: 94 °C for 5 min followed by 30 amplification cycles, each consisting of 94 °C for 1 min, 51 °C for 1 min, 72 °C for 1 min, and 72 °C for 5 min for final extension.

Total RNA from 4T1-luc cells was extracted using the Nucleospin RNA kit (Macherey-Nagel, Hoerd, France) according to the manufacturer's instructions. For quantitative real-time PCR analysis of Snail, Twist, VEGF, or RPL13A (ribosomal protein 13a) gene expression, 1 µg of total RNA was reverse-transcribed using the iScript cDNA synthesis kit (BioRad, Marnes-la-Coquette, France). The cDNAs were PCR-amplified using GoTaq polymerase and the master mix kit from Promega (Charbonnieres, France). Amplification of mouse Snail mRNA was

performed using the forward 5'-TGT GTG GAG TTC ACC TTC CAG-3' and reverse 5'-AGA GAG TCC CAG ATG AGG GT-3' primers. The size of the amplified fragment was 116-bp. Amplification of mouse Twist mRNA was performed using the forward 5'-ACC ATC CTC ACA CCT CTG CAT TCT-3' and reverse 5'-TTC CTT TCA GTG GCT GAT TGG CAC-3' primers. The size of the amplified fragment was 143-bp. Amplification of mouse VEGF mRNA was performed using the forward 5'-AAT GAT GAA GCC CTG GAG TGC-3' and reverse 5'-C TTT CGT TTT TGA CCC TTT CCC-3' primers. The size of the amplified fragment was 201-bp. Amplification of mouse RPL13A mRNA was performed using the forward 5'-CCC TCC ACC CTA TGA CAA GA-3' and reverse 5'-TTC TCC TCC AGA GTG GCT GT-3'. The size of the amplified fragment was 153-bp for the RPL13A transcript. The amplification conditions for all tested genes were as follows: 95 °C for 2 min followed by 40 amplification cycles, each consisting of 95 °C for 3 sec and 60 °C for 30 sec for extension.

#### *Protein overexpression and purification*

High expression BL21 (DE3) *Escherichia coli* codon+-competent cells (Agilent Technologies, Massy, France) were transformed with pET15b plasmids encoding either Flag-ZnC, Flag-R9-ZnC or Flag-R9-ZnC<sup>S334D</sup> constructs and were grown in LB Broth medium in the presence of ampicillin (100 µg/ml) and chloramphenicol (50 µg/ml).

Protein expression was induced by adding isopropyl-1-thio-β-D-galactopyranoside (IPTG) concentrations (0.1 mM) at 30 °C for 4 h. When starter cultures reached an optimal absorbance of 0.6, bacterial cell pellets were harvested by centrifugation (3500 x g at 4 °C for 30 min). The pellets were re-suspended with 50 mM Tris-HCl pH 7.4 buffer containing 500 mM NaCl, 2% Triton X-100, 4 M urea and 100 µM ZnCl<sub>2</sub>, then incubated for 5 min in the presence of 0.1 mg/ml lysozyme, and a protease inhibitor cocktail (Sigma-Aldrich, Saint-Quentin Fallavier, France). Cells were lysed by repeated 10 freeze/thawing cycles. Homogenates were further sonicated then centrifuged at 13000 x g for 10 min at 4 °C. Supernatants were diluted to achieve concentrations of 150 mM NaCl and 1 M urea. Anti-Flag M2 affinity resin (Sigma-Aldrich, Saint-Quentin Fallavier, France) was prepared according to the manufacturer's instructions. Ready-to-use resin was incubated with cell lysate for 1 h at 4 °C under overhead shaking then loaded on empty columns. Then the column was washed with TBS and elution performed with 1 M Arg-HCl pH 7. Purity of Flag-ZnC, Flag-R9-ZnC or Flag-R9-ZnC<sup>S334D</sup> proteins was examined by Coomassie blue staining following SDS-PAGE analysis. Purified proteins used for further analyses were dialyzed against phosphate-buffer saline (PBS) using 7K or 10K Slide-A-Lyzer

dialysis cassettes (Thermo Fisher, Illkirch, France). Protein concentration was determined using a Micro BCA protein Assay Kit (Thermo Fisher, Illkirch, France) using bovine serum albumin as a standard.

#### *Determination of protein stability under storage*

Equal amounts of purified R9-TIS11b, R9-ZnC and R9-ZnC<sup>S334D</sup> were stored at either -20 °C, 4 °C or 37 °C up to five days. Remaining protein was quantified by Western blot using Flag-tag antibodies. Results are represented as a percentage of protein level in the control sample that was kept at -20 °C.

#### *Cellular internalization of recombinant proteins*

Purified and dialyzed Flag-ZnC, Flag-R9-ZnC and Flag-R9-ZnC<sup>S334D</sup> proteins were first lyophilized then re-suspended in PBS to reach the required protein concentration. The proteins were labelled using the Alexa Fluor 488 Protein Labelling kit (Invitrogen, Saint Aubin, France) according to the manufacturer's instructions with slight modifications. An optimal labelling was obtained with a 4 h co-incubation of Alexa Fluor 488 with fusion proteins at room temperature. Labelled proteins were dialyzed against PBS overnight at 4 °C to remove free dye. ZnC-, R9-ZnC- and R9-ZnC<sup>S334D</sup>-labelled proteins were aliquoted and stored at -20 °C until used.  $3 \times 10^4$  4T1-luc cells were plated on an eight-chamber Lab-Tek Coverglass plate (Thermo Fisher, Illkirch, France) and cultured overnight in RPMI 1640 medium containing 10% fetal bovine serum, 100 U/ml of penicillin and 100 µg/ml of streptomycin. The day after, medium was removed and RPMI 1640 containing 2 % fetal bovine serum and 50 ng of Alexa 488-labeled proteins were added to cells for 16 h at 37 °C. Then cells were washed twice (5 min) with PBS prior to the addition of Hoechst 33342 30 min to stain nuclei and Alexa Fluor 594 wheat germ agglutinin (Invitrogen, Saint Aubin, France) for 10 min to label cell plasma membrane. After two 5 min-final washes in PBS, cells were fixed for 15 min in 4 % paraformaldehyde (PFA). Uptake and intracellular localization of labelled proteins were assessed by inverted fluorescence microscopy (Zeiss, Imager Z1) as well as laser confocal microscopy (Leica, TCS-SP2). For deconvolution fluorescence microscopy, image scans were acquired in series by a CCD camera. All data sets were subjected to deconvolution and subsequently used for image reconstructions. Stacking each of the individual sections produces a 3-dimensional image on a 2-dimensional background, resulting in an image projection.

In experiments designed to determine the effect of R9-ZnC and R9-ZnC<sup>S334D</sup> fusion proteins on VEGF expression levels, 4T1-luc cells were cultured for 24 h in 24-well plates (1.5 x 10<sup>5</sup> cells /well) as described in "Cell culture" section. Purified fusion proteins were added at a final concentration of 100 nM in serum-free RPMI 1640 for 24 h. At the end of the incubation period, the culture medium was collected to measure secreted VEGF and cells were lysed to isolate total RNA or for further Western blot analyses.

#### *Enzyme-linked immunosorbent assay*

For VEGF protein measurement, 4T1-luc cells were incubated with 100 nM of purified recombinant R9-ZnC and R9-ZnC<sup>S334D</sup> proteins. Culture medium was collected after 24 h. VEGF (splice variants VEGF-165 and VEGF-121) content of the supernatants (100 µl) was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Peprotech, Neuilly-sur-Seine, France) with horseradish peroxidase detection in accordance with the manufacturer's instructions. The absorption was read at 405 and 650 nm (reference) in a microplate reader (Multiskan EX, Thermo LabSystems). In each assay, the recombinant human VEGF was used to generate the standard curve. Standards as well as samples were assayed as duplicates. The minimum limit of detection was 16 pg/ml. Results are represented as pg/ml.

#### *Proliferation assay (WST-1)*

Cell proliferation was measured using the WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene disulfonate) (Roche, Meylan, France) colorimetric assay. 1.0 x 10<sup>4</sup> 4T1-luc cells per well were seeded in a 96-well microplates in a final volume of 100 µl of appropriate growth medium. Cells were starved overnight using 100 µl/well of serum-free culture medium. Before starting the proliferation experiment, culture medium was replaced by fresh complete growth medium (RPMI 1640 + 10 % fetal bovine serum) containing 12.5, 25 or 50 nM of recombinant proteins, respectively. Cells were allowed to proliferate over time and the experiment was terminated after 24 h and 48 h by adding 10 µl per well of WST-1 (Roche, Meylan, France). After 2 h incubation at 37 °C, the absorbance of each well was measured at 450 nm. All experiments were performed in duplicates.

### *Wound healing migration assay*

4T1-luc cells were seeded in 12-well plates and incubated until confluence. The cell monolayer was scratched and the wound healing process was pursued in the presence of growth medium containing the cell proliferation inhibitor mitomycin (2 µg/ml) and 50 nM of recombinant proteins. Wound closure was monitored by phase-contrast microscopy at t=0 h and t=16 h. At each time point, the distance between the wound edges was measured.

### *Matrigel invasion assay*

For the Matrigel invasion assay,  $5 \times 10^4$  4T1-luc cells in growth medium supplemented with 2 % serum were seeded in the upper chamber of the transwell inserts containing Matrigel™ (24-well plate; 8 µm pore size; Becton Dickinson, Le Pont de Claix, France). Culture medium containing 15 % serum was added to the lower chamber as chemoattractant. Cells were incubated for 24 h (for luciferase measurement) or 48 h (for Crystal Violet staining) at 37°C, 5% CO<sub>2</sub> in the presence of 50 nM final concentration of R9-ZnC and R9-ZnC<sup>S334D</sup>. Cells that did not invade through the pores were removed using a cotton swab. Invaded cells were either stained with crystal violet (Sigma-Aldrich, Saint-Quentin Fallavier, France) or lysed for Firefly luciferase measurement using the luciferase reporter assay system (Promega, Charbonnières, France) on a LUMAT LB 9507 luminometer (EGG-Berthold). Results are represented as a percentage of the luciferase activity of control cells.

### *Soft agar colony formation assay*

Anchorage-independent cell growth of 4T1-luc cells was assayed in 12-well plates coated with 0.6 % soft agar (Becton Dickinson, Le Pont de Claix, France) in growth medium as the bottom layer.  $1.5 \times 10^4$  4T1-luc cells per well were re-suspended in 0.3 % soft agar in growth medium supplemented with 50 nM of recombinant proteins or PBS respectively, and plated as a top layer. 250 µl of growth medium were added once a week. Colony formation was monitored over a period of 14 days. For colony counting, growth medium was replaced by 500 µl of 0.0001 % crystal violet in 10 % ethanol. After 2 h, the staining solution was removed and each well was carefully rinsed several times with tap water until the soft agar became nearly transparent. The total number of colonies above 6 µm was then counted.

### *HUVEC tubulogenesis assay*

48-well plates were coated with 150  $\mu$ l of diluted Matrigel<sup>TM</sup> (85 % Matrigel<sup>TM</sup> (Becton Dickinson, Le Pont de Claix, France) and 15 % EGM-2 medium (Lonza, Levallois-Perret, France) per well and left for 30 min at 37 °C to allow matrigel polymerization.  $3 \times 10^5$  HUVEC cells per well were seeded then treated with 50 nM of purified recombinant R9-ZnC and R9-ZnC<sup>S334D</sup> proteins immediately after cell attachment. Formation of capillary-like structures was observed by phase-contrast microscopy at 24 h post-treatment. The cell-covered area was measured using ImageJ software and the results are presented as a percentage of the control.

### *Statistical analyses*

Results are expressed as means  $\pm$  s.e.m. The mean values were compared by ANOVA using Dunnett's or Bonferroni's multiple comparison test. Statistical significance is indicated as \* for  $P \leq 0.05$ ; \*\* for  $P \leq 0.01$ ; \*\*\* for  $P \leq 0.001$ .

## **Results**

### *The N- and C-terminal domains of TIS11b are active in mRNA decay*

The TIS11b protein consists of three domains: the N-terminal domain, the tandem zinc finger domain and the C-terminal domain. To determine the minimal active domain of TIS11b, we generated different truncated versions of the protein. We either kept the N-terminal domain (NZn<sub>(1-195)</sub>) or the C-terminal domain (ZnC<sub>(109-339)</sub>), each associated to the two zinc fingers, or just the tandem zinc finger domain (Zn<sub>(99-195)</sub>) (Supplementary figure 1A). Previous studies using transfection of TIS11b expression vector and reporter gene construct in which the Firefly luciferase coding sequence was cloned upstream of the 3'UTR of VEGF mRNA (Luc-3'UTR) allowed us to show a TIS11b-mediated decrease in luciferase activity which was accompanied by a destabilization of the chimeric Luc-3'UTR transcript (*Ciais et al., 2004; Planel et al., 2010*). To analyse the activity of the truncated TIS11b protein constructs, COS7 cells were co-transfected with increasing doses of eukaryotic expression vectors and Luc-3'UTR plasmid. Supplementary figure 1B shows that full-length TIS11b and ZnC were the most active proteins when compared to NZn and Zn truncated forms. The expression levels of our protein constructs were checked by RT-PCR. All constructs were expressed in a dose-dependent manner (Supplementary figure 1C), indicating that the observed differences in luciferase activity were

not biased by different expression levels. As ZnC was the most active truncated form of TIS11b, we performed our further studies with this construct.

#### *R9-ZnC and R9-ZnC<sup>S334D</sup> fusion proteins reduce luciferase reporter gene activity through VEGF-3'UTR*

We have previously reported that substitution of serine 334 (S334) by an aspartate (D) in the TIS11b sequence markedly increases TIS11b protein stability (*Rataj et al., submitted, Article 1*). We therefore generated the fusion constructs R9-ZnC and R9-ZnC<sup>S334D</sup> (Figure 1A) and tested their activity in luciferase assays (Figure 1B). Luciferase activity was significantly reduced by R9-TIS11b ( $30 \pm 2.5$  % of control), R9-ZnC ( $42 \pm 2.3$  % of control) and R9-ZnC<sup>S334D</sup> ( $34 \pm 4.4$  % of control). No significant difference between R9-ZnC and R9-ZnC<sup>S334D</sup>-mediated luciferase activity was observed. Western blot analyses of COS7 cell lysates confirmed equal expression level of each construct (Figure 1B). Multiple bands correspond to different phosphorylation levels of the proteins as reported previously (*Cao & Lin, 2008*). Due to deletion of the N-terminal part of TIS11b, R9-ZnC/ R9-ZnC<sup>S334D</sup> fusion proteins were detected at 38 kDa. To further confirm the mRNA-destabilizing activity of our constructs, we analysed the effect of R9-TIS11b, R9-ZnC and R9-ZnC<sup>S334D</sup> on endogenous VEGF mRNA by Northern blot (Figure 1C). All three fusion proteins reduced VEGF mRNA steady state levels compared to the control level. The truncated proteins of TIS11b fused to R9 were more active than the full-length TIS11b fusion protein. This effect was even more pronounced for the truncated mutant R9-ZnC<sup>S334D</sup>. Altogether, these data indicate that the function of TIS11b in ARE-mediated decay is neither impaired by truncating the protein, nor by replacing the S334 residue by an aspartate.

#### *Deletion of the N-terminal domain of TIS11b doubles protein half-life in cellulo*

R9-TIS11b fusion protein was shown to be rather unstable. We next evaluated the effect of the N-terminal deletion of R9-TIS11b on protein stability *in cellulo*. COS7 cells were transfected with R9-TIS11b, R9-ZnC or R9-ZnC<sup>S334D</sup> expression constructs then treated with cycloheximide (CHX) for various periods of time. Protein levels were assessed in cell lysates by Western blot at different times. Figure 1D shows representative results of several independent experiments. The half-life of full-length TIS11b (4 hours) was doubled following the deletion of its amino-terminal domain and the exchange of S334 by an aspartate to mimick a permanent phosphorylation of the protein by this residue. However, already the truncation of TIS11b (R9-ZnC) seems to have a



stabilizing effect as the slope of the protein degradation curve is less abrupt compared to TIS11b full-length protein. These results led us to overexpress and purify these truncated fusion proteins.

#### *R9-ZnC and R9-ZnC<sup>S334D</sup> protein overexpression and purification*

We generated prokaryotic expression vectors for R9-TIS11b, R9-ZnC and R9-ZnC<sup>S334D</sup> containing a Flag-tag. Recombinant proteins were produced and purified using affinity chromatography as described in Material and Methods. Figure 2 illustrates the different steps of the purification process as well as Western blot analyses of eluted recombinant proteins. Due to the absence of serine/threonine protein kinases in bacteria, purified proteins were expected at 38 kDa for R9-TIS11b and 29 kDa for R9-ZnC and R9-ZnC<sup>S334D</sup>. By contrast, R9-TIS11b, R9-ZnC and R9-ZnC<sup>S334D</sup> were detected in mammalian cells at 51 kDa and 38 kDa, respectively (Figure 1B).

#### *Deletion of the N-terminal domain of TIS11b increases the stability of the purified protein under storage*

We next evaluated the effect of TIS11b truncation on the protein stability under storage. Equal amounts of purified recombinant R9-TIS11b, R9-ZnC and R9-ZnC<sup>S334D</sup> were incubated at 4 °C or 37 °C up to five days, then analysed by Western blot. The same amounts of protein were stored at -20 °C as controls. A five-day incubation of the three recombinant proteins at 4 °C led to a degradation of about 40 % R9-TIS11b, whereas truncated TIS11b forms remained intact (Figure 3A). Around 50 % of R9-TIS11b full-length protein was lost after a one day-incubation at 37 °C (Figure 3A). In contrast, truncated proteins displayed an increased stability compared to R9-TIS11b. R9-ZnC protein was stable for at least two days at 37 °C then a decrease of 40 % was observed after a five days-incubation. Interestingly, R9-ZnC<sup>S334D</sup> protein was almost totally preserved after five days-incubation at 37 °C, indicating that the stabilizing effect was more pronounced when the S334 residue was replaced by an aspartate. Taking advantage of the significantly improved protein stability, further *in vitro* studies focused on R9-ZnC and R9-ZnC<sup>S334D</sup>.

### *R9-ZnC and R9-ZnC<sup>S334D</sup> are efficiently internalized into living cells*

Internalization of recombinant Alexa-Fluor 488-labelled R9-TIS11b has been previously demonstrated (Planel *et al.*, 2010). We thus tested the cellular uptake of the novel truncated recombinant R9-ZnC and R9-ZnC<sup>S334D</sup> proteins in living luciferase-expressing murine breast cancer cells (4T1-luc). Alexa Fluor 488-labelled ZnC without R9 cell-penetrating peptide was used as a negative non-penetrating control. Fluorescence microscopy analysis showed efficient cellular internalization of Alexa Fluor 488-labelled R9-ZnC or R9-ZnC<sup>S334D</sup> (green fluorescent signal) compared to the non-permeable form Alexa Fluor 488-labelled ZnC (Figure 3B upper panel). These results confirm the efficient cellular uptake in the presence of the cell-penetrating peptide R9. Both proteins were predominantly dispersed throughout the cytoplasm with a punctate appearance. The intracellular localization of labelled recombinant proteins was checked using confocal microscopy. Deconvolution of these images along the *z* axis indicated a cytoplasmic localization of Alexa Fluor 488-labelled R9-ZnC or R9-ZnC<sup>S334D</sup> (Figure 3B lower panel). In line with protein stability experiments, fluorescence signal of Alexa Fluor 488-labelled R9-ZnC or R9-ZnC<sup>S334D</sup> was still detectable in 4T1-luc cells 4 days after treatment (data not shown). These results led us to further evaluate the effect of R9-ZnC and R9-ZnC<sup>S334D</sup> on the tumorigenic phenotype of 4T1-luc cells.

### *Purified R9-ZnC and R9-ZnC<sup>S334D</sup> proteins decrease VEGF mRNA and protein expression*

Tumor angiogenesis is mainly driven by the pro-angiogenic factor VEGF. In order to investigate the destabilizing-activity of purified R9-ZnC and R9-ZnC<sup>S334D</sup> proteins on endogenous VEGF mRNA as well as on VEGF protein levels in living cells, 4T1-luc cells were treated with 100 nM of recombinant protein for 24 h in serum-free medium. R9-ZnC and R9-ZnC<sup>S334D</sup> decreased VEGF mRNA level by 55 and 82 %, respectively, compared to the control (Figure 4A).

The observed effect on the mRNA level was correlated to VEGF protein level (Figure 4B). A 35 % decrease in secreted VEGF was detected in the presence of R9-ZnC compared to the control. Interestingly, VEGF protein was undetectable upon R9-ZnC<sup>S334D</sup> treatment. These data suggest that our novel protein constructs are potent inhibitors of VEGF expression in 4T1-luc cells.

### *R9-ZnC<sup>S334D</sup> inhibits proliferation and migration of breast cancer cells in vitro*

To examine the effect of R9-ZnC and R9-ZnC<sup>S334D</sup> on the proliferation of cancer cells, 4T1-luc cells were treated up to 48 h with 12.5, 25 or 50 nM of recombinant protein. Cell proliferation was measured at t=0 h, t=24 h and t=48 h of treatment. The highest proliferation rate was observed the controls (Figure 5A). 4T1-luc cell proliferation was reduced by 30 % in the presence of R9-ZnC for 48 h compared to control. This effect was dose-independent. Interestingly, we observed a 50 %-inhibition of cell proliferation in the presence of 25 or 50 nM of R9-ZnC<sup>S334D</sup>.

4T1-luc cells are known to be highly aggressive and to form spontaneous metastasis *in vivo* (Lou *et al.*, 2008). These cells are the murine equivalent of the triple negative human breast cancer cell lines (Pulaski & Ostrand-Rosenberg, 2001; Tao *et al.*, 2008). Migration of cancer cells is an initial event during the formation of metastasis. Therefore, we tested the effect of 50 nM of R9-ZnC and R9-ZnC<sup>S334D</sup> on the migration capacities of 4T1-luc cells using the wound healing assay. A significant inhibition of wound closure was observed at 16 h post-treatment in the presence of R9-ZnC<sup>S334D</sup> compared to the control whereas R9-ZnC had no significant effect (Figure 5B).

Altogether, these results suggest that the proliferation and migration of 4T1 breast cancer cells was impaired only by the R9-ZnC<sup>S334D</sup> mutant.

### *R9-ZnC and R9-ZnC<sup>S334D</sup> reduce cancer cell invasion and anchorage-independent cell growth*

To further investigate the anti-tumoral effect of R9-ZnC and R9-ZnC<sup>S334D</sup>, invasion and anchorage-independent growth assays were performed using 4T1-luc cells. As shown in Figure 6A, cell invasion in a matrigel-coated Boyden chamber assay was significantly reduced by  $56.1 \pm 8.2$  % and by  $52.9 \pm 14.4$  % in the presence of either R9-ZnC or R9-ZnC<sup>S334D</sup>, respectively, compared to the control. No significant difference was observed between R9-ZnC and the truncation mutant R9-ZnC<sup>S334D</sup>. On the other hand, soft-agar colony formation assays revealed that R9-ZnC did not alter 4T1-luc-triggered colony formation while a marked decrease of the total 4T1 colony number was observed upon R9-ZnC<sup>S334D</sup> treatment. (Figure 6B). These data indicate that both R9-ZnC and R9-ZnC<sup>S334D</sup> inhibit cancer cell invasion. Nevertheless, anchorage-independent growth appears to be specifically impaired by R9-ZnC<sup>S334D</sup>.

### *R9-ZnC and R9-ZnC<sup>S334D</sup> downregulate the expression of EMT-markers*

The inhibitory effect of R9-ZnC and R9-ZnC<sup>S334D</sup> on 4T1-luc cell invasion led us to analyse the expression of epithelial-mesenchymal transition (EMT) markers, as EMT is a crucial cellular process which enables cancer cells to invade. The effect of R9-ZnC and R9-ZnC<sup>S334D</sup> on the expression of the EMT upstream regulators Twist and Snail was determined by QPCR analysis (Figure 7A). We detected a decrease of Twist mRNA level in R9-ZnC<sup>S334D</sup>-treated cells, whereas R9-ZnC showed no effect. By contrast, both proteins reduced Snail mRNA level. Based on these results, we analysed the expression of E-Cadherin, N-Cadherin and Vimentin as they are downstream targets of the transcription factor Twist and Snail. Figure 7B shows that N-Cadherin and Vimentin were decreased by 20 to 30 % after treatment of 4T1-luc cells with 100 nM recombinant R9-ZnC<sup>S334D</sup> protein whereas E-Cadherin expression was not affected (Figure 7B). These results, which remain to be confirmed, suggest that R9-ZnC and R9-ZnC<sup>S334D</sup> proteins negatively regulate the expression of certain EMT markers and identify this novel TIS11b-derivatives as potential repressors of 4T1 cell aggressiveness.

### *R9-ZnC and R9-ZnC<sup>S334D</sup> impair the formation of pseudo capillaries by endothelial cells*

The formation of new blood vessels under physiological and pathological conditions requires the migration and reorganization of endothelial cells to form a vascular network. To test if R9-ZnC and R9-ZnC<sup>S334D</sup>, as potential anti-angiogenic agents, impair endothelial cell organization into pseudo-capillaries, HUVEC cells were plated at high density (confluence) on matrigel then treated with either 50 nM of recombinant R9-ZnC or R9-ZnC<sup>S334D</sup>. As shown in Figure 8, tube formation was inhibited in both conditions when compared to the control. A delayed organization of endothelial cells was already observed 6 h post-treatment (data not shown). Control cells were observed to orient and elongate while R9-ZnC or R9-ZnC<sup>S334D</sup>-treated cells remained rounded and show little orientation. Quantification of the pseudo-capillary network revealed that the endothelial cell-occupied area remained 50 % larger upon treatment with R9-ZnC or R9-ZnC<sup>S334D</sup> than in their absence (control), confirming that the truncated forms of TIS11b proteins are able to disrupt the functional organization of endothelial cells.

## Discussion

During the last decade, evidence has accumulated that the control of mRNA stability plays a critical role in cellular homeostasis. The functional relevance of post-transcriptional gene regulation is highlighted by several pathologies which are associated with a dysregulation of mRNA stability, including chronic inflammation and cancer (*Benjamin and Moroni, 2007*). We have previously brought the proof of concept of an anti-angiogenic and anti-tumoral therapy based on destabilization of short-lived mRNAs by the zinc finger protein TIS11b (*Planel et al., 2010*). Because the stability of proteins is paramount for their therapeutic use and thus, a major challenge for protein engineering, we aimed at improving the stability and activity of our therapeutic molecule. We integrated N-terminal truncation and C-terminal mutation of a specific serine as a novel TIS11b engineering strategy and demonstrated that these novel proteins are indeed potent inhibitors of several tumorigenesis hallmarks in breast cancer cells.

Like the two other members of the TIS11 family (TTP and TIS11d), TIS11b protein structure consists of three domains: the N-terminal domain, the tandem zinc finger domain and the C-terminal domain. The tandem zinc finger domain is crucial for the binding of TIS11b to the ARE located in the 3' untranslated region of the target mRNAs (*Lai et al., 2002*), but cannot induce mRNA decay by itself (*Rigby et al., 2005*). The N-terminal and C-terminal domain of TIS11b have been suggested to function as binding platforms for components of the mRNA decay machinery (*Lykke-Andersen & Wagner, 2005*). To determine the minimal active domain of TIS11b, we generated three truncated protein versions (NZn, Zn and ZnC) and tested their ability to induce ARE-mediated decay of a luciferase reporter gene mRNA fused to VEGF mRNA 3'UTR (Luc-3'UTR). Importantly the activity of the ZnC deletion mutant was comparable to that of the full-length TIS11b while the NZn mutant was significantly less active as measured by VEGF-3'UTR-driven luciferase activity. These results are in agreement with those reported by others (*Lykke-Andersen & Wagner, 2005*). Indeed, tethered N-terminal domain of TIS11b was shown to be less efficient than the C-terminal domain in the activation of  $\beta$ -globin reporter mRNA decay. The differences between NZn- and ZnC-induced mRNA destabilization suggest that these TIS11b protein domains interact with distinct components of the mRNA-decay machinery.

TIS11 proteins are target of several kinases and therefore hyperphosphorylated proteins (*Cao & Lin, 2008*). These post-translational modifications modulate their activity, stability and subcellular localization. Unpublished data of our team demonstrated the importance of the phosphorylatable C-terminal serine 334 (S334) in the control of TIS11b protein stability and

activity (Rataj, *et al.*, submitted, Article 1). We thus replaced the S334 by an aspartate (D) to mimic a permanent phosphorylation at this residue in order to potentiate the stabilizing effect of the N-terminal domain deletion of TIS11b. Both truncated proteins ZnC and ZnC<sup>S334D</sup> were successfully fused to the cell-penetrating peptide R9, composed of nine arginine residues, for their subsequent intracellular delivery.

Overexpression experiments as well as purified protein storage analyses demonstrated that the protein half-life in living cells and the stability of the purified truncated protein R9-ZnC at 37° C were indeed enhanced when compared to the full-length TIS11b. These effects were even more pronounced for the R9-ZnC<sup>S334D</sup> mutant. In addition, transduction experiments showed that our novel protein constructs were stable for several days in living cells (data not shown). It remains to be determined whether the stabilizing effect of TIS11b truncation/mutation is due to resistance to proteasomal degradation or to interaction with stabilizing factors, as both processes are involved in TIS11b protein turnover (Benjamin *et al.*, 2006). Although degradative ubiquitination of the TIS11 protein family is not well described so far, the fact that the N-terminal domain of proteins is used as the ubiquitination acceptor site might explain the stabilization of R9-ZnC (Ciechanover & Ben-Saadon, 2004). Benjamin *et al.* demonstrated that phosphorylation-dependent interaction between 14-3-3 protein and TIS11b prevents its proteasomal degradation (Benjamin *et al.*, 2006). In addition, it was shown very recently that TTP is degraded through ubiquitin-independent and proteasome-dependent mechanisms (Vo Ngoc *et al.*, 2014). The authors detected intrinsically disordered regions in the primary sequence of all three TIS11 proteins. These sequences do not harbour stable 3-D structures and could serve as proteasome recognition motifs (Tompa *et al.*, 2008). One could assume that deletion of intrinsically disordered regions located in the N-terminal domain of TIS11b increases protein stability.

As the fusion of cell-penetrating peptides to their cargo may alter the biological activity of the cargo, we checked the ability of R9-ZnC and R9-ZnC<sup>S334D</sup> to induce degradation of Luc-3'UTR reporter gene when overexpressed *in vitro*. Both protein constructs were as active as the full-length TIS11b protein, thus excluding an inhibitory effect of R9. Efficient cellular uptake of purified R9-TIS11b protein in living cells was demonstrated in our previous study (Planel *et al.*, 2010). Here, we showed that our novel protein constructs R9-ZnC and R9-ZnC<sup>S334D</sup> were also efficiently delivered into murine breast cancer cells.

We were first to identify the angiogenic cytokine VEGF as a direct target of TIS11b (Ciais *et al.*, 2004). We further demonstrated that TIS11b inhibits VEGF expression in Lewis Lung carcinoma cells and the development of vascularized tumors in mice (Planel *et al.*, 2010). On the other hand,

TTP was shown to decrease RasVal12-dependent VEGF expression in breast cancer cells (Essafi-Benkhadir *et al.*, 2007). In the present study, we bring evidence that the truncated form of TIS11b, R9-ZnC strongly reduced VEGF expression in breast cancer cells while R9-ZnC<sup>S334D</sup> completely obliterated VEGF expression, thus confirming the initially observed anti-angiogenic activity of the full-length TIS11b. TTP was shown to target, at least *in vitro*, multiple factors implicated in almost every step of tumor development (Ross *et al.*, 2012; Sanduja *et al.*, 2012). By contrast, a few studies have addressed the link between TIS11b and tumorigenesis (Hodson DJ *et al.*, 2010; Planel *et al.*, 2010). We tested the effect of our novel protein constructs on several hallmarks of tumorigenesis using the highly tumorigenic and invasive cell line 4T1-luc. R9-ZnC<sup>S334D</sup> appeared more efficient in the inhibition of 4T1-luc VEGF expression, proliferation, migration and anchorage-independent growth when compared to the non-mutated R9-ZnC. These observations suggest that R9-ZnC<sup>S334D</sup> is the most-promising candidate for therapeutic purposes. It is worth mentioning that the effects of both mutants were observed at low concentrations of purified proteins (50-100 nM).

There is increasing evidence that TIS11 protein family members negatively regulate cell proliferation. Indeed, depletion of TTP in non-invasive human breast cancer cells accelerates significantly their proliferation (Milke *et al.*, 2013). Treatment of PC3 human prostate cancer cells with siRNA against TTP increased their proliferation (Lee *et al.*, 2014). It was shown subsequently shown that TTP acts through suppression of E2F1, a transcription factor involved in G1 to S transition. TTP also promotes mouse embryonic fibroblast cell-cycle arrest by targeting the cyclin-dependent kinase inhibitor p21 (Al-Hajj *et al.*, 2004). On the other hand, restoring TTP levels by protein overexpression inhibited significantly human cervix or breast cancer cell proliferation *in vitro* (Brennan *et al.*, 2009; Griseri *et al.*, 2011). Our results show that the TIS11b-derived mutant R9-ZnC<sup>S334D</sup> decreases 4T1-luc cell proliferation. Nevertheless, target mRNAs of R9-ZnC<sup>S334D</sup> in this context remain to be identified.

Al-Souhibani *et al.* demonstrated that induction of TTP expression in human MDA-MB-231 breast cancer cells significantly reduced the migration abilities of these cells *in vitro* (Al-Souhibani *et al.*, 2014). In addition, the authors reported that the restoration of TTP expression in MDA-MB-231 cells reduces the mRNA stability of MMP1, uPA and uPAR, three key factors involved in invasion and metastasis, by directly interacting with the 3'UTR of these mRNAs (Al-Souhibani *et al.*, 2010). These results are in line with our observations showing that R9-ZnC<sup>S334D</sup> inhibits migration and invasion capacities of 4T1-luc cells and support the idea that TIS11 protein family underexpression promotes the aggressive phenotype of cancer cells.

In contrast to normal adherent cells, tumor cells have the capacity to grow without binding to a substrate. Anchorage-independent proliferation is a hallmark of tumor cell malignancy. Interestingly, we observed a significant decrease of colony growth in the presence of R9-ZnC<sup>S334D</sup> and not with R9-ZnC. This result suggests that R9-ZnC<sup>S334D</sup> may have additional mRNA targets that are involved in the malignant phenotype of 4T1-luc cells. In the same line, epithelial-to-mesenchymal transition is the main underlying mechanism of migration, invasion and further formation of metastasis by cancer cells. EMT is a morphologic conversion process which is initiated by transcription factors such as Snail or Twist. Their activation alters dramatically the gene expression profile of cancer cells including the repression of the epithelial cell adhesion molecule E-Cadherin and the induction of mesenchymal markers such as N-Cadherin and Vimentin. These factors promote remodelling of the cytoskeleton, loss of epithelial cell polarity and cell-cell contacts leading to a migratory cell phenotype (*Lamouille et al., 2014*). We report for the first time the inhibitory effect of a TIS11b-derived truncation (R9-ZnC<sup>S334D</sup>) on the expression of EMT-markers such as Twist, Snail and N-Cadherin. These observations suggest that this novel protein construct could favour the epithelial, non-metastatic phenotype of cancer cells. These data further corroborate the observed negative effects of R9-ZnC<sup>S334D</sup> on migration, invasion and anchorage-independent growth of 4T1-luc cells. However, the observed effects were rather modest at 24h post-treatment and additional experiments using longer incubation- periods in the presence of recombinant proteins are required. As TIS11b induces mRNA destabilization through the binding of AREs located in the 3'UTR of target mRNAs, we used the database AREsite to predict the presence of AREs in the tested EMT markers (*Gruber et al., 2011*). Indeed, AUUUA pentamers are abundant in all transcripts tested (E-Cadherin, N-Cadherin, Snail, Twist and Vimentin) (data not shown), indicating their potential to be post-transcriptionally regulated by TIS11b. However, the direct interaction of TIS11b and its derivatives with these mRNAs needs to be confirmed. In contrast to the current paradigm, we did not observe an increase of E-Cadherin expression concomitant to the decrease of N-Cadherin. *Lou et al.* characterized the expression of EMT markers in several 4T1-luc cell clones which differ in their invasiveness (*Lou et al., 2008*). Interestingly, the authors also observed E-Cadherin expression in metastatic 4T1-luc cells. *Gebeshuber et al.* demonstrated that downregulation of TTP promotes epithelial-to-mesenchymal transition and metastasis in Ras-transformed mammary epithelial cells. Conversely, restored expression of TTP increased E-Cadherin expression and reduced Vimentin mRNA and protein levels (*Gebeshuber et al., 2009*). Since R9-TIS11b-treated Lewis lung carcinoma tumors were significantly less vascularized than non-treated tumors (*Planel et al., 2010*), we investigated the effect of the truncated R9-ZnC and



R9-ZnC<sup>S334D</sup> proteins on endothelial cell function *in vitro*. We show that the ability of human endothelial cells (HUVEC) to form tube-like structures was impaired in the presence of R9-ZnC and R9-ZnC<sup>S334D</sup>, thus strengthening the anti-angiogenic effect of TIS11b. Furthermore, these results indicate that R9-ZnC and R9-ZnC<sup>S334D</sup> can potentially target several cell types within a tumor. Indeed, we have previously demonstrated that TTP targets HIF-1 $\alpha$  in endothelial cells exposed to hypoxia, leading to downregulation of the major HIF-1 $\alpha$  downstream target CAIX, a cancer-related enzyme involved in the regulation of pH homeostasis, cell proliferation and adhesion (*Chamboredon et al., 2011*). The endothelial mRNA targets of our truncated forms of TIS11b remain to be identified.

Altogether, our findings provide further evidence that mRNA stability regulators such as TIS11b, in addition to well described transcription factors, regulate tumorigenic processes. Based on our results, R9-ZnC and R9-ZnC<sup>S334D</sup> appear as anti-tumoral agents which inhibit several hallmarks of cancer progression and need to be evaluated in *in vivo* studies.

### **Conflict of interest**

The authors declare no conflict of interest.

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## Legends of the figures

### Figure 1: Characterization of the novel TIS11b truncated forms R9-ZnC and R9-ZnC<sup>S334D</sup>

- (A) Schematic representation of R9-ZnC and R9-ZnC<sup>S334D</sup> fusion proteins.
- (B) Inhibitory effect of overexpressed R9-TIS11b, R9-ZnC and R9-ZnC<sup>S334D</sup> on VEGF mRNA 3'UTR fused to luciferase reporter gene. COS7 cells showed significantly decreased luciferase activity when transfected with 100 ng of R9-TIS11b, R9-ZnC and R9-ZnC<sup>S334D</sup> pTarget-vectors. pTarget-R9-empty plasmid was used as control. Upper panel: Reporter gene activity was determined 24 h post-transfection as described in Material and Methods. Results are presented as relative light units of Firefly luciferase and are represented as a percentage of luciferase activity in control cells. In the presence of R9-TIS11b, R9-ZnC and R9-ZnC<sup>S334D</sup> Firefly luciferase activity was  $30.2 \pm 2.5$  % for R9-TIS11b,  $42.2 \pm 2.3$  % for R9-ZnC and  $34 \pm 4.4$  % for R9-ZnC<sup>S334D</sup> of controls (n = 4-7). Transfections were performed in duplicates or triplicate and results are means  $\pm$  s.e.m. Each value was compared to control using One-way ANOVA with Bonferroni's multiple comparison post-test. \*\*\*, significantly different from control with  $P < 0.001$ . Lower panel: Western blot analysis using anti-TIS11b antibodies confirmed equal expression of R9-TIS11b, R9-ZnC and R9-ZnC<sup>S334D</sup> pTarget-plasmids. Due to truncation of the full-length R9-TIS11b fusion protein (51 kDa), ZnC and ZnC<sup>S334D</sup> were detected at 38 kDa.
- (C) COS7 cells were transfected with R9-TIS11b, R9-ZnC or R9-ZnC<sup>S334D</sup> pTarget-vectors. 24 h post-transfection, cells were lysed and total RNA was extracted for Northern blot analyses. Upper panel: The membrane was hybridized with a radiolabelled VEGF 3'UTR probe and re-hybridized for 18S RNA detection. Shown is a representative Northern blot of two independent experiments. Lower panel: Quantification of VEGF mRNA signal intensities in this particular experiment. VEGF mRNA values were normalized to 18S RNA values and plotted as a percentage of VEGF mRNA level in control cells.
- (D) COS7 cells were transfected with 100 ng of R9-TIS11b, R9-ZnC or R9-ZnC<sup>S334D</sup> pTarget-vectors. Twenty four hours post-transfection, the half-life for each fusion protein was determined in time-course experiments by treating the cells with the translation inhibitor Cycloheximide (CHX) (10  $\mu$ g/ml). Left panel: Remaining protein was analysed by Western blot using anti-TIS11b antibodies. The membrane was re-probed with anti-Actin antibodies. Protein level was quantified using ImageJ software and normalized to Actin protein values. Right panel: Protein half-life of R9-TIS11b, R9-ZnC and R9-ZnC<sup>S334D</sup> is plotted as a percentage of the initial value over time. Data are representative of three independent experiments. Protein half-life was  $t_{1/2} \approx 4$  h for R9-TIS11b,  $t_{1/2} \approx 6$  h for R9-ZnC and  $t_{1/2} = 8$  h for R9-ZnC<sup>S334D</sup>.

**Figure 2: Overexpression and purification of recombinant R9-ZnC proteins in *Escherichia coli*.**

- (A) R9-ZnC protein expression was induced in *E.coli* by IPTG ( $\beta$ -D-1-thiogalactopyranoside) (NI, non-induced; I, induced) and purified by anti-Flag affinity-based chromatography (FT, flow through; W1/W10, washing step 1 or 10). The same procedure was used for R9-TIS11b, R9-ZnC and R9-ZnC<sup>S334D</sup> purification.
- (B) Purified R9-TIS11b, R9-ZnC and R9-ZnC<sup>S334D</sup> were analysed by SDS-PAGE and visualized by Coomassie Brilliant Blue staining. The apparent molecular weight of the recombinant proteins was slightly higher than the predicted one (40 kD for R9-TIS11b and 30 kD for R9-ZnC/R9-ZnC<sup>S334D</sup>).
- (C) Specificity of eluted proteins was further validated by Western blot using anti-Flag antibody.

**Figure 3: Increased stability under storage and efficient cellular uptake of purified R9-ZnC proteins.**

- (A) Left panel: Equal amounts of purified R9-TIS11b, R9-ZnC, R9-ZnC<sup>S334D</sup> recombinant proteins were stored at either 4 °C or 37 °C up to five days. Samples kept at -20 °C were used as reference. Remaining protein was quantified by Western blot using the Flag-tag antibody. Arrows indicate purified R9-TIS11b, R9-ZnC, R9-ZnC<sup>S334D</sup>. Bands below are products of protein degradation. Results are representative of two independent experiments. Right panel: Protein level was quantified using ImageJ software and normalized to -20 °C control samples. The data are percentage of respective controls for both temperature conditions. After five days-incubation at 4 °C, 63  $\pm$  9.5 % of R9-TIS11b, 88  $\pm$  16.0 % of R9-ZnC and 92  $\pm$  3.2 % of R9-ZnC<sup>S334D</sup> initial protein quantity were detected (n = 2-3). Following five day-storage at 37 °C, 47  $\pm$  5.2 % of R9-TIS11b, 56  $\pm$  14.0 % of R9-ZnC and 79  $\pm$  2.2 % of R9-ZnC<sup>S334D</sup> initial protein quantity were detected (n = 2-3).
- (B) 4T1-luc cells were incubated for 16 h in the presence of 50 ng Alexa 488-labeled purified ZnC, R9-ZnC or R9-ZnC<sup>S334D</sup> followed, by the addition of Hoechst 33342 for 30 min to stain nuclei and Alexa Fluor 594 wheat germ agglutinin for 10 min to label cell plasma membrane. Laser confocal microscopy visualized internalization of ZnC (non-penetrating control), R9-ZnC or R9-ZnC<sup>S334D</sup> (upper panel). Deconvolution of laser confocal microscopy images along the *x-y* axes (central), *x-z* axes (bottom) and *y-z* axes (right) confirmed internalization of Alexa 488-labeled ZnC, R9-ZnC or R9-ZnC<sup>S334D</sup> (lower panel). White lines mark axes along which deconvolution

was done. Green fluorescence signal along the *z*-axis indicates efficient cellular uptake of labelled proteins. Scale bar, 37.5  $\mu\text{m}$ .

**Figure 4: Inhibitory effect of R9-ZnC and R9-ZnC<sup>S334D</sup> on VEGF expression in 4T1-luc breast cancer cells.**

$2.0 \times 10^5$  4T1-luc cells were seeded in a 12-well plate and incubated for 24 h in the presence of 100 nM purified R9-ZnC or R9-ZnC<sup>S334D</sup> protein.

- (A) Quantitative real time-PCR analysis of VEGF mRNA in treated cells. RPL13A was used as housekeeping gene and results are normalized to control. Results are means  $\pm$  SD of two independent experiments. VEGF mRNA expression was  $58.2 \pm 8.8$  % of control for R9-ZnC and  $27.4 \pm 8.5$  % of control for R9-ZnC<sup>S334D</sup>.
- (B) Culture media of the same experiments were used to determine VEGF protein content using ELISA. 215 pg VEGF/ml and 142 pg/ml were measured in control and R9-ZnC-treated cells, whereas VEGF protein was undetectable after R9-ZnC<sup>S334D</sup> treatment. Presented results are means  $\pm$  SD of duplicates.

**Figure 5: R9-ZnC<sup>S334D</sup> inhibits proliferation and migration of 4T1-luc tumor cells.**

- (A) To perform proliferation assays,  $1.0 \times 10^4$  4T1-luc cells were plated in 96-well plates and treated for 48 h with 12.5, 25 or 50 nM of R9-ZnC or R9-ZnC<sup>S334D</sup> in complete growth medium. Equal amount of vehicle served as control. At  $t = 0$  h,  $t = 24$  h and  $t = 48$  h, WST-1 was added and absorbance at 450 nm was determined after 2 h incubation at 37 °C. The highest proliferation activity was observed for control. Shown is a representative experiment of  $n = 3$  independent ones.
- (B) 4T1-luc cells were seeded in 12-well plates and incubated until confluence then the monolayer was scratched. Wound healing was monitored over 16 h in the presence of 50 nM of R9-ZnC, R9-ZnC<sup>S334D</sup> or vehicle. Distance between wound edges was measured at  $t = 0$  h and  $t = 16$  h. Light microscopy acquisitions on the left side are representative of three independent experiments. Data on the right are percentages of wound closure 16 h post-treatment normalized to  $t = 0$  h. While no effect of R9-ZnC was observed, migration of 4T1-luc cells was significantly inhibited by 30 % compared to control. Results are means  $\pm$  s.e.m. of three independent experiments. Each value was compared to control using One-way ANOVA with Bonferroni's multiple comparison post-test. \*, significantly different from control with  $P < 0.05$ . Scale bar, 200  $\mu\text{m}$ .

**Figure 6: Both R9-ZnC and R9-ZnC<sup>S334D</sup> inhibits invasion whereas anchorage-independent growth of tumor cells is impaired by R9-ZnC<sup>S334D</sup> only.**

- (A) Invasive properties of 4T1-luc cells were analysed using the Boyden chamber matrigel invasion assay as described in Material and Methods. Upper panel: 4T1-luc cells were treated for 48 h with 50 nM of recombinant proteins using the transwell system. Invaded cells were stained with Crystal Violet and cell occupied area was measured using ImageJ software. Images represent the whole transwell surface (scale bar, 1 mm). Lower panel: Invaded cells were lysed in appropriate assay buffer and Firefly luciferase was measured. Results are presented as percentage of control. In the presence of R9-ZnC and R9-ZnC<sup>S334D</sup>, Firefly luciferase activity was  $43.9 \pm 8.2$  % of control for R9-ZnC and  $47.1 \pm 14.4$ % of control for R9-ZnC<sup>S334D</sup>. Results are means  $\pm$  s.e.m. of three independent experiments. Each value was compared to control using One-way ANOVA with Bonferroni's multiple comparison post-test. \*, significantly different from control with  $P < 0.05$ .
- (B) Effect of R9-ZnC and R9-ZnC<sup>S334D</sup> on anchorage-independent growth of 4T1-luc cells. Soft agar colony formation assay was performed as described in Material and Methods. Colonies were stained with Crystal Violet 14 d after plating and were counted. The graphs illustrate the total number of counted colonies ( $> 6 \mu\text{m}$ ) of the whole agar surface for the experiment shown. Two independent experiments showed similar results. Scale bar, 0.2 mm.

**Figure 7: Effect of R9-ZnC and R9-ZnC<sup>S334D</sup> proteins on EMT-markers in 4T1-luc breast cancer cells.**

- (A) Quantitative real-time PCR analysis of Snail and Twist mRNA in treated 4T1-luc cells. A decrease of Twist mRNA level was detected in the presence of R9-ZnC<sup>S334D</sup> only, whereas both R9-ZnC or R9-ZnC<sup>S334D</sup> reduced Snail mRNA level. RPL13A was used as housekeeping gene and results are normalized to control. Presented results are means  $\pm$  SD of duplicates.
- (B) Western Blot analysis using anti-E-Cadherin, anti-N-Cadherin and anti-Vimentin antibodies showed a decreased Vimentin and N-Cadherin expression after treatment of 4T1-luc cells with 100 nM recombinant R9-ZnC or R9-ZnC<sup>S334D</sup> proteins. Protein level values were quantified using Chemidoc ImageLab software and normalized to Tubulin protein values. Results are presented as normalized values against control and are means  $\pm$  SD of two independent experiments.



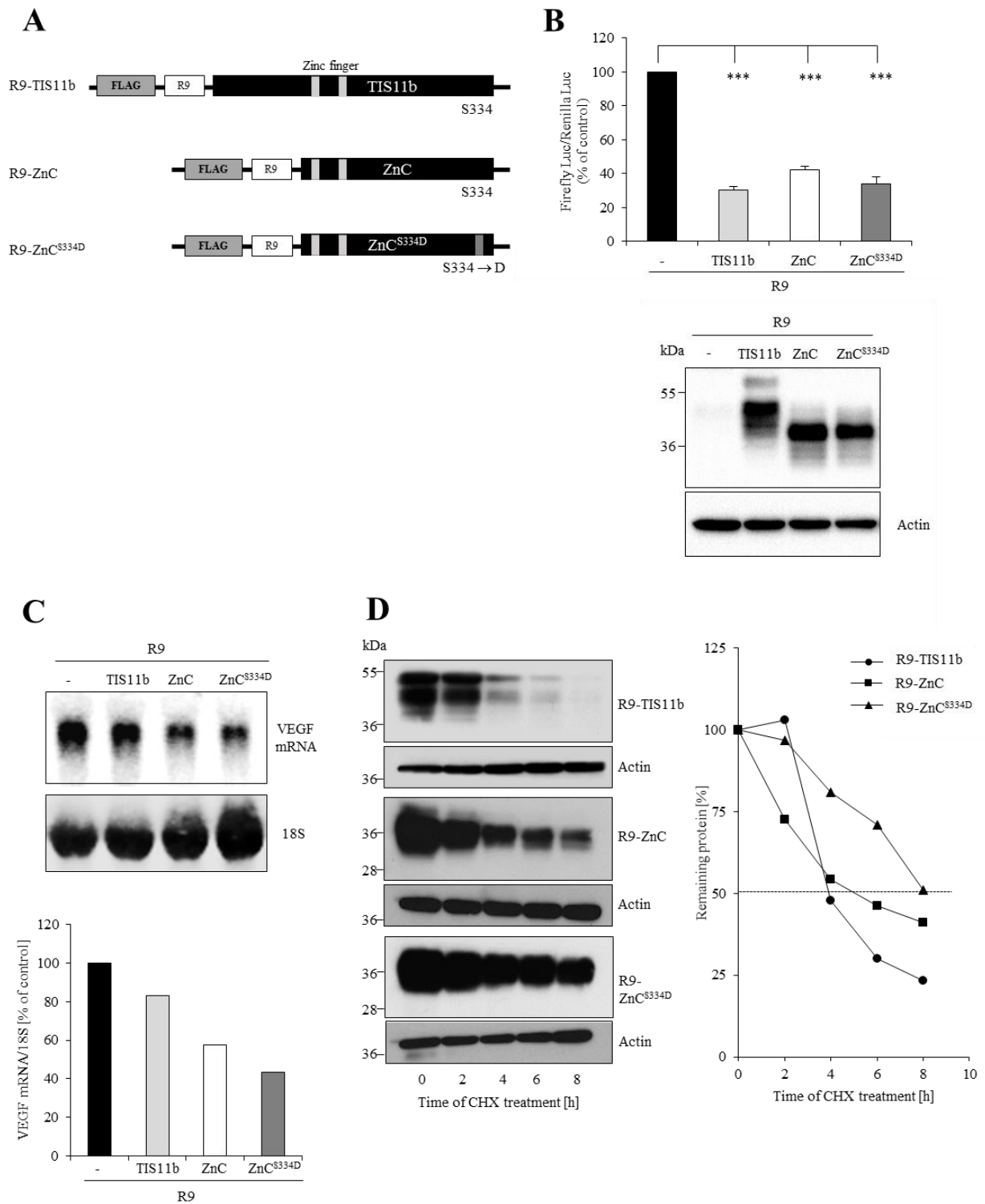
**Figure 8: Effect of purified R9-ZnC and R9-ZnC<sup>S344D</sup> proteins on endothelial cells.**

Formation of a pseudo-capillary network using HUVEC endothelial cells was performed as described in Material and Methods. Images are representative of three independent experiments. Cell-occupied area was determined using ImageJ software. The area covered by HUVEC was  $56 \pm 14\%$  and  $54 \pm 7\%$  higher in the presence of R9-ZnC and R9-ZnC<sup>S344D</sup>, respectively, than that covered by non-treated cells. Results are means  $\pm$  s.e.m. of three independent experiments. Each value was compared to control using One-way ANOVA with Bonferroni's multiple comparison post-test. \*, significantly different from control with  $P < 0.05$ .

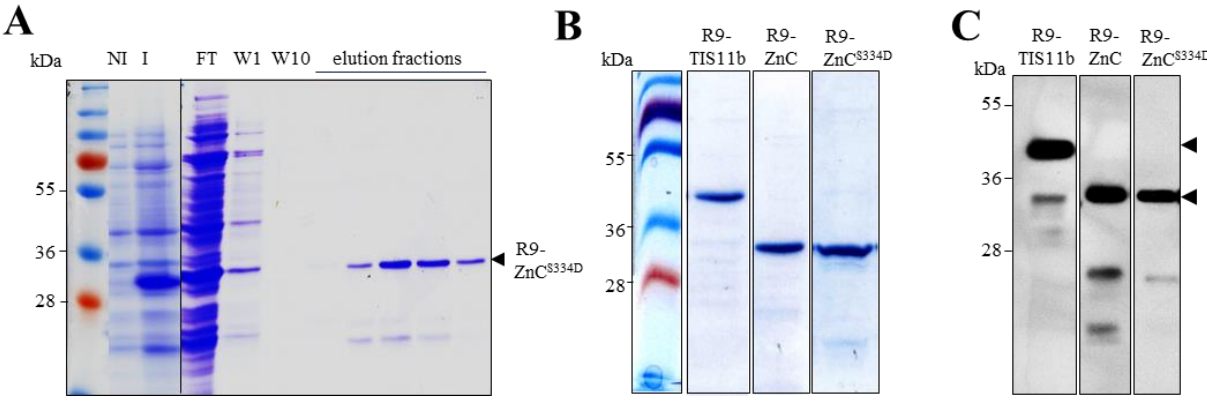
**Supplementary figure 1: Truncation of TIS11b does not impair its mRNA-destabilizing activity**

- (A) Schematic representation of truncated TIS11b proteins NZn<sub>(1-195)</sub>, Zn<sub>(99-195)</sub> and ZnC<sub>(109-339)</sub>.
- (B) Effect of overexpressed TIS11b and its truncated forms NZn, Zn and ZnC on the luciferase activity of a luciferase reporter gene fused to the 3'UTR of VEGF mRNA. pTarget-R9-empty plasmid was used as control. Reporter gene activity was determined 24 h post-transfection as described in Material and Methods. COS7 cells showed a significant, dose-dependent decrease in luciferase activity in the presence of TIS11b and ZnC. The NZn domain of TIS11b is less active than the ZnC while the Zn domain displays the lowest activity. Results are presented as relative light units of Firefly luciferase over Renilla luciferase and normalized against the control pTarget-empty plasmid. Transfections were performed in triplicates and results are means  $\pm$  s.e.m. of two to three independent experiments. Each value was compared to control using One-way ANOVA with Dunnett's multiple comparison post-test. \*, significantly different from control with  $P < 0.05$ ; \*\*, significantly different from control with  $P < 0.01$ . All values were compared to each other using One-way ANOVA with Bonferroni's multiple comparison post-test. #, Zn significantly different from full-length TIS11b  $P < 0.05$ .
- (C) RT-PCR confirmed a dose-dependent expression of TIS11b, NZn, Zn and ZnC in COS7 cells. HPRT was used as housekeeping gene.

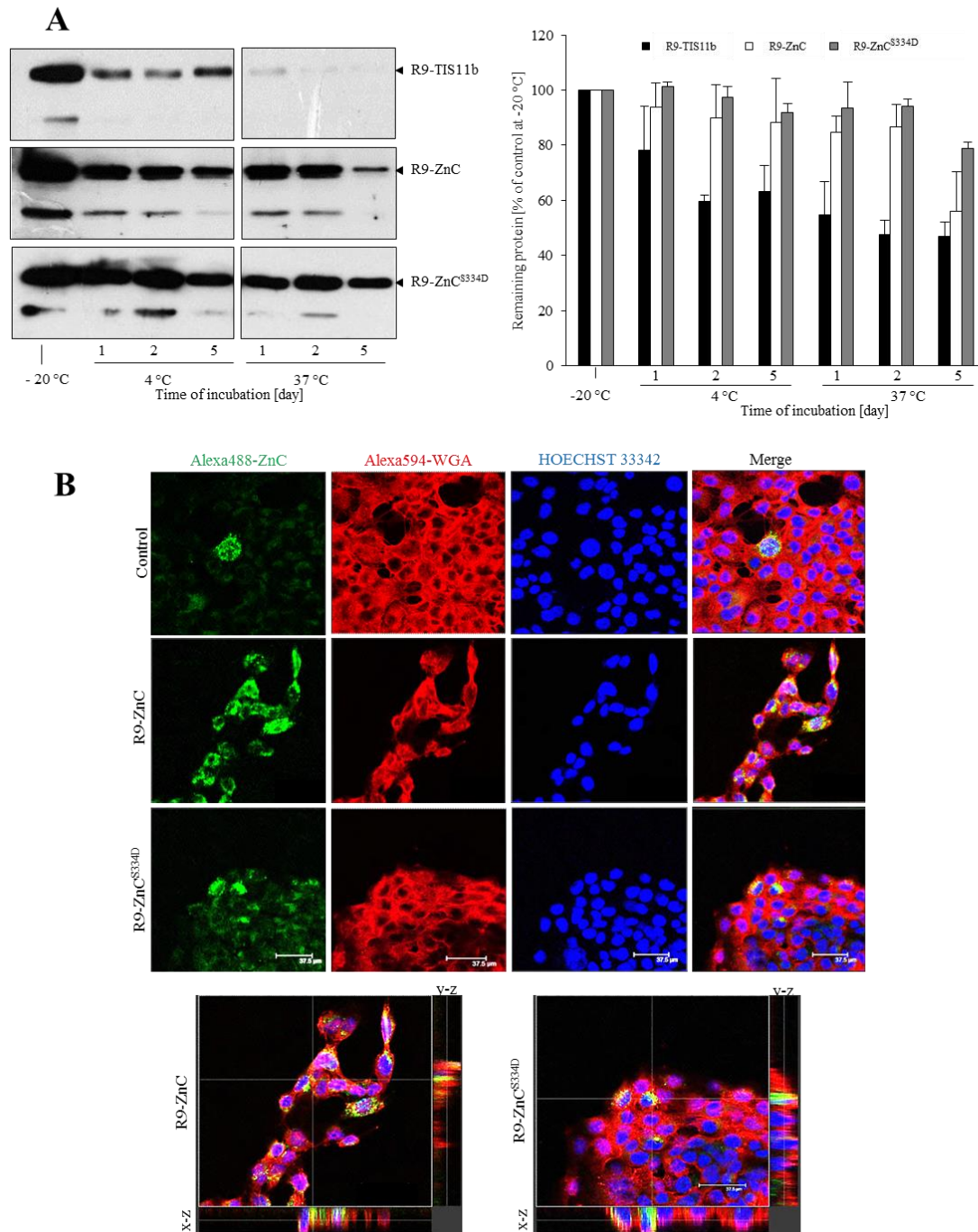
**FIGURE 1**



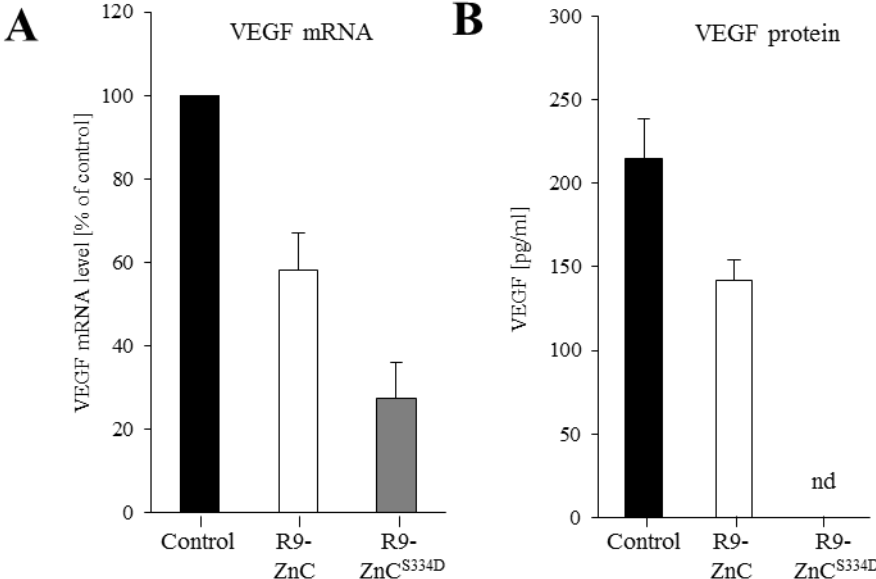
**FIGURE 2**



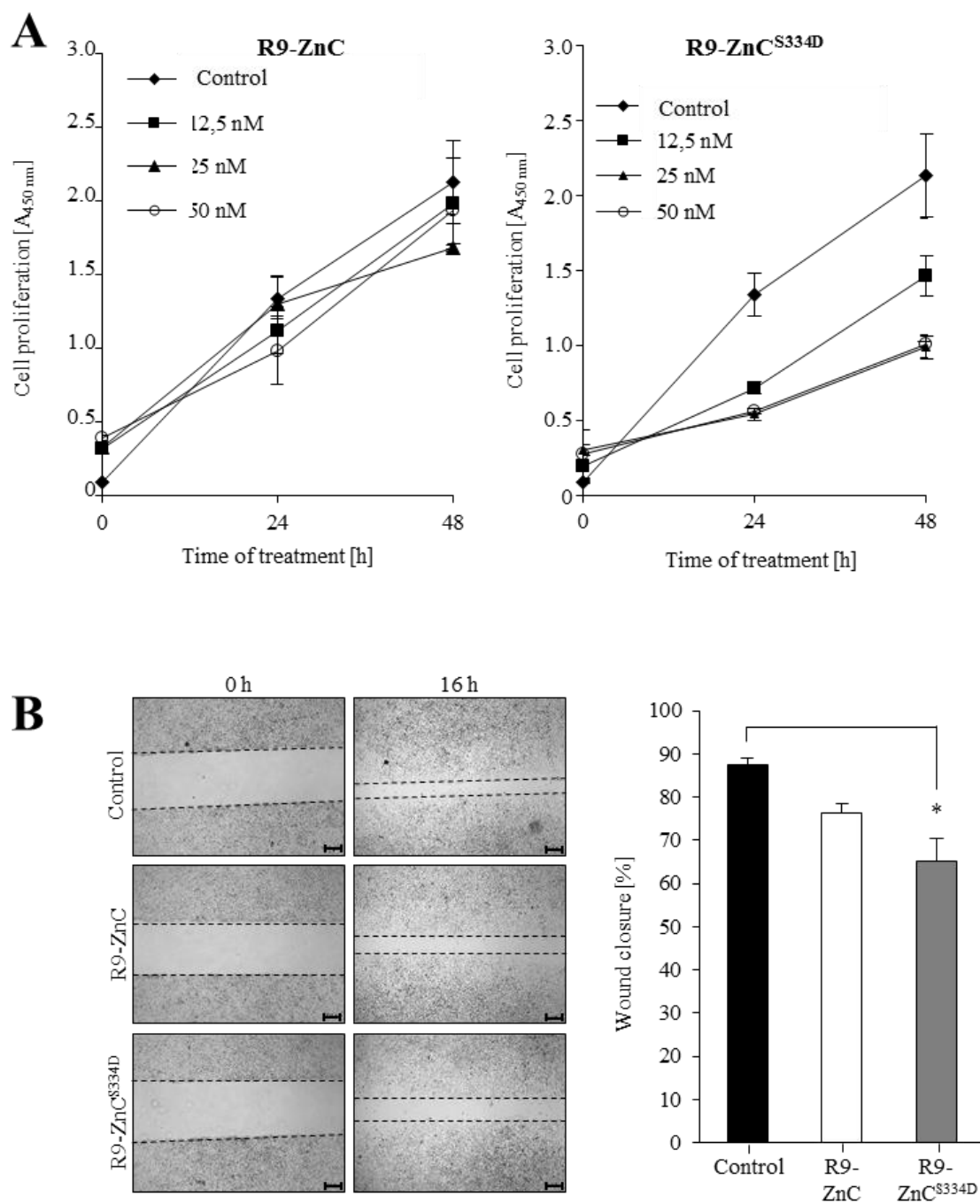
**FIGURE 3**



**FIGURE 4**

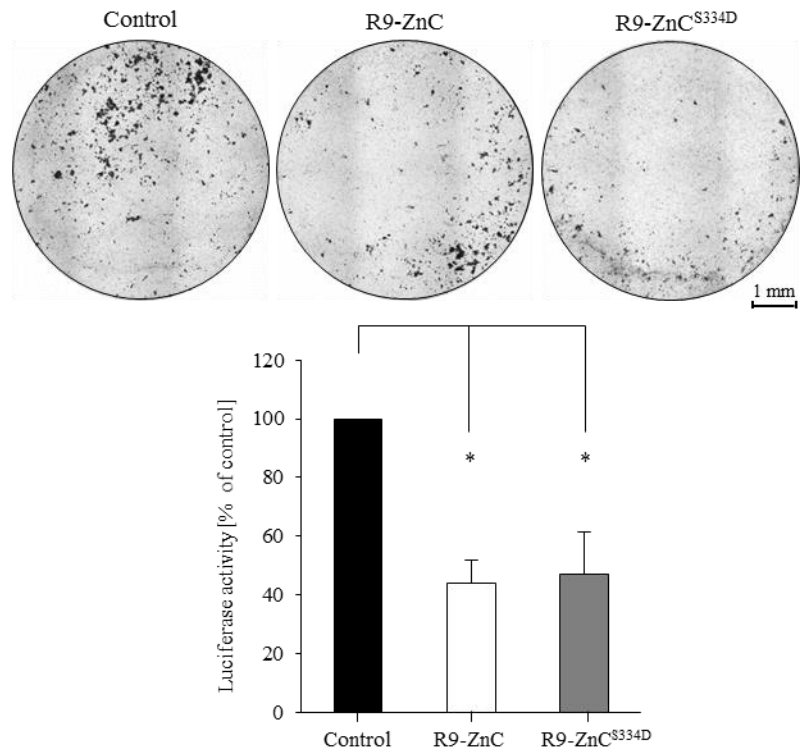


**FIGURE 5**

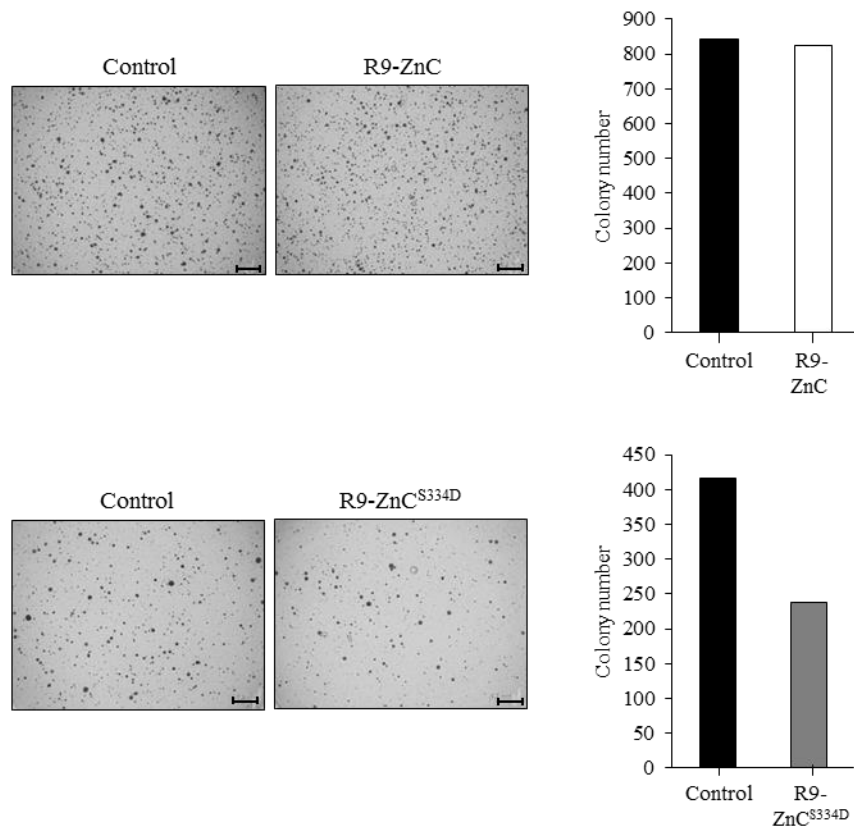


**FIGURE 6**

**A**

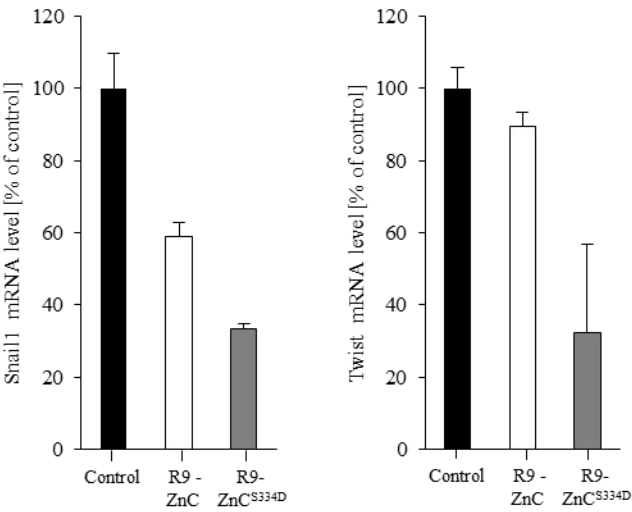


**B**

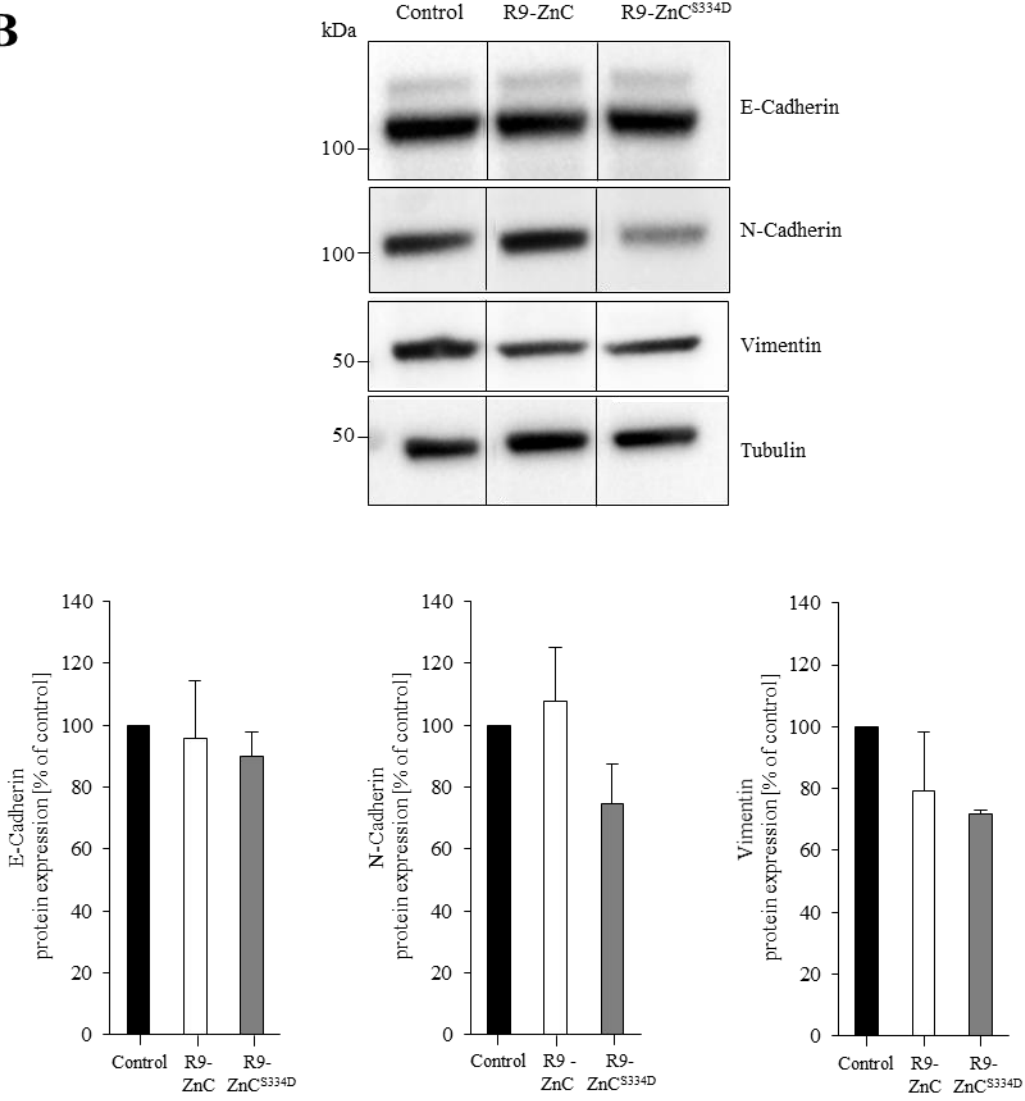


**FIGURE 7**

**A**

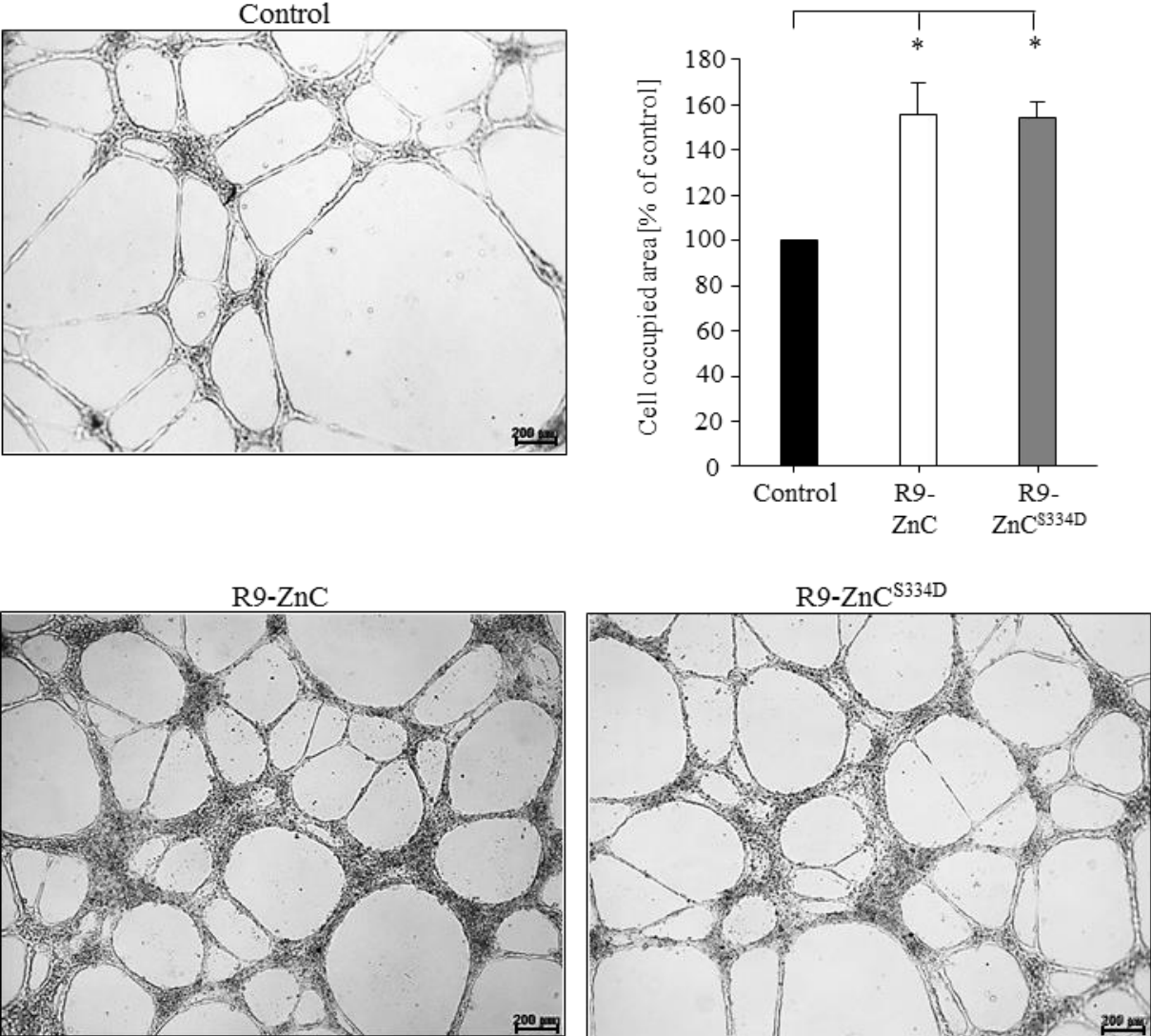


**B**

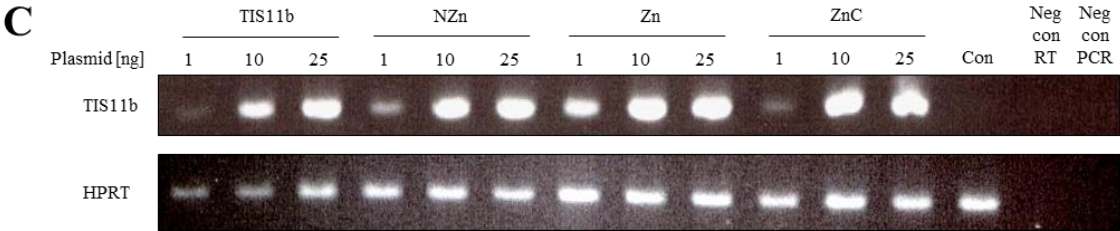
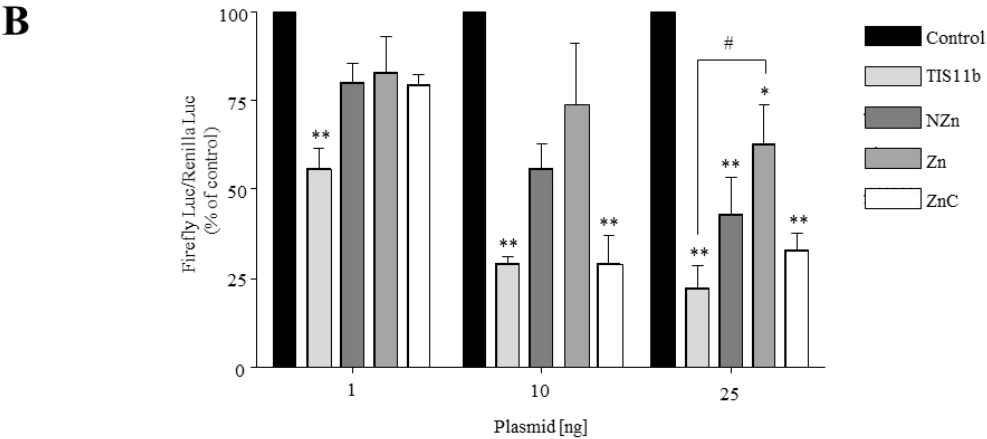
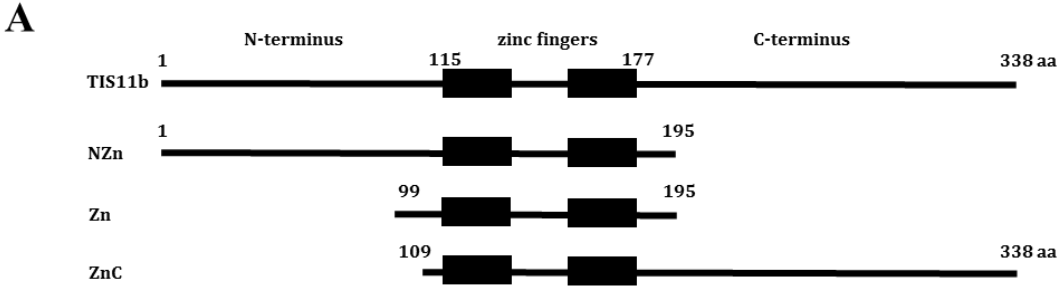




**FIGURE 8**



**SUPPLEMENTARY FIGURE 1**





**Introduction**

Encouraged by the previously described *in vitro* results which suggested an inhibitory effect of R9-ZnC and R9-ZnC<sup>S334D</sup> purified protein on several cancer cell characteristics, we performed *in vivo* studies using mouse preclinical models. The aims of these experiments were to determine the effect of R9-ZnC and R9-ZnC<sup>S334D</sup> on several hallmarks of tumor progression including tumor growth, tumor vascularization and tumor invasion capacities as well as to identify their potential mRNA targets.

We decided to use the syngeneic mouse 4T1 breast tumor model for several reasons (*Pulaski & Ostrand-Rosenberg, 2001*). The 4T1 mammary carcinoma cell line was initially isolated by *Fred Miller* and colleagues from a spontaneously grown mammary tumor in a BALB/c mouse (*Dexter et al., 1978; Aslakson & Miller, 1992*). 4T1 cells are highly tumorigenic and invasive and give rise to palpable tumors within a short period. When orthotopically injected into the fat pad of the mammary gland of immunocompetent mice, these cells are able to form both a primary tumor and spontaneous metastases in several distant organs, such as lung, bone, brain, liver and lymph nodes. This model is very similar to that of human breast cancer and mimics late stage triple negative human breast cancer. 4T1 tumors were described to be hypoxic as a high expression of carbonic anhydrase (CAIX), a HIF-1 $\alpha$  downstream target was detected in these tumors (*Lou et al., 2008*). 4T1 tumors are also highly vascularized, thus representing a good model to explore the therapeutic potential of novel anti-angiogenic therapies *in vivo*. Indeed, among other studies, *Walti et al.* have described recently the unexpected effect of the tyrosine kinase inhibitor sunitinib on 4T1 lung metastases (*Walti et al., 2012*). Sunitinib increased the number of pulmonary metastases due to the reduced pericyte coverage of lung vessels which enhanced the extravasation of 4T1 cells and formation of metastatic lesions. By contrast, the synthetic tyrosine kinase inhibitor 194-A suppressed 4T1 metastasis due to the inhibition of VEGFRs/FGFRs signalling (*Chien et al., 2013*). In addition, the 4T1 model has been used to explore several chemotherapeutic treatments. Among others, Paclitaxel was intensively studied during the last years (*Jiang et al., 2010; Ho et al., 2012; Meyer-Losic et al., 2013; Sharma et al., 2013*). Given the importance of the tumor environment in cancer cell behaviour, the syngeneic 4T1 model was also used to investigate the involvement of the immune system in cancer progression and to evaluate anti-tumor immunotherapeutics. For example, a very recent study demonstrated that the blockade of the cytokine Oncostatin M and the chemokine Eotaxin by neutralizing antibodies

reduced the content of tumor-promoting M2-macrophages in 4T1 tumors, leading to tumor regression and decreased intratumoral vascularization (*Tripathi et al., 2014*).

## Background

Taking the advantage of the murine 4T1 model, we initially injected luciferase-expressing 4T1-luc cells in the fat pad of the mammary gland. These studies were limited by the fast orthotopic tumor growth and the restricted accessibility of tumors for size measurements with caliper. The monitoring of tumor development was performed exclusively by bioluminescence *in vivo* imaging and we have faced an important heterogeneity in tumor development within the different groups.

Based on previous successful *in vivo* studies of our laboratory, in which pre-established subcutaneous Lewis lung cancer tumors (LLC) in nude mice were treated by intratumoral injections of the cell-permeable R9-TIS11b fusion protein, we decided to continue our *in vivo* studies by implanting 4T1-luc cells subcutaneously (*Planel et al., 2010*). Overall, the major drawback of our 4T1-luc *in vivo* experiments was the spontaneous regression of pre-established tumors. Strikingly, this regression was more frequent in the control group. *Tao et al.* demonstrated a biphasic growth of 4T1-luc tumors with a rapid growth during the first two weeks post-implantation then a regression between week 2 and 4, and a re-growth in weeks 5 and 6 (*Tao et al., 2008*). The authors revealed a correlation between the observed tumor regression and a host-immune response as they observed necrosis and leukocyte infiltration. This correlation was further strengthened by the absence of the biphasic tumor growth of 4T1-luc cells in athymic nude or SCID BALB/c mice. In addition, antibodies directed against 4T1 antigens were found in the serum of tumor-bearing mice at week 6. *DuPré et al.* reported that 40-50 % of the 4T1 tumor mass were immune cells, underlining the importance of the host immune system in this murine tumor model (*DuPre et al., 2007*). 4T1 tumors seem to be heterogeneous as highly invasive and non-metastatic clones could be obtained from the same tumor (*Tao et al., 2008*).

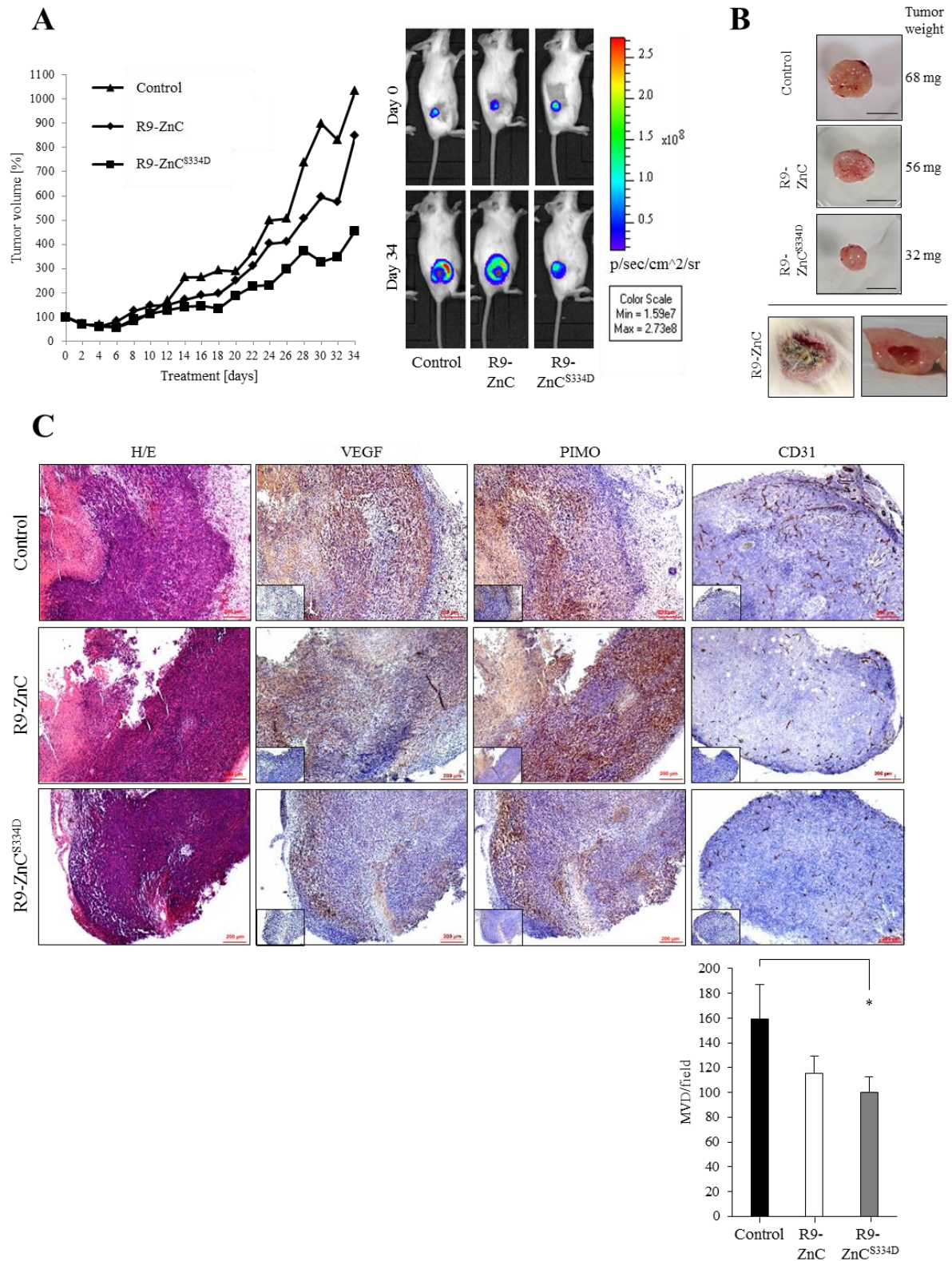
In the present work, we illustrate and describe a part of the results of a pilot study, which was performed to determine the best dose of our novel protein constructs for the treatment. In this experiment, we tested the anti-tumoral effect of three concentrations (50 ng, 100 ng and 200 ng) of R9-ZnC or R9-ZnC<sup>S334D</sup>. We observed that a dose of 200 ng of purified R9-ZnC or R9-ZnC<sup>S334D</sup>/injection decreased tumor volume and impaired several hallmarks of tumorigenesis. Although the results presented here are rather encouraging, the data need to be confirmed in future experiments using a statistically significant number of animals.

## Results & Discussion

### *Intratumoral injection of R9-ZnC or R9-ZnC<sup>S334D</sup> inhibits 4T1 luc tumor growth*

To evaluate the anti-tumoral effect of our novel protein constructs R9-ZnC/ R9-ZnC<sup>S334D</sup> *in vivo*, luciferase-expressing 4T1-luc cells were subcutaneously injected into immunocompetent female BALB/c mice. Based on our *in vitro* studies on the protein half-life and protein stability of purified R9-ZnC or R9-ZnC<sup>S334D</sup> proteins, pre-established tumors (minimal size 50 mm<sup>3</sup>) were treated every other day by intratumoral injections of either 200 ng of R9-ZnC or R9-ZnC<sup>S334D</sup> or vehicle as control. As shown in Figure 2-1 A, tumor volume was decreased by 18 % (R9-ZnC) or 56 % (R9-ZnC<sup>S334D</sup>) after 34 days of treatment compared to the control, indicating that tumor growth inhibition was more pronounced in the presence of R9-ZnC<sup>S334D</sup>. *In vivo* bioluminescence imaging, which detects the luciferase activity of living 4T1-luc cells, confirmed this inhibitory effect (8.8 x 10<sup>8</sup> photons/s for R9-ZnC<sup>S334D</sup>-treated tumor versus 2.7 x 10<sup>9</sup> photons/s for control tumor). At day 34 of treatment, no significant difference was observed between the luciferase activity of R9-ZnC-treated tumors and the control. However, differences in tumor weight correlated with differences in tumor volume (Figure 2-1 B). We observed an important macroscopic necrosis of R9-ZnC and R9-ZnC<sup>S334D</sup>-treated tumors (Figure 2-1 B). Even if ulceration is described as a characteristic of the 4T1 breast cancer model, necrosis appeared earlier during tumor progression in the presence of our novel protein constructs. During tumor dissection, the centre of the treated tumors was filled with a viscous liquid, whereas tissue control tumors remained compact. Furthermore, the R9-ZnC<sup>S334D</sup>-treated tumor appeared to be paler than the control, suggesting weaker intratumoral vascularization.

**Figure 6**



**Figure 2-1: R9-ZnC or R9-ZnC<sup>S334D</sup> inhibits 4T1-luc tumor growth, tumor vascularization and VEGF expression as well as tumor hypoxia.**

4T1-luc cells were subcutaneously implanted into immunocompetent BALB/c mice (n=1 per group). Pre-established tumors were treated by injecting 200 ng of R9-ZnC, R9-ZnC<sup>S334D</sup> or vehicle directly into the tumor every other day.

- (A) Tumor growth was monitored by either sequential determination of tumor volume using caliper or measurement of emitted bioluminescence at day 0 and 34 of treatment. Tumor volume is as percentage of tumor volume at the first day of treatment. Bioluminescence signals of *in vivo* imaging are expressed as photons/sec/cm<sup>2</sup>/sr.
- (B) Tumor weight confirmed *in vivo* imaging signals at day 34 of treatment and tumor volume measurements. Scale bar, 1 cm. Images in the lower panel illustrate macroscopic ulceration and necrosis observed for R9-ZnC-treated tumors.
- (C) Histochemical hematoxylin/eosin staining and immunohistochemical detection of intratumoral VEGF expression, hypoxic areas (PIMO, Pimonidazole hydrochloride) and tumor vascularization (CD31) after 34 days of treatment. The insets on the left lower corner present antibody negative control to exclude nonspecific immunohistochemical staining. The graph represents quantification of microvessel density (MDV) in several stained tumor sections of control, R9-ZnC and R9-ZnC<sup>S334D</sup>-treated mice. Results are means  $\pm$  SD. Each value was compared to control using One-way Kruskal-Wallis test and Dunnett's Multiple comparison post-test. \*, significantly different from control with  $P < 0.05$ . Scale bar, 200  $\mu$ m.

*R9-ZnC or R9-ZnC<sup>S334D</sup>-treatment of 4T1-luc tumors decreases VEGF expression and microvessel density as well as hypoxic areas*

To further analyse the inhibitory effect of R9-ZnC or R9-ZnC<sup>S334D</sup> on tumor growth *in vivo*, we performed immunohistochemical analyses (Figure 2-1 C). Macroscopic necrosis was confirmed by Haematoxylin & Eosin staining of tumor sections which indicated a necrotic centre of the tumor (pink staining) due to eosinophilic structures and a peripheral rim of living tumor cells (blue staining). Compared to the control tumor, R9-ZnC- or R9-ZnC<sup>S334D</sup>-treated tumors showed a completely disorganized intratumoral tissue structure. In addition, fibrin-like structures as another indicator of necrosis were present in R9-ZnC- or R9-ZnC<sup>S334D</sup>-treated tumors.

To investigate the effect of R9-ZnC or R9-ZnC<sup>S334D</sup> on tumor vasculature, tumor sections were stained for CD31, a marker of blood vessels (Figure 2-1 C). The microvessel density (brown staining) was markedly reduced as compared to the control, even though the inhibitory effect was more pronounced in the presence of R9-ZnC<sup>S334D</sup>. We quantified a mean of  $115 \pm 14$  (R9-ZnC) and  $100 \pm 12$  (R9-ZnC<sup>S334D</sup>) versus  $160 \pm 28$  (control) microvessels per tumor section.

As VEGF is the key factor in the formation of new blood vessels, we further determined the effect of our novel protein constructs on intratumoral VEGF expression. Immunohistochemical staining demonstrated decreased VEGF protein levels (brown staining) in R9-ZnC- or R9-ZnC<sup>S334D</sup>-treated tumors as compared to the control (Figure 2-1 C). Again, R9-ZnC<sup>S334D</sup> was more effective in the inhibition of VEGF expression than R9-ZnC. These results are in



agreement with those reported previously by our lab and showing that full-length R9-TIS11b also decreases LLC tumor vascularization in nude mice (*Planel et al., 2010*).

As hypoxia is a major stimulus inducing angiogenesis, we evaluated the presence of hypoxic areas in R9-ZnC- or R9-ZnC<sup>S334D</sup>-treated tumors. Pimonidazole hydrochloride (PIMO) was injected intraperitoneally before sacrificing the animals. Pimonidazole hydrochloride is reduced and activated in hypoxic cells and forms covalent adducts with thiol groups of proteins, peptides and amino acids. Immunohistochemical detection of these adducts (brown staining) demonstrated a dramatically decreased intratumoral hypoxia upon R9-ZnC<sup>S334D</sup>-treatment *in vivo* as compared to the control (Figure 2-1 C). Staining of hypoxic areas and VEGF shows that they were co-localized confirming that hypoxia is a major inducer of tumor angiogenesis. Interestingly, treatment of tumors with R9-ZnC did not alter the level of tumor hypoxia. If we succeed in confirming these observations in our future experiments, this could be of great interest. Indeed, hypoxia regulates transcription of various genes implicated in tumor cell metabolism, angiogenesis and EMT through the major action of HIF-1 (*Ruan et al., 2009; De Bock et al., 2011; Casazza et al., 2014*). Therefore, hypoxia signalling is involved in almost every step of tumorigenesis and impacts tumor cells as well as other cell types of the tumor microenvironment. In addition, hypoxia is associated with therapy resistance and disease progression as it favours the most invasive tumor cells in various cancers. Our laboratory identified HIF-1 $\alpha$  mRNA as a target of TTP in endothelial cells (*Chamboredon et al., 2011*). In this study, TIS11b was also able to destabilize HIF-1 $\alpha$  mRNA *in vitro*. One can hypothesize that intratumoral injection of R9-ZnC<sup>S334D</sup> mimics the *in vitro* action of TIS11b on HIF-1 $\alpha$ . This question remained to be answered in further investigations.

*Decreased expression of several angiogenic and inflammatory cytokines upon R9-ZnC- or R9-ZnC<sup>S334D</sup> treatment in vivo*

The results of the immunohistochemical analyses led us to further dissect the anti-tumoral effect of R9-ZnC or R9-ZnC<sup>S334D</sup> using a mouse angiogenesis antibody array. The expressions of 53 angiogenesis-related proteins in tissue lysates of the control, R9-ZnC and R9-ZnC<sup>S334D</sup> tumors were simultaneously detected (Figure 2-2 A). We observed the highest detection for the Coagulation Factor III, CXCL16, Endostatin, Insulin Growth Factor Binding Protein 3 (IGFBP-3), Interleukin-1 $\alpha$  (IL-1 $\alpha$ ), CXCL1, Matrix-metalloprotease 3 (MMP-3), MMP-9, Osteopontin, Platelet Factor 4, Placental Growth Factor 2 (PIGF-2), Serpin E1 and Thrombospondin-2. No expression was detected for the following proteins: Angiopoietin-3 (Ang-3), Epidermal Growth Factor (EGF), Keratinocyte Growth Factor (KGF), Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF), IL-1 $\beta$ , IL-10, Platelet-Derived Endothelial Cell Growth Factor (PD-ECGF), Prolactin and Tissue inhibitor of metalloprotease 4 (TIMP-4). In contrast to our previously described *in vitro* analyses of VEGF expression in 4T1-luc cells at the mRNA and protein level and our immunohistochemical analysis of VEGF in 4T1 tumors *in vivo*, VEGF and VEGF-b were undetectable using antibody arrays. It is worth mentioning that during the quantification of soluble VEGF using ELISA, we noticed a low basal VEGF secretion by 4T1-luc cells as compared to another tumor cell line such as MDA-MB-231 human breast cancer cells.

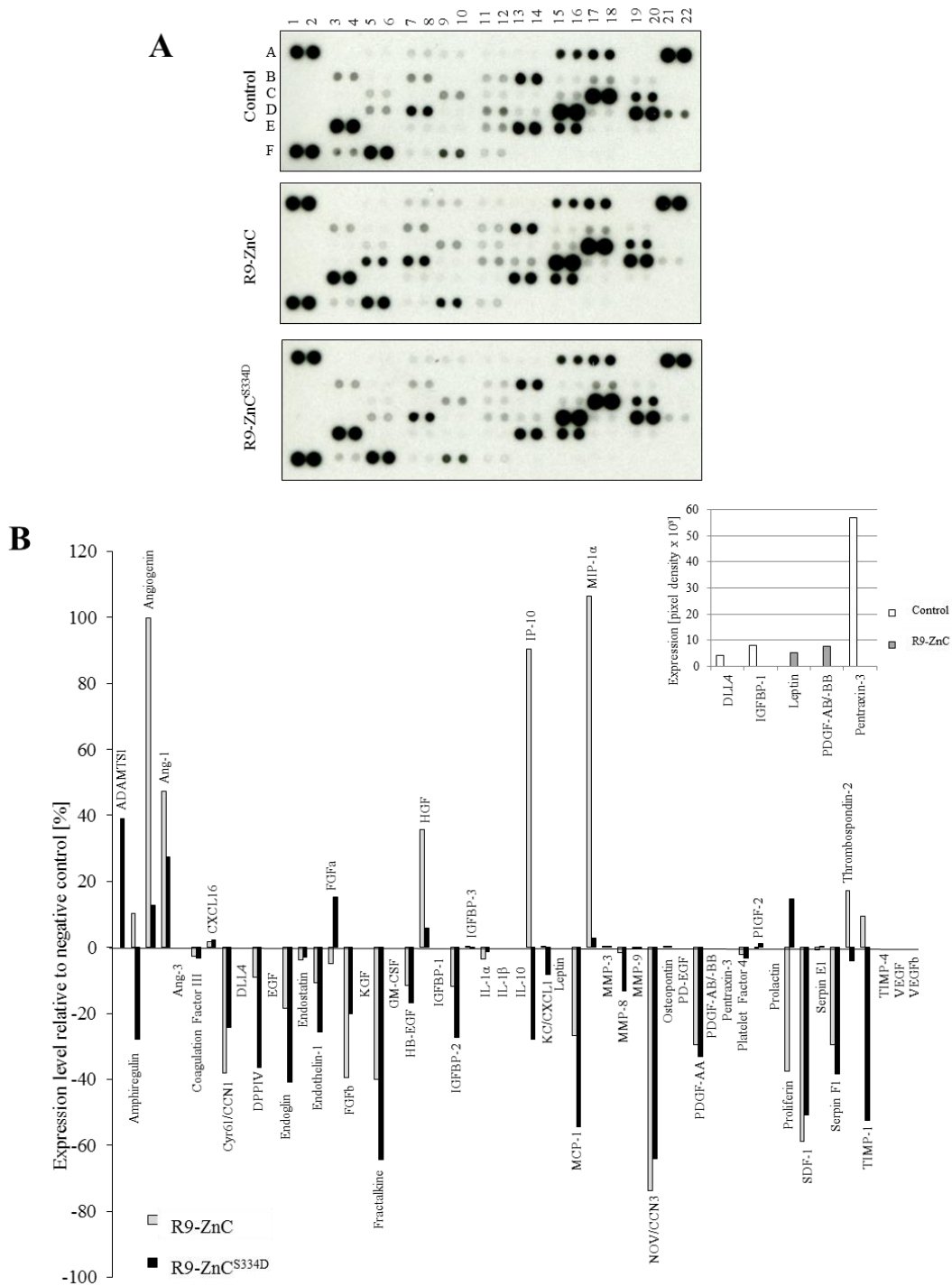


Figure 2-2: **Multi-target action of ZnC proteins on tumor angiogenesis factors *in vivo*.**

- (A) Mouse angiogenesis antibody array analysis of control and R9-ZnC/ R9-ZnC<sup>S334D</sup>-treated tumors mice (n=1 per group). The table on the right side indicates arrangement of tested antibodies as well as expression level of detected proteins compared to control.
- (B) Representation of the expression level of angiogenic proteins normalized against control. Antibody arrays illustrated in (A) were scanned and pixel density was quantified using ImageJ software. The embedded graph in the right upper corner depicts angiogenic factors which were detected just in one of the three tissue extracts.

es	Protein	Expression-fold relative to control [%]	
		R9-ZnC	R9-ZnC <sup>S334D</sup>
A5,A6	ADAMTS1	ne	38,97
A7,A8	Amphiregulin	10,16	-27,77
A9,A10	Angiogenin	99,72	12,87
A11,A12	Ang-1	47,19	27,55
A13,A14	Ang-3	ne	ne
A15,A16	Coagulation Factor III	-2,75	-3,38
A17,A18	CXCL16	1,67	2,34
B3,B4	Cyr61/CCN1	-38,20	-24,15
B5,B6	Dll4	*	*
B7,B8	DPPIV	-9,10	-36,32
B9,B10	EGF	ne	ne
B11,B12	Endoglin	-18,43	-40,84
B13,B14	Endostatin	-3,95	-2,92
B15,B16	Endothelin-1	-10,86	-25,70
B17,B18	FGFa	-4,79	15,25
B19,B20	FGFb	-39,37	-20,14
C3,C4	KGF	ne	ne
C5,C6	<b>Fractalkine</b>	<b>-40,02</b>	<b>-64,25</b>
C7,C8	GM-CSF	ne	ne
C9,C10	HB-EGF	-11,62	-16,74
C11,C12	HGF	35,78	5,86
C13,C14	IGFBP-1	*	*
C15,C16	IGFBP-2	-11,92	-27,41
C17,C18	IGFBP-3	0,16	-0,01
C19,20	IL-1 $\alpha$	-3,48	-1,36
C21,C22	IL-1 $\beta$	ne	ne
D3,D4	IL-10	ne	ne
D5,D6	IP-10	90,34	-27,93
D7,D8	KC/CXCL1	0,03	-8,31
D9,D10	Leptin	**	ne
D11, D12	<b>MCP-1</b>	<b>-26,88</b>	<b>-54,37</b>
D13,D14	MIP-1 $\alpha$	106,48	2,73
D15,D16	MMP-3	0,16	0,19
D17,D18	MMP-8	-1,65	-13,28
D19,D20	MMP-9	-0,03	-0,03
D21,D22	<b>NOV/CCN3</b>	<b>-73,73</b>	<b>-64,03</b>
E3,E4	Osteopontin	0,43	0,23
E5,E6	PD-ECGF	ne	ne
E7,E8	PDGF-AA	-29,44	-33,22
E9,E10	PDGF-AB/-BB	**	ne
E11,12	<b>Pentraxin-3</b>	*	*
E13,E14	Platelet Factor 4	-2,22	-3,21
E15,E16	PIGF-2	-0,17	1,08
E17,E18	Prolactin	ne	ne
E19,E20	Proliferin	-37,61	14,72
F3,F4	<b>SDF-1</b>	<b>-58,96</b>	<b>-50,84</b>
F5,F6	Serpin E1	-0,90	0,41
F7,F8	Serpin F1	-29,44	-38,47
F9,F10	Thrombospondin-2	17,28	-3,99
F11,12	<b>TIMP-1</b>	<b>9,51</b>	<b>-52,38</b>
F13,F14	TIMP-4	ne	ne
F15,F16	VEGF	ne	ne
F17,F18	VEGF-b	ne	ne
A1,A2	reference spots		
A21,A22	reference spots		
F1,F2	reference spots		
F19,F20	negative control		

Table 2-1: **Mouse angiogenesis antibody array analysis of control and R9-ZnC- or R9-ZnC<sup>S334D</sup>-treated tumor tissue extracts.**

This table indicates arrangement of tested antibodies as well as expression fold of detected proteins compared to control (con). ne, not expressed; \*, only expressed in control; \*\* only expressed in R9-ZnC-treated tumor extract.

With few exceptions, the overall expression of the tested angiogenic factors was repressed in R9-ZnC- or R9-ZnC<sup>S334D</sup>-treated tumors compared to the control (Figure 2-2 B; Table 2-1). Unexpectedly, for some genes, an antagonistic effect was observed between R9-ZnC and R9-ZnC<sup>S334D</sup> (e.g. Amphiregulin, IP-10, Proliferin, TIMP-1) with R9-ZnC promoting and R9-ZnC<sup>S334D</sup> repressing mRNA and/or protein expression. These observations suggest that the mutation of the Serine 334 might either extend the TIS11b target mRNA-target repertoire or potentiate TIS11b function. For further discussion, we will concentrate on Fractalkine (CX3CL1), Monocyte chemoattractant protein 1 (MCP-1/CCL2), nephroblastoma overexpressed (NOV/CCN3), Pentraxin 3 (PTX3), Stromal-derived factor 1 (SDF-1/CXCL12) and Tissue inhibitor of metalloprotease 1 (TIMP-1) as these factors were inhibited by more than 50 % upon R9-ZnC- or R9-ZnC<sup>S334D</sup>-treatment *in vivo* (Figure 2-2 B; Table 2-1).

**Fractalkine (CX3CL1)** is the only member of the CX3C chemokine family known so far (*Ferretti et al., 2014*). The membrane-anchored form of this chemokine acts as an adhesion molecule to maintain leukocytes adhesion to endothelial cells. Soluble fractalkine efficiently attracts mainly T and B lymphocytes, natural killer cells and monocytes via the activation of the CX3C receptor (CX3CR). Fractalkine expression has been reported for several cell types such as endothelial and epithelial cells, lymphocytes, neurons, microglial cells and osteoblasts. In cancer, fractalkine harbours a dual function as chemoattractant for leukocytes and adhesion molecule for tumor cells. However, the function of this chemokine in cancer is controversial. The pro- or anti-tumoral function of fractalkine highly depends on the type of cancer and the cell type within tumor tissue. In breast cancer, CX3CR expression is induced, allowing tumor cells to migrate towards high fractalkine concentrations released by bone stromal cells and neurons to form brain and skeleton metastasis. Less skeletal dissemination of human breast cancer cells was demonstrated in CX3CL1<sup>-/-</sup> mice (*Jamieson-Gladney et al., 2011*). *Reed et al.* reported that the inhibition of the fractalkine-CX3CR-axis decreases macrophage infiltration and angiogenesis in the mammary cancer cells *in vivo* (*Reed et al., 2012*). By contrast, *Park et al.* reported a correlation of high fractalkine levels and good prognosis in breast cancer patients as this chemokine induced a strong immune response (*Park et al., 2012*).

**MCP-1 (CCL2)** is overexpressed in the primary tumor or metastatic sites of human breast cancer (*Soria & Ben-Baruch, 2008*). This inflammatory cytokine play causative roles in breast cancer and is associated with tumor progression as well as cancer cell aggressiveness. MCP-1 released by tumor cells promotes tissue remodelling and activates their migratory phenotype. In addition, tumor cell-derived MCP-1 attracts monocytes and promotes their differentiation into

pro-tumoral tumor-associated macrophages (TAMs). MCP-1 produced by several cell types of the tumor microenvironment, such as TAMs or cancer-associated fibroblasts, create a milieu favouring angiogenesis and suppressing immunity. Indeed, *Ueno et al.* reported a correlation between MCP-1 and angiogenic factors, such as IL-8, TNF $\alpha$  and VEGF in human breast cancer (*Ueno et al., 2000*). Furthermore, MCP-1 release is involved in the osteoclastogenesis at bone metastatic sites facilitating the colonization of disseminated cancer cells. A recent study, using the 4T1 breast cancer model aimed to investigate the role of stromal-derived MCP-1 in tumor progression (*Yoshimura et al., 2013*). 4T1 cells were injected in wild type and MCP-1 deficient mice. Although tumor size was unaffected, early necrosis and larger necrotic areas as well as reduced macrophage infiltration and reduced angiogenesis was observed in tumors of MCP-1 deficient mice. As already described, we observed an important macroscopic necrosis in our R9-ZnC- and R9-ZnC<sup>S334D</sup>-treated 4T1-luc tumors (Figure 2-1 B). Furthermore, *Yoshimura et al.* observed reduced spontaneous lung metastasis in the absence of stroma-derived MCP-1. In addition, MCP-1 mRNA in 4T1 cells was nearly undetectable *in vitro* supporting the hypothesis that stromal cells are the major source of MCP-1 in those tumors. Interestingly, *Sadri et al.* observed elevated MCP-1 mRNA and protein levels in AUF1-deficient mice (*Sadri & Schneider, 2009*).

**NOV (CCN3)** was originally identified in myeloblastosis-associated virus-induced nephroblastoma due to its overexpression (*Joliot et al., 1992*). High NOV levels are associated with fast proliferating tumors, metastasis and poor outcome in renal cell carcinoma (*Bleau et al., 2005*). *Ouellet et al.* demonstrated the role of NOV in the formation of bone-metastasis using the 4T1 tumor model (*Ouellet et al., 2011*). Overexpression of NOV in weakly bone metastatic breast cancer cells enhances their potential to form bone metastasis. In addition, NOV favoured osteoclast differentiation facilitating the growth of cancer cells in bone environment. Due to multiple protein domains, NOV is interacting with several factors present in the extracellular matrix regulating cell adhesion and migration. NOV was postulated as angiogenic inducer as it promotes endothelial cell adhesion by interacting with integrin receptors, endothelial cell survival and migration (*Bleau et al., 2005*). Recently, *Chen et al.* observed that prostate cancer cell-derived NOV recruits macrophages, induces their differentiation into TAMs and induces tumor angiogenesis *in vivo* (*Chen et al., 2014*). *López-Silanes et al.* described the binding of the mRNA-stabilizing protein HuR to the 3'UTR of NOV mRNA in a human colorectal carcinoma cell line (*Lopez de Silanes et al., 2004*). However, the pro- or anti-tumoral role of NOV is controversially discussed and seems to be cancer type-dependent.

**Pentraxin (PTX3)** is a modulator of tumor-associated inflammation. PTX3 is mainly expressed by vascular endothelium and smooth muscle cells, but is also synthesized by myeloid dendritic cells, mononuclear macrophages/phagocytes, fibroblasts, adipocytes and other cell types (*Cieslik & Hrycek, 2012*). Its expression is under the control of several mediators, including the pro-inflammatory cytokine TNF $\alpha$ . In addition to its role in inflammation, PTX3 is involved in angiogenesis and tissue remodelling (*Presta et al., 2007*). For example, a recent study demonstrated that TNF $\alpha$ -induced PTX3 expression in human bone metastatic breast cancer cells stimulated the differentiation and activation of osteoclasts facilitating the formation of bone metastasis (*Choi et al., 2014*). In addition, the authors observed an enhanced migratory potential of human breast cancer cells and of macrophages towards high levels of PTX3. Furthermore, high levels of PTX3 expression were detected in bone metastasis samples of breast cancer patients. A positive correlation between high PTX3 expression in primary tumors and poor survival was determined. Moreover, high PTX3 levels are associated with advanced clinical stages of lung cancer, glioma, prostate carcinoma and pancreatic cancer (*Ravenna et al., 2009; Diamandis et al., 2011; Kondo et al., 2013; Locatelli et al., 2013*).

**SDF-1 (CXCL12)** acts via CXCR4, the most frequently overexpressed receptor in cancer (*Domanska et al., 2013*). Stromal cells in the tumor microenvironment constitutively express CXCL12, which promotes the growth and survival of CXCR4-expressing tumor cells via paracrine signalling. Furthermore, CXCR4-positive tumor cells migrate towards high CXCL12 levels in distant organs to eventually form metastasis. A human breast cancer mouse model demonstrated increased infiltration of CXCR4-expressing inflammatory, vascular and stromal cells into the tumor due to high CXCL12 expression of cancer cells and tumor-associated stromal cells (*Orimo et al., 2005*). Tumor cells benefit from their CXCR4 expression to enter CXCL12-rich bone marrow microenvironment leading to chemotherapy resistance. In tumors, CXCL12-CXCR4-axis promotes VEGF-mediated angiogenesis via AKT signalling (*Liang et al., 2007*). Indeed, inhibition of CXCR4 led to dramatically reduced VEGF expression in a breast cancer mouse model (*Hassan et al., 2011*). CXCR4-positive bone marrow-derived progenitor cells are recruited to hypoxic tumor tissue by the local high CXCL12 level, thus promoting tumor angiogenesis (*Aghi et al., 2006*).

**TIMP-1** regulates the activity of metalloproteases which are critical for tissue remodelling and further invasion of cancer cells. The classical consideration of TIMP-1 as tumor suppressor has changed during the last years, because further MMP-independent functions of TIMP-1 were discovered including signal transduction, adhesion and proliferation of tumor cells. Indeed, TIMP-1 overexpression is associated with poor prognosis in human breast cancer (*Wurtz et al., 2008*). Besides its MMP-inhibitory activity, TIMP-1 induces epithelial-mesenchymal transition in human breast epithelial cells (*D'Angelo et al., 2014*). In this study, TIMP-1 interaction with the CD36/integrin signalling complex induced the expression of Twist, a key transcription factor in EMT, leading to a fibroblast-like cell shape of breast epithelial cells. *Hekmat et al.* performed proteomic studies of high or low TIMP-1-expressing breast cancer cells and observed dramatic changes in the protein expression profile (*Hekmat et al., 2013*). This study aimed at getting a molecular insight in TIMP-1-associated resistance to chemotherapy (topoisomerase inhibitors). Indeed, hyper-phosphorylation of the major DNA topoisomerases was detected in TIMP-1 overexpressing cells, which showed decreased sensitivity to topoisomerase inhibitors *in vitro*.

Nevertheless, it need to be mentioned that the expression of certain angiogenic factors, such as Angiogenin, Angiopoietin 1 (Ang-1), Interferon gamma-induced protein 10 (IP-10/CXCL10) and Macrophage inflammatory protein 1 $\alpha$  (MIP-1 $\alpha$ /CCL3), was induced *in vivo* upon R9-ZnC-treatment compared with control (Figure 2-2 B; Table 2-1). Very interestingly, IP-10 was increased by R9-ZnC and decreased by R9-ZnC<sup>S334D</sup> while MIP-1 $\alpha$  was not changed by R9-ZnC<sup>S334D</sup>. Briefly, **Ang-1** is an anti-angiogenic factor that is involved in the recruitment of pericytes during blood vessel maturation. **IP-10 (CXCL10)** is angiostatic and anti-tumoral via its receptor CXCR3 as it inhibits endothelial cell proliferation and motility. Stromal-derived IP-10 attracts natural killer cells in a mouse model of acute myeloid leukaemia inducing anti-tumoral immunity (*Saudemont et al., 2005*). **Angiogenin** is described as a secreted pro-angiogenic factor, which promotes tumor progression. As we did not find any ARE in the 3'UTR of the Angiogenin mRNA, which is essential for TIS11b-mediated mRNA decay, the mechanism of induction of Angiogenin remains to be elucidated.

Indeed, as TIS11b and its truncated forms R9-ZnC and R9-ZnC<sup>S334D</sup> promote AU-rich element-mediated mRNA decay, we searched for occurrence of AREs in the mRNA of tested angiogenic factors which could explain the inhibitory effect of our proteins constructs. We used the AREsite database to determine the abundance of AU-rich motifs located in the 3'UTR of human Fractalkine, MCP-1, NOV, Pentraxin 3, SDF-1 and TIMP-1 mRNA (Table 2-2) (*Gruber et al., 2011*). ARE motif conservation is indicated as we used murine 4T1-luc cells. Except for TIMP-



1, all of these angiogenic and/or inflammatory factors harbour AREs in their 3'UTR, suggesting a potential post-transcriptional regulation of these transcripts by ARE-binding proteins. To the best of our knowledge, nothing is known so far concerning the interaction between TIS11b and these factors. The inhibition of TIMP-1 in the absence of ARE motifs could be due to alternative functions of TIS11b-truncated forms or to a secondary effect through their action on an upstream regulator of TIMP-1.

The role of the TIS11 protein family in the mRNA stability regulation of key factors involved in angiogenesis and inflammation is well-established in the literature (*Benjamin & Moroni, 2007; Baou et al., 2009*). Overall, our results demonstrate a significant inhibitory effect of the novel R9-ZnC and R9-ZnC<sup>S334D</sup> protein constructs on major factors implicated in angiogenesis and inflammation. These novel data deserve further confirmation in additional *in vivo* experiments.

Gene	3'UTR length	ARE motifs			
		AUUUA pentamer	WWAUUUAWW nonamer	WUAUUUAUW nonamer	UUAUUUAU U nonamer
Angiogenin		-	-	-	-
Fractalkine/CX3CL1	2 016 nt	2 •	2 •	1 •	1 •
IP-10	810 nt	1 •	1 •	-	-
MCP-1/CCL2	376 nt	1 •	-	-	-
MIP-1 $\alpha$	416 nt	4	3	1	-
NOV/CCN3	1 306 nt	7 •	-	-	-
Pentraxin-3	649 nt	4 •	1 •	1 •	-
SDF-1/CXCL12	3 163 nt	4 •	1	-	-
TIMP-1		-	-	-	-

Table 2-2: AREsite database prediction of the abundance of ARE motifs in the 3'UTR of factors implicated in tumor angiogenesis and inflammation such as Angiogenin, Fractalkine (CX3CL1), Interferon gamma-induced protein 10 (IP-10), Monocyte chemoattractant protein 1 (MCP-1/CCL2), Macrophage inflammatory protein 1 $\alpha$  (MIP-1 $\alpha$ ), nephroblastoma overexpressed (NOV/CCN3), Pentraxin-3 (PTX3), Stromal-derived factor 1 (SDF-1/CXCL12) and Tissue inhibitor of metalloprotease 1 (TIMP-1). • conserved motif in *Mus musculus* (*Gruber et al., 2011*).

*R9-ZnC and R9-ZnC<sup>S334D</sup> reduces expression of EMT-markers in vivo*

The inhibitory effect of R9-ZnC and R9-ZnC<sup>S334D</sup> on several chemokines implicated in tumor progression and formation of metastasis as identified by the above antibody array experiments, led us to hypothesize that our novel protein constructs could have an effect on key factors of the epithelial-mesenchymal transition (EMT), an early event of the metastatic cascade. In addition, we already observed a decrease in N-Cadherin and Vimentin expression upon R9-ZnC or R9-ZnC<sup>S334D</sup>-treatment of 4T1-luc cells *in vitro* (Rataj *et al.*, Article 2).

As gene expression can differ between *in vitro* and *in vivo* conditions, we determined the protein expression of E-Cadherin, N-Cadherin, Vimentin, Snail and Twist in 4T1-luc tumor tissue lysates (Figure 2-3). In line with our *in vitro* results, **E-Cadherin** expression levels were not changed upon R9-ZnC or R9-ZnC<sup>S334D</sup> treatments when compared to controls. The maintenance of E-Cadherin expression was already described in pre-clinical metastatic breast cancer models including 4T1 cells (Lou *et al.*, 2008; Erin *et al.*, 2013). In addition, this epithelial marker is present in tumors of breast cancer patients. These results suggest that cancer cells may maintain epithelial characteristics, while simultaneously acquiring the invasive phenotype to better control the reversible EMT-MET processes. Furthermore, these results underline the heterogeneity of tumors. Interestingly, a recent study confirmed further functions of E-Cadherin beyond its role as cell-adhesion molecule (Chu *et al.*, 2013). This study revealed that E-Cadherin expression was required for 4T1 tumor growth *in vivo*.

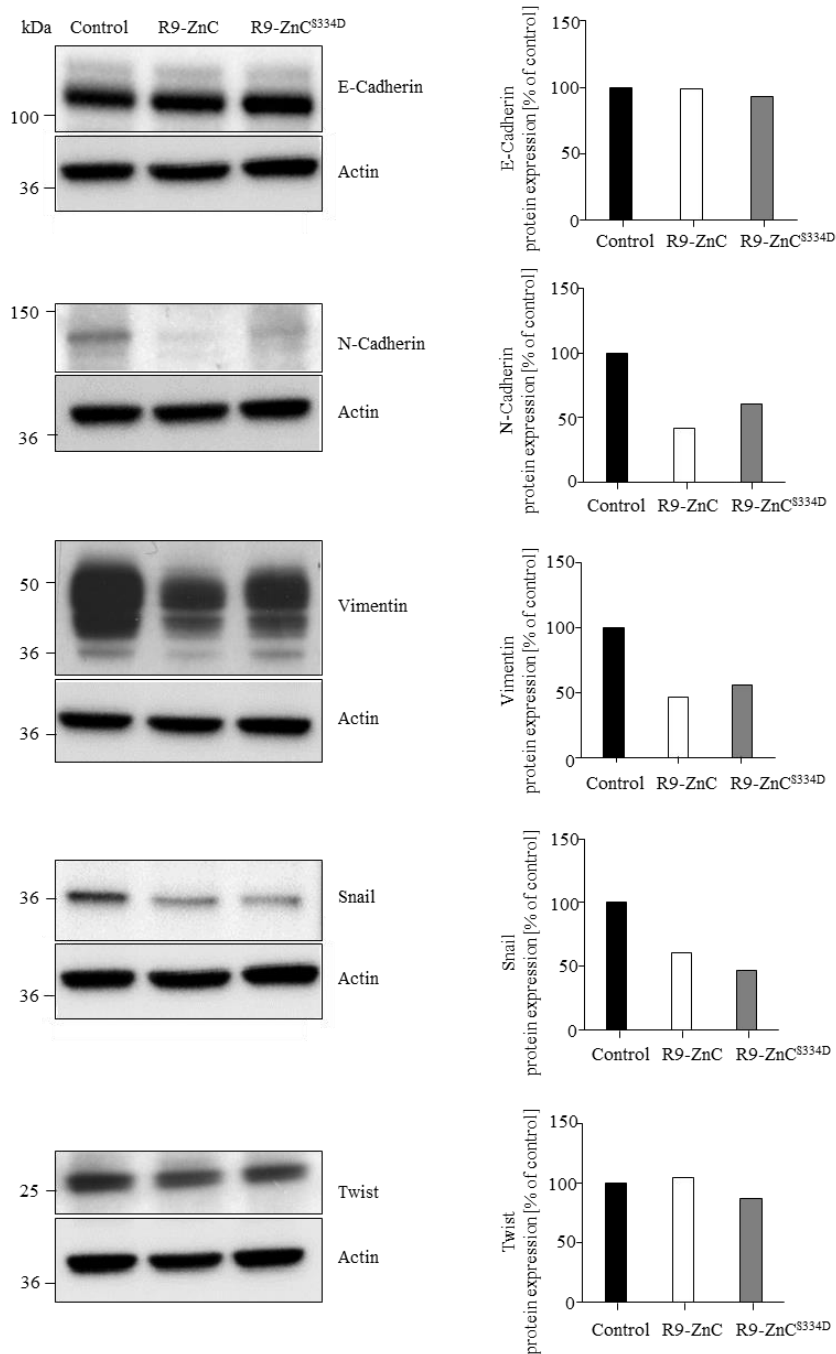


Figure 2-3: **Effect of R9-ZnC and R9-ZnC<sup>S334D</sup> proteins on EMT-markers in 4T1-luc breast tumors *in vivo*.**

Western Blot analysis using anti-E-Cadherin, anti-N-Cadherin, anti-Vimentin, anti-Snail and anti-Twist antibodies showed a decreased N-Cadherin, Vimentin and Snail expression after treatment of 4T1-luc tumors *in vivo* with 200 ng of recombinant R9-ZnC- or R9-ZnC<sup>S334D</sup> protein mice (n=1 per group). E-Cadherin and Twist protein expression remained unchanged. Protein level was quantified as pixel densities using Chemidoc ImageLab software and normalized to Actin protein values detected on the same membrane to compensate for protein loading differences.

The levels of **N-Cadherin** in our tumor lysates were 41 % of control for R9-ZnC and 60 % of control for R9-ZnC<sup>S334D</sup>, thus confirming our *in vitro* data. This mesenchymal cell marker promotes human breast cancer cell migration, invasion and metastasis, independently of E-Cadherin expression (Nieman *et al.*, 1999).

Furthermore, in agreement with our *in vitro* data, we observed that **Vimentin** expression was of 46 % and 56 % of control for R9-ZnC and R9-ZnC<sup>S334D</sup>, respectively. Vimentin is considered as a canonical mesenchymal marker of EMT as it increases migration and invasion of breast cancer cells (Korsching *et al.*, 2005; Satelli & Li, 2011). Its expression is induced by transcription factors other than Snail and Twist. One could hypothesize that TIS11b targets transcription factors such as Slug (Snail2), resulting in decreased Vimentin expression (Vuoriluoto *et al.*, 2011; Lamouille *et al.*, 2014).

Indeed, changes in gene expression that contribute to the phenotypic alterations during EMT include early activated transcription factors such as Snail and Twist. We detected 60 % and 47 % of control **Snail** expression in R9-ZnC and R9-ZnC<sup>S334D</sup>-treated tumors, respectively. Snail induces N-Cadherin expression. Thus, the observed decrease in N-Cadherin expression could be due to the loss of the activating transcription factor Snail upon treatment with our novel protein constructs. We detected almost unaltered **Twist** protein expression (104 % and 87 % of control Twist expression for R9-ZnC for R9-ZnC<sup>S334D</sup>, respectively). In cancer cells, Twist represses E-Cadherin transcription and enhances N-Cadherin expression independently of Snail. Interestingly, the transcription of Snail and Twist is induced by HIF-1 $\alpha$  (Yang & Wu, 2008; Yang *et al.*, 2008).

During the last years, several post-transcriptional mechanisms have emerged as critical steps in the control of EMT in cancer. RNA-binding proteins regulate EMT via different processes, including mRNA maturation as well as mRNA export, turnover, localization and translation. Therefore, we used again the AREsite database to determine the presence of ARE motifs located in the 3'UTR of human E-Cadherin, N-Cadherin, Vimentin, Snail and Twist mRNA (Table 2-3) (Gruber *et al.*, 2011). Importantly, for all tested EMT proteins, ARE motifs were predicted in their 3'UTR, suggesting a potential post-transcriptional control of these transcripts by ARE-binding proteins. As far as we know, no data concerning the effect of TIS11b on these EMT markers is available. Gebeshuber *et al.* demonstrated that the overexpression of TTP in Ras-expressing mammary epithelial cells increased E-Cadherin expression, reduced Vimentin mRNA/protein levels as well as lung metastases *in vivo* (Gebeshuber *et al.*, 2009). Snail mRNA was described as target of HuR (Dong *et al.*, 2007). In this study, Snail mRNA stability was

increased by HuR in response to hydrogen peroxide, leading to enhanced cell migration due to repressed E-Cadherin expression.

Gene	3'UTR length	ARE motifs			
		AUUUA pentamer	WWAUUUAWW nonamer	WUAUUUAUW nonamer	UUAUUUAU U nonamer
E-Cadherin	2 035 nt	3	1	-	-
N-Cadherin	1 187 nt	1 •	-	-	-
Snail	830 nt	3	1	-	-
Twist	706 nt	3 •	1 •	1 •	1 •
Vimentin	322 nt	1	-	-	-

Table 2-3: AREsite database prediction of the abundance of ARE motifs in the 3'UTR of factors implicated in epithelial-mesenchymal transition (EMT) such as E-Cadherin, N-Cadherin, Vimentin, Snail and Twist. • conserved motif in *Mus musculus* (Gruber et al., 2011).

In summary, we observed a 50 % decrease of the mesenchymal markers N-Cadherin and Vimentin as well as of the EMT-inducing transcription factor Snail upon treatment of 4T1-luc tumors with our novel protein constructs R9-ZnC or R9-ZnC<sup>S334D</sup> (Figure 2-3). It remains to be confirmed in additional experiments whether these results are statistically significant. The observed simultaneous expression of epithelial and mesenchymal markers suggests an intermediate phenotype of 4T1-luc cancer cells and indicates intratumoral heterogeneity *in vivo*. If N-Cadherin, Snail and Vimentin are direct or indirect targets of TIS11b deserves further investigations as these factors are also regulated by several other cellular processes (Lamouille et al., 2014).

## Conclusion

In the present pilot study, using the syngeneic 4T1-luc breast cancer model, we demonstrated reduced tumor growth upon intratumoral injections of our novel protein constructs R9-ZnC and R9-ZnC<sup>S334D</sup>. Immunohistochemical analyses confirmed the macroscopically observed necrosis and revealed reduced intratumoral VEGF expression as well as microvessel density. Furthermore, we detected an important decrease of intratumoral hypoxia upon R9-ZnC<sup>S334D</sup> treatment. Given the deleterious effects of tumor hypoxia, one could speculate that this novel protein construct could have a therapeutic potential. Protein profiling of R9-ZnC- or R9-ZnC<sup>S334D</sup>-treated tumors using antibody arrays demonstrated the inhibition of various angiogenic

or inflammatory cytokines and pro-metastatic chemokines, among which are Fractalkine, MCP-1, NOV, SDF-1, Pentraxin and TIMP-1. In addition, we show for the first time a link between EMT markers and TIS11b, which is supported by the presence of AU-rich elements in the 3'UTR of these EMT-marker transcripts. However, a direct interaction of TIS11b or its truncated forms with these mRNAs remains to be demonstrated.

Even though these data need further confirmation, R9-ZnC and R9-ZnC<sup>S334D</sup> seem to have an anti-tumoral multi-target effect on tumor progression *in vivo* based on our antibody array results and immunohistochemical analyses. The diversity of the affected key steps in tumorigenesis suggests that R9-ZnC and R9-ZnC<sup>S334D</sup> could impact several cell types including tumor cells, endothelial cells, immune cells and other components of the tumor microenvironment. A further anti-metastatic action of our novel cell-permeable fusion proteins could be hypothesised. **It is worth-mentioning that most of the anti-tumoral effects that we observed *in vitro* and *in vivo* were more pronounced with the mutant R9-ZnC<sup>S334D</sup>, indicating that phosphorylation of the Serine 334 within TIS11b potentiate its mRNA-destabilizing activity.** In this respect, we believe it is appropriate to use both proteins in parallel in our *in vitro* and *in vivo* approaches.

## Material & Methods

### *Cell culture, SDS-PAGE, Western Blot and Protein overexpression and purification*

All these methods were performed as previously described in Article 2 (Rataj *et al.*, Article 2). In addition, blots were probed with rat anti-Snail (1:1000, Ozyme, Montigny-le-Bretonneux, France) and rabbit anti-Twist (1:500, Santa Cruz, Heidelberg, Germany).

### *In vivo experiments*

*In vivo* experiment protocols were approved by the institutional guidelines and the European Community for the Use of Experimental Animals. 6-weeks-old female BALB/c mice were purchased from Janvier Labs and maintained in the Animal Resources Centre of our department.  $5.0 \times 10^5$  4T1-luc cells were re-suspended in 50  $\mu$ l of growth medium and mixed with 50  $\mu$ l of Matrigel (Becton Dickinson, Le Pont de Claix, France). The cell suspension was then subcutaneously injected into the hind flank of the animals. Tumors appeared 5 to 8 days after implantation. Tumor growth was recorded by either sequential determination of tumor volume using caliper or measurement of emitted bioluminescence at day 1 and 34 of treatment. Tumor

volume was calculated according to the formula  $V = 0.5ab^2$  (a, largest diameter; b, smallest diameter). Results are illustrated as percentage of the tumor volume at the first day of treatment. In addition, tumor growth was followed by non-invasive *in vivo* imaging. Animals were subcutaneously injected with 150 mg of D-Luciferin/kg bodyweight (Perkin Elmer, Courtaboeuf, France) and acquisitions were performed 15 min post-injection (IVIS Lumina II, Xenogen, Caliper Life Science). Data were analysed using Xenogen Living Image software version 3.0. Bioluminescence signals, which reflect the number of living tumor cells, are presented as photons/sec/cm<sup>2</sup>/sr. When tumor volume reached 50 mm<sup>3</sup>, animals were randomly divided into three groups. *In vivo* treatment of pre-established tumors was performed by intratumoral injection of 200 ng of R9-ZnC or R9-ZnC<sup>S334D</sup> in a final volume of 10 µl every other day. Control mice were injected with 10 µl of vehicle. Mice were sacrificed after 34 days of treatment through cervical dislocation. A maximal tumor volume of 1 cm<sup>3</sup> was our end point criterion. Twenty minutes before death, 1.5 mg of Pimonidazole hydrochloride (Hypoxprobe) was intraperitoneally injected for tumor hypoxia analyses. Tumors were collected carefully, weighted and either fixed overnight in 4 % paraformaldehyde (PFA) or formalin-free fixation solution (FFF) (Sigma-Aldrich, Saint-Quentin Fallavier, France) and embedded in paraffin, or conserved at -80 °C for total RNA and protein isolation.

### *Immunohistochemistry*

For H & E staining, 5-µm-thick paraffin sections of PFA-fixed tumors were deparaffinised and rehydrated. Nuclei were stained with haematoxylin and eosinophilic structures with eosin (Sigma-Aldrich, Saint-Quentin Fallavier, France). Sections were rapidly dehydrated and mounted.

For VEGF immunodetection, 5-µm-thick paraffin sections of PFA-fixed tumors were deparaffinised, rehydrated and microwaved in 10 mM citrate buffer pH 6 at 800 W for 2 x 5 min. Endogenous peroxidase activity was blocked by incubating sections with 1 % H<sub>2</sub>O<sub>2</sub> in methanol for 20 min. Slides were then sequentially incubated for 10 min in TBS buffer containing 0.1 % Tween 20, for 20 min in TBS buffer containing 5 % goat serum (DAKO A/S, Les Ulis, France) and 2 % BSA, and for 1 h with 4 µg/ml of rabbit polyclonal anti-human VEGF antiserum which recognizes the N-terminus of VEGF-121, VEGF-165 and VEGF-189 isoforms (Santa Cruz Biotechnology, Heidelberg, Germany). After two washes of 5 min in TBS containing 0.1 % Tween 20, sections were sequentially incubated for 1 h with biotinylated goat anti-rabbit secondary antibodies and for 45 min with an avidin/biotinylated horseradish peroxidase complex

(DAKO A/S, Les Ulis, France). Peroxidase activity was revealed using diaminobenzidine tetrachloride as a chromogen (DAKO A/S, Les Ulis, France). Sections were briefly counterstained with haematoxylin (Sigma-Aldrich, Saint-Quentin Fallavier, France), dehydrated and mounted.

For CD31 immunodetection, 5- $\mu$ m-thick paraffin sections of FFF-fixed tumors were deparaffinised, rehydrated and incubated with a 1 mg/ml aqueous trypsin solution (Sigma-Aldrich, Saint-Quentin Fallavier, France) for 10 min at 37 °C. Endogenous peroxidase activity was blocked by incubating sections with 1 % H<sub>2</sub>O<sub>2</sub> in methanol for 20 min. Slides were then sequentially incubated for 10 min in TBS buffer containing 0.1 % Tween 20, for 20 min in TBS buffer containing 5 % rabbit serum (DAKO A/S, Les Ulis, France) and 2 % BSA, and for 1 h with 156 ng/ml of rat anti-mouse CD31 antibodies (Becton Dickinson, Le Pont de Claix, France). After two washes of 5 min in TBS containing 0.1 % Tween 20, sections were sequentially incubated for 1 h with biotinylated rabbit anti-rat secondary antibodies and for 45 min with an avidin/biotinylated horseradish peroxidase complex (DAKO A/S, Les Ulis, France). Peroxidase activity was revealed using diaminobenzidine tetrachloride as a chromogen (DAKO A/S, Les Ulis, France). Sections were briefly counterstained with haematoxylin (Sigma-Aldrich, Saint-Quentin Fallavier, France), dehydrated and mounted. To determine microvessel density, CD31-stained vessels of three to five random fields (x 5) per tumor section were counted and illustrated as microvessel density per field.

For hypoxia immunodetection, 5- $\mu$ m-thick paraffin sections of PFA-fixed tumors were deparaffinised, rehydrated and microwaved in 10 mM citrate buffer pH 6 at 800 W for 2 x 5 min. Endogenous peroxidase activity was blocked by incubating sections with 1 % H<sub>2</sub>O<sub>2</sub> in methanol for 20 min. Slides were then sequentially incubated for 10 min in TBS buffer containing 0.1 % Tween 20, for 20 min in TBS buffer containing 5 % goat serum (DAKO A/S, Les Ulis, France) and 2 % BSA, and for 1 h with 1.2  $\mu$ g/ml of mouse monoclonal anti-Pimonidazol hydrochloride antibodies (Hypoxyprobe, Burlington, Massachusetts, USA). After two washes of 5 min in TBS containing 0.1% Tween 20, sections were sequentially incubated for 1 h with biotinylated goat anti-mouse secondary antibodies and for 45 min with an avidin/biotinylated horseradish peroxidase complex (DAKO A/S, Les Ulis, France). Peroxidase activity was revealed using diaminobenzidine tetrachloride as a chromogen (DAKO A/S, Les Ulis, France). Sections were briefly counterstained with haematoxylin (Sigma-Aldrich, Saint-Quentin Fallavier, France), dehydrated and mounted.



### *Angiogenesis antibody array*

Tissue fragments of control and R9-ZnC- or R9-ZnC<sup>S334D</sup>-treated tumors were lysed in PBS containing protease inhibitor cocktail (Sigma-Aldrich, Saint-Quentin Fallavier, France) and 1 % Triton X-100 according to the manufacturer instructions (R&D Systems, Lille, France). Membranes of the Proteome Profiler Mouse Angiogenesis Array Kit (R&D Systems, Lille, France) were hybridized using 300 µg of total protein extracts according to the manufacturer guidelines. The arrays were imaged together and BioMax Kodak films (Sigma-Aldrich, Saint-Quentin Fallavier, France) were scanned afterwards to quantify hybridization signals as pixel densities using ImageJ software. Values of duplicate spots representing one angiogenic protein were averaged and the background signal was subtracted. Interarray comparability was verified by measuring equal pixel intensity of positive control spots on each array. Results are illustrated as protein expression level compared to the control [%].

**DISCUSSION**  
**&**  
**CONCLUSIONS**



Gene expression is a tightly controlled mechanism in eukaryotic cells. Transcriptional and post-transcriptional processes maintain the balance between the synthesis and the degradation rate of a gene product, thereby determining its final amount within the cell. Post-transcriptional mechanisms such as pre-mature mRNA processing, nuclear mRNA export, RNA interference, mRNA sequestration, codon usage, translational repression by miRNAs or proteins and the control of mRNA turnover have emerged as key steps in the regulation of gene expression.

mRNA stability is controlled by *cis*-acting sequences located in the 3' untranslated region (3'UTR) of the mRNA and *trans*-acting factors which recognize these motifs. Among others, AU-rich elements (ARE) are *cis*-acting sequences of great importance as they are highly abundant in the mammalian transcriptome (Gruber *et al.*, 2011). AREs are recognized by either stabilizing RNA-binding proteins (ARE-BPs) or destabilizing ARE-BPs. The TIS11 protein family plays a key role in ARE-mediated mRNA decay. Three members of this CCCH tandem zinc-finger protein family are known in humans: TTP, TIS11b and TIS11d. Although these proteins showed redundancy *in vitro* as they could target the same ARE-containing mRNAs, murine knock out models demonstrated their unique role *in vivo* (Taylor *et al.*, 1996; Carballo *et al.*, 1998; Stumpo *et al.*, 2004; Bell *et al.*, 2006; Hodson *et al.*, 2010). The repertoire of mRNAs targeted by the TIS11 protein family includes factors such as TNF $\alpha$ , VEGF and multiple transcripts involved in inflammation, angiogenesis and cancer. During the last years, an important link between the deregulation of mRNA turnover and cancer has appeared (Benjamin & Moroni, 2007). In many types of cancer, TIS11 protein family is underexpressed while tumor-promoting transcripts are highly stabilized suggesting a potential tumor suppressor role of these proteins (Brennan *et al.*, 2009). TIS11 proteins harbour several serine residues and are, indeed, hyperphosphorylated. Upon several stimuli, these proteins are target of different kinases such as the p38MAPK kinase-activated MK2, ERK and PKB/AKT. These post-translational modifications modulate the expression, activity, stability and subcellular localization of the TIS11 protein family. Our team was first to show that TIS11b is a physiological regulator of VEGF mRNA in adrenocortical cells challenged with the cAMP-mobilizing hormone ACTH (Gaillard *et al.*, 2000).

VEGF is known to be the key driver of physiological and pathological angiogenesis (Ferrara & Davis-Smyth, 1997). On the other hand, TIS11b-deficient mice die at E10.5 due to major vascular defects caused by high VEGF levels (Stumpo *et al.*, 2004; Bell *et al.*, 2006). The *in vivo* TIS11b-KO model confirmed our previous *in vitro* results demonstrating that VEGF

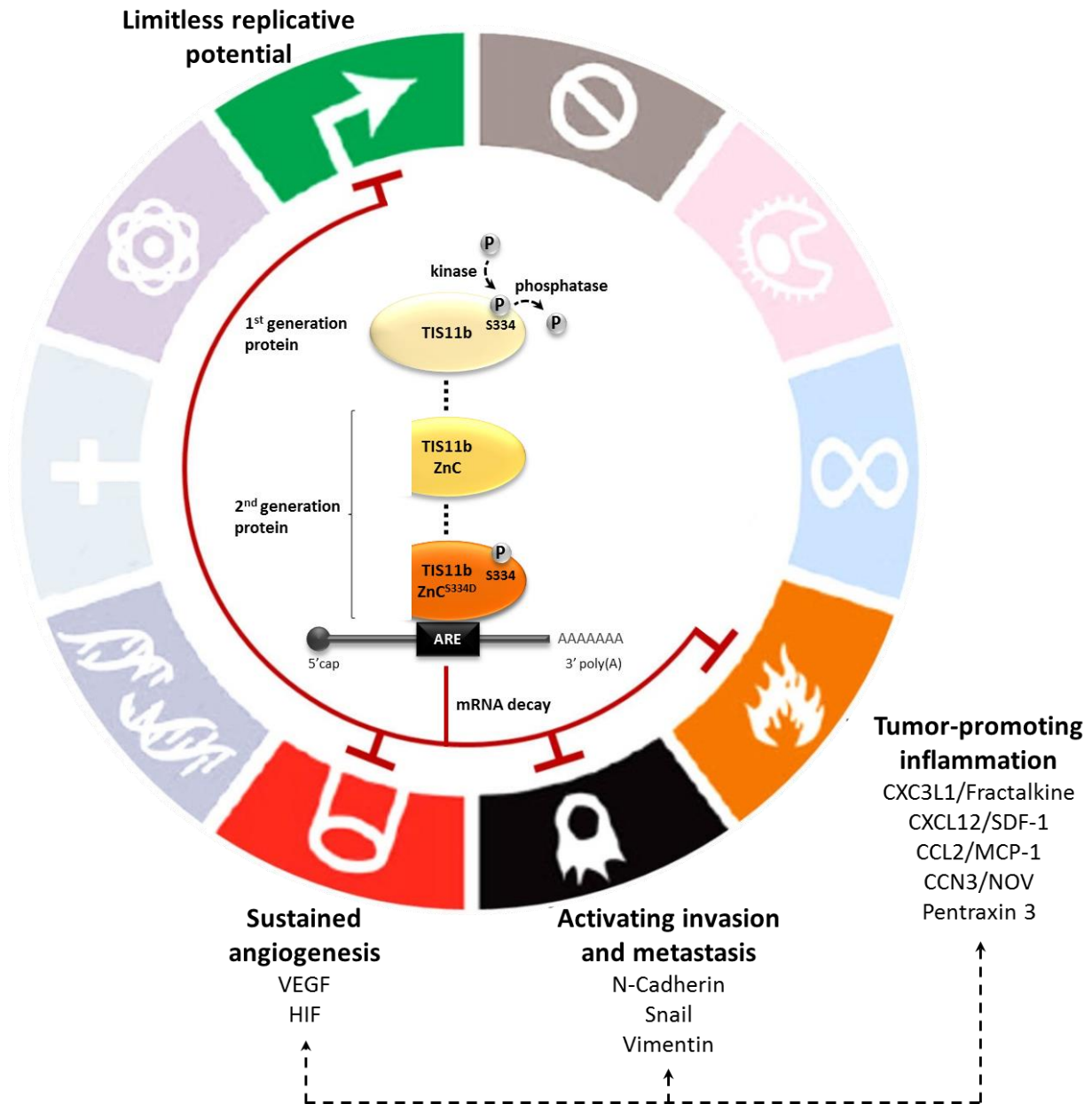


Figure 42: Multi-target effect of truncated/mutated TIS11b fusion proteins on cancer hallmarks. First generation full-length TIS11b protein fused to the cell-penetrating peptide R9 (not illustrated) was truncated by the deletion of the N-terminal protein domain to improve TIS11b protein stability (R9-ZnC, second generation proteins). Increasing colour intensity corresponds to enhanced protein stability. As phosphorylation at serine 334 improves TIS11b protein stability, we generated a truncation mutant R9-ZnC<sup>S334D</sup> in which we mimicked a permanent phosphorylation at S334. Both second generation proteins R9-ZnC and R9-ZnC<sup>S334D</sup> were shown to inhibit different hallmarks of tumor progression *in vitro* and *in vivo* including cancer cell proliferation, tumor angiogenesis, tumor-promoting inflammation and invasion/metastasis formation in a murine breast cancer model. Further key events in tumorigenesis (transparent colour) were not studied. Dotted lines indicate that the listed factors favour different hallmarks in cancer progression.

mRNA is a direct target of TIS11b-mediated mRNA destabilization (Ciais *et al.*, 2004). The fact that VEGF is a direct target of TIS11b is of great interest as tumors produce high amounts of this pro-angiogenic factor to induce the formation of new blood vessels in their surrounding tissue (Bergers & Benjamin, 2003; Bell *et al.*, 2006). Due to the increasingly reported resistance of tumors to existing anti-angiogenic therapies, the development of new strategies to overcome this phenomenon is urgently needed (Bergers & Hanahan, 2008; Vasudev & Reynolds, 2014). Our group has developed a promising experimental anti-angiogenic and anti-tumoral cancer therapy which was based on TIS11b-induced degradation of VEGF and other short-lived mRNAs (Planel *et al.*, 2010). However, the instability of the purified recombinant R9-TIS11b fusion protein was a limiting factor for the continuation of this study. We therefore hypothesized that genetic engineering of alternative variants of TIS11b protein might improve its protein stability. As mentioned in Article 1, the phosphorylation at Serine 334 of TIS11b primary sequence plays an important role in the stabilization of the protein (Rataj *et al.*, submitted, Article 1).

In the present work, we show that deletion of the first hundred amino acids of the N-terminal domain of TIS11b which is 338 amino acids long, highly increases TIS11b protein stability without affecting its activity. This mutant was called ZnC. The stabilizing effect was even more pronounced when a permanent phosphorylation was mimicked at Serine 334 in the C-terminal domain of TIS11b (ZnC<sup>S334D</sup>). As we have determined the minimal domain required for protein activity and improved the stability of our proteins, other protein engineering strategies to improve protein robustness would be a further step. The usage of uncommon amino acids which could not be recognized by mammalian proteases might address this question.

Both purified fusion proteins R9-ZnC and R9-ZnC<sup>S334D</sup> efficiently transduced living cells. To characterize the effect of our novel protein constructs, we used the 4T1-luc murine breast cancer cell line as it is considered as a clinical relevant model (Pulaski & Ostrand-Rosenberg, 2001). *In vitro* and *in vivo* studies indicate a multi-target anti-angiogenic and anti-tumoral effect of both R9-ZnC and R9-ZnC<sup>S334D</sup> as summarized in Figure 42. However, it is worth-mentioning that **most of the anti-tumoral effects that we observed *in vitro* and *in vivo* were more pronounced with the mutant R9-ZnC<sup>S334D</sup>**, suggesting that phosphorylation of Serine 334 within TIS11b potentiate its function in mRNA decay.

We detected a significant decrease of VEGF expression in cancer cells and an inhibition of the organization of endothelial cells into pseudo-capillaries in the presence of R9-ZnC and R9-ZnC<sup>S334D</sup> *in vitro*. In addition, we observed reduced VEGF expression and less vascularization in our *in vivo* tumor model upon treatment indicating an anti-angiogenic effect of our proteins. However, VEGF was still present in R9-ZnC-treated tumors. Alternative splicing VEGF<sub>xxx</sub>b

isoforms have been described and reported to act as anti-angiogenic and anti-tumoral isoforms of VEGF (Bates *et al.*, 2002; Rennel *et al.*, 2008; Merdzhanova *et al.*, 2010; Delcombel *et al.*, 2013). Catena *et al.* demonstrated that VEGF<sub>xxx</sub> and VEGF<sub>xxx</sub>b protein levels were significantly higher in breast cancer tumors compared to normal human breast tissue, suggesting the co-expression of both VEGF isoforms in these tumors (Catena *et al.*, 2010). The antibodies and QPCR primers used in our study do not distinguish between VEGF<sub>xxx</sub> and VEGF<sub>xxx</sub>b isoforms. One could hypothesize that anti-tumoral VEGF<sub>xxx</sub>b isoforms are expressed in R9-ZnC-treated tumors thus explaining VEGF detection even though tumor vascularization and growth were inhibited. However, AREs located in the 3'UTR of VEGF are identical for both isoforms. To our knowledge, nothing is known about the destabilizing effect of TIS11b protein on VEGF<sub>xxx</sub>b mRNA so far.

Interestingly, decreased VEGF expression was accompanied by a concomitant decrease of intratumoral hypoxic areas in R9-ZnC<sup>S334D</sup>-treated tumors *in vivo*. Hypoxia signalling is involved in almost every step of tumorigenesis and impacts tumor cells as well as cells of the tumor microenvironment. The anti-tumoral effect of our novel protein construct might also proceed through alteration of these mechanisms. In addition, in various but not in all cancers, hypoxia is associated with therapy resistance and disease progression as it favours the most invasive tumor cells. It would be therefore interesting to test in our preclinical models whether R9-ZnC<sup>S334D</sup> improve the response to chemotherapeutic agents such as paclitaxel or others anti-cancer agents.

In addition to the inhibitory effect on tumor angiogenesis, R9-ZnC<sup>S334D</sup> impaired other major hallmarks of cancer progression as it reduced the proliferation, the migration, the invasion and the anchorage-independent growth of murine breast cancer cells *in vitro*. *In vivo*, we demonstrated that tumor growth was delayed when pre-established murine breast cancer tumors were treated R9-ZnC<sup>S334D</sup>. Strikingly, while the effect of R9-ZnC was less significant *in vitro* and *in vivo*, the expression of several pro-angiogenic and pro-inflammatory cytokines such as Fractalkine, MCP-1, NOV, SDF-1, Pentraxin and TIMP-1 was altered upon both R9-ZnC and R9-ZnC<sup>S334D</sup> treatments. On the other hand, we found that few angiogenic factors were exclusively upregulated by R9-ZnC. This observation may help to understand why this mutant is less efficient in the inhibition of tumor growth. In this context, it would be exciting to investigate the molecular mechanisms used by R9-ZnC and R9-ZnC<sup>S334D</sup> to mediate mRNA decay and how they could be specific for certain mRNA targets.

Fractalkine, MCP-1, NOV, SDF-1, Pentraxin and TIMP-1 are produced by several cell types of the primary tumor such as tumor cells, endothelial cells, cancer-associated fibroblasts or tumor-

associated macrophages, thereby creating a tumor microenvironment which favours angiogenesis, suppresses immunity and facilitates tumor cell invasion. We believe that further analyses of the effect of our novel proteins on the tumor microenvironment as well as on tumor's acquired capability to evade destruction by the host immune system would be of high interest and could be addressed in future studies.

As Fractalkine, MCP-1, NOV, SDF-1, Pentraxin also favour metastasis formation by either attracting tumor cells or facilitating the colonization of circulating cancer cells in distant organs, their inhibited expression in our *in vivo* study indicate a potential anti-metastatic effect of R9-ZnC and R9-ZnC<sup>S334D</sup>. Our *in vitro* data demonstrating the negative effect of our proteins on tumor cell invasiveness and anchorage-dependent cell growth support this hypothesis. In addition, expression of N-Cadherin, Vimentin and Snail, which are markers of the mesenchymal cancer cell phenotype and associated with the formation of metastasis, was reduced *in vitro* and *in vivo*. Based on this data, it would be interesting to further investigate the anti-metastatic effect of our novel protein constructs using appropriate *in vivo* murine models. As we have started our study with 4T1 cells which are known to be highly metastatic when transplanted into the mammary fat pad, these cells might be a good preclinical model. High expression of factors such as CXCL12 and TIMP-1 are associated with resistance to chemotherapy (*Hekmat et al., 2013*). Both factors were markedly reduced upon treatment with R9-ZnC or R9-ZnC<sup>S334D</sup> *in vivo*. Even though the combination of chemotherapy and anti-angiogenic therapies is still controversially discussed, it would be interesting to test how R9-ZnC or R9-ZnC<sup>S334D</sup> would impair tumor progression in combination with chemotherapeutics in these settings. Finally, as TIS11 proteins might impair other hallmarks of cancer progression than those that we have tested, it would be of high interest to determine how R9-ZnC and R9-ZnC<sup>S334D</sup> might influence tumor metabolism or the resistance to apoptosis for example (*Ross et al., 2012*).

The major drawback of our therapeutic approach so far is the failure to target exclusively cancer cells and components of the tumor microenvironment. The fusion of R9-ZnC and R9-ZnC<sup>S334D</sup> to the RAFT(cRGD)<sub>4</sub> vector might solve this inconvenience as this molecule binds to the  $\alpha\beta 3$  integrin receptor which plays an important role in angiogenesis, cell migration, invasion and formation of metastases (*Garanger et al., 2005*). The  $\alpha\beta 3$  integrin receptor is expressed on tumor cells and other cell types (including endothelial cells). Alternatively, ZnC and ZnC<sup>S334D</sup> fused to the positively charged R9 cell penetrating peptide could be further linked to an anionic inhibitory sequence via an MMP-cleavable linker (*Jiang et al., 2004*). As MMPs are highly abundant in tumor stroma, the linker sequence would be specifically cleaved in this tissue leading to the dissociation of the anionic inhibitory sequence and the internalization of R9-ZnC



and R9-ZnC<sup>S334D</sup>. Hence, the vectorization of our novel proteins would allow their systemic or intraperitoneal injection *in vivo*. This step opens challenging prospects for the future.

Of course, our data need to be confirmed in pre-clinical studies using highly invasive human breast cancer cells such as MDA-MB-231 cells to exclude phylogenetic differences between human and mouse. Nevertheless, **our promising results identify R9-ZnC and R9-ZnC<sup>S334D</sup> as novel multi-target anti-tumoral agents. Our data support the emerging link between mRNA stability and cancer and provide novel concepts for the development of innovative anti-cancer therapies.**

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# APPENDIX





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# Molecular Mechanisms of Angiogenesis

From Ontogenesis to Oncogenesis

 Springer



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# Contents

## Part I Angiogenesis During Embryonic Development

- 1 **Emergence of Endothelial Cells During Vascular Development . . . .** 3  
Anne Eichmann and Luc Pardanaud
- 2 **Lymphatic Vascular Morphogenesis . . . . .** 25  
Florence Tatin and Taija Makinen

## Part II The Physiological Angiogenic Signal: Cellular and Molecular Mechanisms

- 3 **Finding New Partnerships: The Function of Individual Extracellular Receptor Domains in Angiogenic Signalling by VEGF Receptors . . . . .** 47  
Caroline A.C. Hyde, Philipp Berger, and Kurt Ballmer-Hofer
- 4 **Wnt/Frizzled Signaling in the Vasculature . . . . .** 77  
Thierry Couffignal, Pascale Dufourcq, and Cécile Dupl a
- 5 **BMP9, BMP10, and ALK1: An Emerging Vascular Signaling Pathway with Therapeutic Applications. . . . .** 99  
Sabine Bailly
- 6 **Apelin Signaling in Retinal Angiogenesis . . . . .** 121  
Yves Audigier, Lo c van den Berghe, and Bernard Masri
- 7 **Emerging Role of the Two Related Basic Helix-Loop-Helix Proteins TAL1 and LYL1 in Angiogenesis. . . . .** 149  
Val rie Pinet, Virginie Deleuze, and Dani le Mathieu

### Part III Hypoxia, Ischemia and Angiogenesis

- 8 Hypoxia and Extracellular Matrix Remodeling** . . . . . 171  
Laurent Muller, Catherine Monnot, and Stéphane Germain
- 9 Sphingosine-1-Phosphate in Hypoxic Signaling** . . . . . 199  
Olivier Cuvillier
- 10 Reciprocal Crosstalk Between Angiogenesis and Metabolism** . . . . . 219  
Gaëlle Laurent and Fatima Mechta-Grigoriou
- 11 Endothelial Progenitor Cells and Cardiovascular Ischemic Diseases: Characterization, Functions, and Potential Clinical Applications** . . . . . 235  
David M. Smadja, Bernard I. Lévy, and Jean-Sébastien Silvestre

### Part IV Tumor Angiogenesis

- 12 Endothelial Cell Reactions to Oxygen: Implications for Cancer** . . . . . 267  
Rodrigo Leite de Oliveira and Massimiliano Mazzone
- 13 Tumor Angiogenesis and Lymphangiogenesis: Microenvironmental Soil for Tumor Progression and Metastatic Dissemination** . . . . . 283  
Jenny Paupert, Maureen Van De Velde, Frédéric Kridelka, and Agnès Noël
- 14 Angiogenesis and Invasion in Malignant Glioma: Friends or Foes?** . . . . . 307  
Thomas Daubon and Andreas Bikfalvi
- 15 Role of Endothelial Cells in Tumor Escape from Immunity** . . . . . 325  
Fabrice Soncin
- 16 Tumor High Endothelial Venules and Lymphocyte Trafficking** . . . . . 339  
Krystle M. Veerman, Fanny Lafouresse, and Jean-Philippe Girard
- 17 Posttranscriptional Regulation of Angiogenesis Through AU-Rich mRNA Degradation: Potential Application in Cancer Therapy** . . . . . 353  
Séverine Planel, Felicitas Rataj, Jean-Jacques Feige, and Nadia Cherradi

### Part V Anti-angiogenic Drugs in Human Cancer Therapy

- 18 Antiangiogenic Drugs in Cancer Therapy: Effect on Advanced Renal Cell Carcinoma** . . . . . 375  
Sylvie Négrier

Contents	vii
<b>19 Anti-angiogenic Therapies in Colorectal Cancer</b> . . . . .	383
Clarisse Eveno and Marc Pocard	
<b>20 Resistance to Antiangiogenic Treatments via Upregulation of Substitution Pathways</b> . . . . .	397
Renaud Grépin, Mélanie Guyot, and Gilles Pagès	
<b>21 VEGF-A Splice Variants: Do They Play a Role in Tumor Responses to Anti-angiogenic Therapies?</b> . . . . .	421
Beatrice Eymin, Asma Boudria, and Cherine Abou-Faycal	
<b>Part VI Biomarkers of Tumor Angiogenesis</b>	
<b>22 Progenitor Endothelial Cells as Biomarkers of Anti-vascular Agents</b> . . . . .	445
Melissa Taylor and Françoise Farace	
<b>23 Soluble Vascular Endothelial (VE)-Cadherin: Toward a Marker of Endothelial Dysfunction</b> . . . . .	461
Adama Sidibé, Helena Polena, Tiphaine Mannic, Barry Stidder, Laurence Bouillet, and Isabelle Vilgrain	
<b>24 The Prognostic and Predictive Value of VEGF Across Various Tumor Types</b> . . . . .	479
Celine Pallaud	
<b>Index</b> . . . . .	493

## Chapter 17

# Posttranscriptional Regulation of Angiogenesis Through AU-Rich mRNA Degradation: Potential Application in Cancer Therapy

S verine Planel, Felicitas Rataj, Jean-Jacques Feige, and Nadia Cherradi

**Abstract** Angiogenesis is a finely tuned process that is tightly regulated in time and space by environmental factors (oxygen levels, extracellular matrix, diffusible growth factors, and cytokines). Expression of angiogenesis effectors and regulators is coordinately regulated at both transcriptional and posttranscriptional levels. Accumulating evidence suggests that regulation of mRNA stability plays a pivotal role in this process. Many AU-rich mRNAs encoding cytokines, growth factors, transcriptional factors, and receptors are involved in cancer and inflammation. Overexpression of these mRNAs in tumors is often correlated with deregulation in their mRNA stability. mRNA decay is regulated by *cis*-regulatory elements represented by the AU-rich elements (AREs) present in the 3'-UTR of target mRNAs and *trans*-acting ARE-binding proteins (ARE-BPs) that control mRNA degradation by diverse ribonucleases. Competition between ARE-BPs will finally determine whether an mRNA is degraded or stabilized. Regulation of mRNA stability is furthermore controlled by signaling pathways that are often overactive in cancer and impact the function of stabilizing or destabilizing factors. In this review, we present examples of angiogenesis genes regulated through ARE-directed mRNA decay with focus on deregulation of these processes in tumor angiogenesis. We finally comment on the modulation of ARE-BP expression and activity as a potential future application in anti-angiogenic and anti-tumorigenic therapies.

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## 17.1 Introduction

Gene expression is a temporally and spatially tightly controlled process that begins with transcriptional initiation and ends with translation of a mature mRNA into protein. In between these two steps, a series of regulatory events that include pre-mRNA processing and splicing, mRNA export from the nucleus to the cytoplasm, quality control assessment, and mRNA decay and/or stabilization coordinately contribute to this finely regulated process. Regulation of mRNA stability is a critical control point of gene expression, particularly for short-lived mRNAs that encode growth factors, angiogenic and inflammatory cytokines, and proto-oncogenes. This regulation involves both *cis* elements, mainly located in the 3'-untranslated regions (3'-UTR) of mRNAs and *trans*-acting factors. The latter include several RNA-binding proteins that specifically recognize the *cis* elements and bind to them in multimolecular complexes that allow or prevent the recruitment of components of the mRNA deadenylation and mRNA degradation machineries (Stoecklin and Muhlemann 2013). Adenosine and uridine (AU)-rich elements are the best characterized *cis* elements that target rapid mRNA decay and control translation. They are most often arranged as repeated pentamers of AUUUA sequences that can eventually overlap (Barreau et al. 2005; Hitti and Khabar 2012). Their frequency is estimated at approximately 8 % of the human transcriptome (Halees et al. 2008). Dysregulation of these mRNA stability control processes, caused either by mutations in the *cis*-regulatory elements or by changes in expression of the *trans*-acting proteins binding to these elements, is observed in various pathologies including human cancer (Dixon et al. 2001; Lopez de Silanes et al. 2007; Mayr and Bartel 2009; Misquitta et al. 2001). Whether they cause the disease or contribute to it as a result of genomic or genetic alterations is still a matter of debate. Nevertheless, this has prompted several research teams to investigate whether interference with these processes could represent a novel multitarget therapeutic approach (Eberhardt et al. 2007; Essafi-Benkhadir et al. 2007; Planel et al. 2010; Stoecklin et al. 2003).

## 17.2 The Regulation of mRNA Stability

Various facets of mRNA decay processes have been previously reviewed (Belasco 2010; Garneau et al. 2007; Wilusz et al. 2001). Here, our main goal is to summarize the relation between AU-rich-mediated mRNA decay and posttranscriptional regulation of angiogenic genes.



### ***17.2.1 Cis-Acting Elements: Adenylate-Uridylate-Rich Elements***

AU-rich elements (AREs) were originally identified as instability determinants in mRNA encoding proteins implicated in the inflammatory response (Caput et al. 1986). Several ARE-containing mRNAs are commonly involved in angiogenesis and cancer. These genes include cytokines, chemokines, growth factors, transcriptional factors, RNA-binding proteins, and others. AREs are found in the 3'-UTR of short-lived mRNAs, characteristic of early and transient regulatory responses, and serve as binding sites for a variety of trans-acting factors that modulate mRNA half-life and translation. They were first divided into three classes based on their sequence characteristics and functional properties. Class I and class II AREs contain various copies of an AUUUA motif, whereas class III AREs do not bear this pentanucleotide. AREs classified as class I are mostly found in early-response-gene mRNAs that encode nuclear transcription factors (Chen and Shyu 1994; Chen et al. 1995). They contain one to three copies of dispersed AUUUA motifs coupled with nearby U-rich sequences or U stretches. All AREs assigned to class II are present in cytokine mRNAs, e.g., the granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), VEGF, and IL-3, and are present as multiple copies of AUUUA that cluster together. The three different classes of ARE appear to direct rapid mRNA decay with distinct kinetics (Xu et al. 1997). The GM-CSF ARE, a representative of the class II AREs, directs asynchronous cytoplasmic deadenylation, while class I AREs, such as the *c-fos* ARE, and class III AREs, such as the *c-jun* ARE, mediate synchronous poly(A) shortening followed by the decay of the mRNA body. More recently, an alternative classification based on a computationally derived 13-base-pair motif WWUUAUUUAUUWW (W=A/U) further clustered ARE-mRNAs into five groups depending on the number of motifs in the ARE stretch. Groups 1–4 contain five, four, three, and two pentameric (AUUUA) repeats, respectively, while Group 5 contains only one repeat within the 13-bp pattern (Bakheet et al. 2003). An AU-rich element database (ARED) has been created and upgraded several times (Bakheet et al. 2003, 2006). More than 6,000 human ARE-mRNAs have been listed in the latest version ([http://brp.kfshrc.edu.sa/ARED/AREDInteg\\_notes.htm](http://brp.kfshrc.edu.sa/ARED/AREDInteg_notes.htm)). To date, the physiological importance of AREs has been evaluated in vivo only for very few mRNAs. Surprisingly, although TNF- $\alpha$  class II ARE was shown to play a major role in the posttranscriptional control of this gene in cellular models (Kontoyiannis et al. 1999), in vivo deletion of the *c-myc* ARE (class I) did not alter *c-myc* mRNA metabolism in healthy transgenic mice (Langa et al. 2001). These observations indicate that class II AREs, characterized by clustered copies of the UUAUUUAUU nonamer, are dominant instability determinants, whereas class I AREs do not necessarily induce mRNA instability. Further complexity in the regulation of mRNA stability via AREs stems

from the fact that, in addition to primary structures, secondary structural changes in the 3'-UTR are required for the correct assembly of ARE-binding proteins (ARE-BPs) (Bevilacqua et al. 2003). For example, the formation of hairpin-like structures by two adjacent AREs is necessary for the recognition of human and rat VEGF mRNA by hypoxia-induced ARE-BPs and is crucial for the regulation of VEGF expression under hypoxic conditions (Claffey et al. 1998).

### ***17.2.2 Trans-Acting Elements: ARE-Binding Proteins***

ARE-BPs may alter mRNA stability either by tagging an mRNA for rapid deadenylation and degradation or by protecting it from nucleases. Competition between stabilizing and destabilizing factors will ultimately determine the overall amount of protein produced by one mRNA. Around 20 ARE-BPs have been identified so far. In this review, we will focus on the best known (Table 17.1).

#### **17.2.2.1 Stabilizing Proteins**

The best characterized mRNA-stabilizing protein is the human antigen R (HuR). HuR was first identified as a tumor antigen in lung carcinoma of individuals with paraneoplastic neurologic disorder (Dalmau et al. 1990). It is a member of the mammalian homologs of the embryonic lethal abnormal vision (ELAV) RNA-binding proteins first described in *Drosophila* (Ma et al. 1996). HuR is ubiquitously expressed. It possesses three RNA recognition motifs through which it binds to target mRNAs bearing AU- or U-rich sequences and subsequently modifies their expression by altering their stability, translation, or both (Brennan and Steitz 2001; Simone and Keene 2013). HuR is predominantly localized in the nucleus of most unstimulated cells, but it can translocate to the cytoplasm upon cell stimulation (Fan and Steitz 1998). The repertoire of HuR target mRNAs is large as demonstrated by profiling of transcripts that are bound to HuR, which includes many cytokines, growth factors, and cell cycle regulators (Lebedeva et al. 2011; Lopez de Silanes et al. 2004). It is worth mentioning that cytoplasmic localization rather than a significant overall increase in HuR expression is important in increasing the stability and translation of ARE-containing mRNAs. The mechanism of stabilization is unknown but is suggested to compete with destabilizing ARE-BPs for the AREs (Cherradi et al. 2006; Lal et al. 2004; Linker et al. 2005).

#### **17.2.2.2 Destabilizing Proteins**

Tristetraprolin (TTP, also named ZFP36, TIS11, NUP475) is the founding member of a family of tandem CCCH-class zinc finger proteins that comprises three members expressed in all mammals (TTP, ZFP36L1 also named TIS11b or BRF1 and

**Table 17.1** Major ARE-binding proteins involved in the control of mRNA stability

ARE-binding protein	Function	RNA-binding domain	Mode of action	Modifications
Tristetraprolin (TTP, TIS 11, ZFP36), TIS11b (BRF1, ZFP36L1), TIS11d (BRF2, ZFP36L2)	Destabilizing	CCCH-type zinc finger	Recruit decay enzymes: Cer4, Dcp1, Exosome (PM/Sci-75, Rrp4)	Phosphorylation by p38-MAPK pathway leads to association With 14-3-3 proteins
AUF1 (hnRNP D) four splice isoforms (p37, p40, p42, p45)	Usually destabilizing, sometimes stabilizing	RRM	Recruits the exosome, RNA remodeling	Phosphorylation leads to modifications in RNA conformation and/or in AUF1 RNA-binding activity
KSRP	Destabilizing	KH domain	Recruits decay enzymes: PARN and exosome	Phosphorylation by p38-MAPK pathway leads to reduced RNA-binding affinity
HuR (ELAV proteins)	Stabilizing	RRM	Competes with destabilizing proteins for ARE-binding; might relocalize mRNAs away from the decay machinery	CARM-1 mediated methylation reduces stabilizing function; phosphorylation by Chk2, PKC $\alpha$ and PKC $\delta$ regulates HuR shuttling and RNA-binding affinity
CUG-BP	Destabilizing	RRM	Recruits PARN	Phosphorylated by myotonic dystrophy protein kinase

Entries in brackets indicate alternative protein names

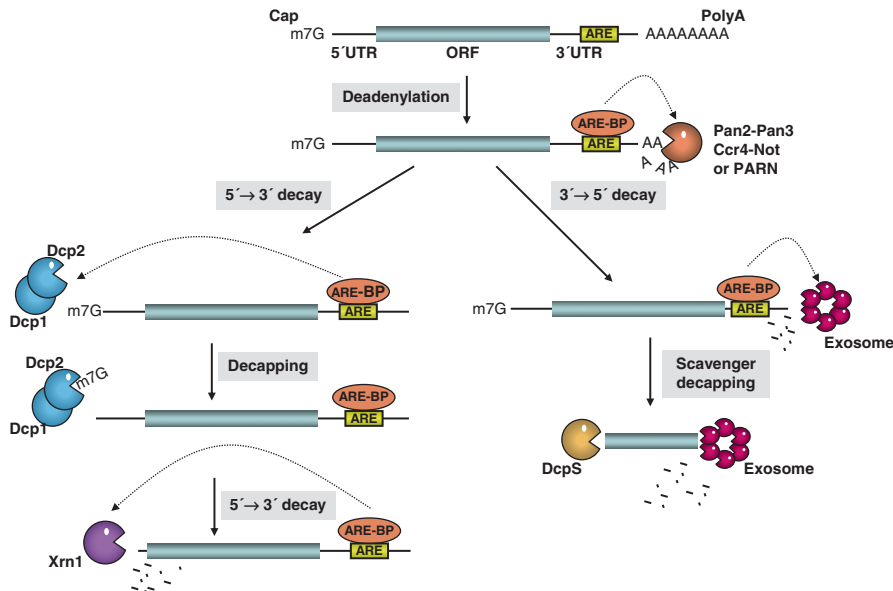
TIS TPA-induced sequence, ZFP zinc finger protein, AUF1 AU-rich binding factor-1, hnRNP heterogeneous nuclear ribonucleoprotein, KSRP KH-splicing regulatory protein, ELAV embryonic lethal abnormal vision, CUG-BP CUG-binding protein, PARN poly(A)-specific ribonuclease, RRM RNA recognition motif, MAPK mitogen-activated protein kinase

ZFP36L2 also named TIS11d or BRF2) and a fourth member only present in rodents (ZFP36L3). All four proteins bind and destabilize ARE-containing mRNAs in vitro (Baou et al. 2009). However, gene knockout (KO) studies in mice have provided evidence for their unique role in vivo (Carballo et al. 1998; Stumpo et al. 2009; Stumpo et al. 2004). The most convincing demonstration came from the phenotype of the TTP-deficient mice, where TNF- $\alpha$  mRNA was significantly stabilized in macrophages, leading to a dramatic increase in circulating TNF- $\alpha$  and a consequent systemic inflammatory syndrome (Carballo et al. 1998). Since the initial description of TNF- $\alpha$  and GM-CSF as physiological TTP targets, numerous other mRNAs have been proposed and, to some degree, validated as targets of TTP (reviewed by Brooks and Blakeshear (2013)). TIS11b/BRF1 plays a crucial role in development since its deletion is embryonic lethal at E10.5 due to abnormal placentation and major vascular defects. This probably arises from a failure to repress the expression of vascular endothelial growth factor (VEGF) during a critical phase of vascular development (Bell et al. 2006; Stumpo et al. 2004). Reported mRNA targets of TIS11b/BRF1 include IL-3, VEGF, c-IAP2, Ier3, and StAR (Ciais et al. 2004; Duan et al. 2009; Lai et al. 2006; Lee et al. 2005; Stoecklin et al. 2002). Deletion of the 29 N-terminal amino acids of TIS11d/BRF2 results in female infertility, whereas the complete inactivation of the gene induces lethality within 2 weeks after birth due to a defective definitive hematopoiesis (Ramos et al. 2004; Stumpo et al. 2009). The central zinc finger domains of these three proteins interact with AREs within the mRNA 3'-UTR, while the N- and C-terminal domains recruit enzymes involved in the mRNA degradation pathway. A crystal structure shows that the TIS11d tandem zinc finger domains directly bind to UAUU motifs (Hudson et al. 2004).

KSRP (KH-splicing regulatory protein) is a multifunctional ARE-BP that modulates many steps of RNA fate including pre-mRNA splicing, ARE-mediated mRNA decay, and maturation of selected miRNAs from precursors (reviewed in Briata et al. (2011)). Studies based on cell and animal models revealed that KSRP is essential for the control of cell proliferation and differentiation and the response to DNA damage. KSRP was also identified as a key regulator of human iNOS mRNA turnover (Linker et al. 2005). AUF1, also known as hnRNP D, was one of the first ARE-BPs identified (Brewer 1991). AUF1 also binds selected ARE with high affinity and has a destabilizing effect on many ARE-mRNAs (reviewed in White et al. (2013)). Differential splicing of AUF1 transcripts yields different mRNAs encoding four different isoforms (p37, p40, p42, p45) with unique individual properties. The mRNA-destabilizing activities of AUF1 are often counteracted by HuR-dependent stabilizing effects (Blaxall et al. 2002; Lal et al. 2004).

### 17.2.3 mRNA Decay Pathways

All eukaryotic mRNAs contain a 7-methyl guanosine cap (m7G-cap) structure at their 5' end, which, in conjunction with the cap-binding protein complex, makes the mRNA resistant to 5' to 3' exonucleases. At the 3' end of mRNAs, a poly(A) tail along with the poly(A) binding protein (PABP) protects the mRNA from 3' to 5' ribonuclease attack (Mangus and van Hoof 2003). Moreover, the



**Fig. 17.1** Mechanisms of ARE-binding protein-mediated mRNA decay. The 5' and 3' ends of a mature transcript are protected by a 7-methylguanosine cap and a polyA tail, respectively. Most mRNAs undergo decay by the deadenylation-dependent pathway. ARE-binding proteins bind to the ARE sequences in the 3'-UTR and recruit deadenylases either directly (Pan2-Pan3 and Ccr4-Not complexes) or indirectly (PARN). Following deadenylation, two mechanisms can degrade the mRNA: either decapping by the enzymes Dcp1/Dcp2 followed by 5' → 3' degradation by the exoribonuclease Xrn1 or 3' → 5' decay triggered by the exosome complex in the presence of the cap structure. In the latter case, the remaining cap structure is hydrolyzed by the scavenger-decapping enzyme DcpS. Dotted arrows represent recruitment of decay enzymes by ARE-binding proteins

5'-m7G-cap/cap-binding complex and the 3' poly(A)/PABP complex can interact with each other to form a closed loop that enhances translation initiation and protects mRNA ends from nuclease attack (Jacobson 1996). Following transport of mRNAs to the cytoplasm, eukaryotic mRNAs undergo decay by a pathway that is initiated by poly(A)-tail shortening, a process termed deadenylation (Brawerman 1981). Deadenylation impacts mRNAs by reducing their translatability and/or inducing their degradation and has been demonstrated to be the major step triggering mRNA decay in eukaryotic cells. Studies using the *c-fos* promoter-driven transcriptional pulsing system showed that AREs induce rapid deadenylation followed by decay of the RNA body (Shyu et al. 1991; Wilson and Treisman 1988). Following deadenylation, either the 5'-cap is removed by a process known as decapping, which allows the mRNA body to be degraded in the 5' → 3' direction by the Xrn1 exoribonuclease, or the unprotected 3' end is attacked by a large complex of 3' → 5' exonucleases known as the exosome (Fig. 17.1). These two pathways are not mutually exclusive, and the relative contribution of each mechanism remains a matter of debate. In the past decade, some ARE-BPs including TTP were found to associate with the exosome complex and/or with the decapping

complex (Chen et al. 2001; Fenger-Gron et al. 2005; Stoecklin et al. 2006). More recently, it has been shown that TTP recruits the multi-subunit CCR4-NOT deadenylase complex to the mRNA (Fabian et al. 2013).

### 17.3 Angiogenesis mRNAs Regulated Through AREs

A number of important ARE genes are commonly involved in angiogenesis. Whereas ARE-mRNAs represent around 8 % of the human transcriptome, a bioinformatic analysis of the angiogenesis-related mRNAs reveals that 25 % of them possess AREs in their 3'-UTR. A non-exhaustive list that includes angiogenic and angiostatic factors, growth factor receptors, ECM components, ECM-degrading enzymes, and specific transcription factors, such as HIF1- $\alpha$ , is presented in Table 17.2. A more extensive screening can be performed using the ARED 3.0

**Table 17.2** Non-exhaustive list of ARE genes involved in both cancer and inflammation

Cellular process	mRNA target		ARE cluster	Function
	Symbol	Name		
Angiogenesis	BAI3	Brain-specific angiogenesis inhibitor-3	V	Angiogenesis inhibitor
	THBS1	Thrombospondin-1	IV	Angiogenesis inhibitor
	THBS2	Thrombospondin-2	IV	Angiogenesis inhibitor
	VASH1	Vasohibin	IV	Angiogenesis inhibitor
	VEGF	Vascular endothelial growth factor A	IV	Angiogenic factor
	ANGPT1	Angiopoietin 1	V	Angiogenic factor
	ANGPTL7	Angiopoietin-related protein 7	V	Angiogenic factor
	FGF2	Fibroblast growth factor-2 (basic)	IV	Angiogenic factor
	EREG	Epreghulin	IV	Angiogenic factor
	CSF1	Colony-stimulating factor 1	IV	Growth factor
	PDGFB	Platelet-derived growth factor, beta polypeptide	IV	Growth factor
	CYR61	Cysteine-rich angiogenic inducer 61	V	Growth factor
	CTGF	Connective tissue growth factor	V	Growth factor
	KDR	Vascular endothelial growth factor receptor 2	V	Growth factor receptor
	FGFRL1	Fibroblast growth factor receptor-like 1	V	Growth factor receptor

(continued)

**Table 17.2** (continued)

Cellular process	mRNA target		ARE cluster	Function
	Symbol	Name		
	JAG1	Jagged 1	III	Receptor
	DLL4	Delta-like 4	IV	Receptor
	ITGB3	Integrin- $\beta$ 3	V	Receptor
	ITGAV	Integrin- $\alpha$ v	V	Receptor
	FZD4	Frizzled homolog 4	III	Receptor
	EPHB4	Eph receptor B4	V	Receptor
	EPHA2	Eph receptor A2	IV	Receptor
	ESM1	Endothelial cell-specific molecule 1 (endocan)	IV	ECM protein
	MMP13	Matrix metalloproteinase 13 (collagenase 3)	IV	ECM protease
	HPSE	Heparanase	I	ECM protease
	SERPINE1	Plasminogen activator inhibitor 1	V	Protease inhibitor
	SERPINE2	Plasminogen activator inhibitor 2	V	Protease inhibitor
	HIF1A	Hypoxia-inducible factor 1- $\alpha$ subunit	III	Hypoxic response
Inflammation	IL-1	Interleukin 1 $\beta$	II	Cytokine
	IL-2	Interleukin 2	III	Cytokine
	IL-3	Interleukin 3	V	Cytokine
	IL-6	Interleukin 6/interferon- $\beta$ 2	IV	Cytokine
	IL-8	Interleukin 8	II	Cytokine
	IL-10	Interleukin 10	V	Cytokine
	IL-15	Interleukin 15	V	Cytokine
	TNF	Tumor necrosis factor- $\alpha$	III	Cytokine
	IFNG	Interferon- $\gamma$	IV	Cytokine
	CSF1	Macrophage colony-stimulating factor 1	IV	Cytokine
	CSF2	Granulocyte-macrophage colony-stimulating factor	I	Cytokine
	CXCL1	Chemokine (C-X-C motif) ligand 1	II	Cytokine
	CXCL2	Chemokine (C-X-C motif) ligand 2	I	Cytokine
	CXCL3	Chemokine (C-X-C motif) ligand 3	III	Cytokine
	CXCL12	Chemokine (C-X-C motif) ligand 12	V	Cytokine
	CX3CL1	Chemokine (C-X3-C motif) ligand 1	IV	Cytokine
	CSF1	Colony-stimulating factor 1	IV	Cytokine
	CTGF	Connective tissue growth factor	V	Growth factor
	TNFRSF12A	Tumor necrosis factor receptor superfamily member 12A	V	Cytokine receptor
	PTGS2	Cyclooxygenase 2 (COX2)	III	Enzyme

ARE clusters are based on ARED Database bioinformatic clustering (<http://brp.kfshrc.edu.sa/AredOrg/>)

database (Bakheet et al. 2006). VEGF-A, the canonical member of the VEGF family, belongs to this list, whereas the related members VEGF-B, VEGF-C, and PlGF do not. A number of *in vitro* experiments conducted in various cell types have established that VEGF-A mRNA stability is decreased in the presence of members of the tristetraprolin family (Brennan et al. 2009; Cherradi et al. 2006; Ciais et al. 2004) and stabilized in the presence of HuR and PAIP2 (Cherradi et al. 2006; Levy et al. 1998; Onesto et al. 2004). VEGF was confirmed to be a major target of ZFP36-L1/TIS11b *in vivo* since genetic invalidation of the latter gene in mice results in embryonic lethality due to angiogenesis defects and VEGF protein overexpression (Bell et al. 2006). However, careful analysis of mouse embryonic fibroblasts derived from TIS11b knockout mice revealed that VEGF upregulation appeared to result from increased translation efficiency rather than from changes in mRNA stability (Bell et al. 2006).

Inflammation is a major inducer of angiogenesis, and both processes are frequently concomitant during tumor growth. Interestingly, as shown in Table 17.2, a number of proinflammatory cytokines and inflammation mediators are also encoded by ARE-mRNAs. These include in particular TNF- $\alpha$ , GM-CSF, and several interleukins.

#### **17.4 Signaling Pathways Regulating Angiogenesis mRNA Stability**

Several stimuli regulate mRNA stability and their number keeps increasing. Cytokines, growth factors, cellular stress inducers, hormones, as well as environmental factors converge to well-known signaling cascades which both regulate the abundance of ARE-BPs and induce their posttranslational modifications. Both events impact the RNA-binding properties and/or the recruitment of the mRNA decay machinery by ARE-BPs. However, whereas a substantial amount of data links ARE-BP expression and/or phosphorylation to the regulation of inflammatory cytokine mRNA stability, little is known on the impact of these modifications on the stability of angiogenic mRNAs. At least three signaling pathways have been shown to regulate ARE-dependent mRNA stability of angiogenic genes, namely, the MAP kinase (MAPK), the AMP kinase (AMPK), and the protein kinase A (PKA) signaling cascades.

In the CCL39 fibroblastic cell line, it was demonstrated that VEGF mRNA stability was increased by anisomycin, a strong activator of stress-activated protein kinases JNK and p38 MAPK (Pages et al. 2000). Such regulation is mediated through an AU-rich region of the VEGF mRNA 3'-UTR located within a stable hairpin structure that binds unknown proteins that are specifically induced by anisomycin treatment. Later on, the same group reported that the regulation of basal VEGF mRNA turnover in normal cells is dependent on the ARE-BP TTP (Essafi-Benkhadir et al. 2007). In addition, the constitutive activation of the ERK pathway in tumor cells was shown to increase VEGF mRNA stability and to induce the expression and the phosphorylation of TTP (Essafi-Benkhadir et al. 2007).



In line with these observations, activation of the p38 MAPK and its downstream kinase MAPK-activated protein kinase 2 (MK2) has been shown to impair the deadenylation of ARE-containing mRNAs *in vivo*, leading to mRNA stabilization (Winzen et al. 1999; Winzen et al. 2007). Macrophages from MK2<sup>-/-</sup> mice show severely reduced levels of TNF- $\alpha$ , IL-1, IL-6, and IFN- $\gamma$  due to decreased cytokine mRNA stability (Kotlyarov et al. 1999; Neininger et al. 2002). A major target of MK2 is the TTP protein, which is directly phosphorylated at serine 52 (Ser52) and serine 178 (Ser178), allowing binding of 14-3-3 adaptor proteins. This interaction reduces the destabilizing activity of TTP (Johnson et al. 2002; Stoecklin et al. 2004). In addition, phosphorylation of TTP on Ser52 and Ser178 by MK2 stabilizes TTP protein by preventing TTP degradation by the proteasome and favors its cytoplasmic localization (Brooks et al. 2002). More recently, it has been shown that TTP phosphorylation by MK2 prevents the recruitment of the deadenylation machinery to the target mRNA (Clement et al. 2011). These studies and others provide strong evidence for p38 MAPK regulation of ARE-containing mRNA stability. The p38 MAPK may also phosphorylate ARE-stabilizing proteins such as HuR, which could compete with the destabilizing proteins for the regulation of VEGF mRNA stability. It has been shown that p38 MAPK regulates HuR localization and subsequently the stability of HuR mRNA targets (Lafarga et al. 2009; Tran et al. 2003).

Glucose deprivation has been shown to induce an increase in VEGF mRNA stability in different carcinoma cell lines, which is mediated by AMP-activated kinase (AMPK) activation, indicating a critical role of AMPK in tumor angiogenesis (Yun et al. 2005). A study in C2C12 myoblasts showed that the AMPK-elicited increase in VEGF expression was mainly due to an increase in VEGF mRNA stability, which may be important for an accelerated angiogenic repair after ischemic damage (Ouchi et al. 2005). Ouchi et al demonstrated that the induction of VEGF expression by AMPK activators in myoblasts depends on p38 MAPK, which is a target of AMPK. Unfortunately, the ARE-binding proteins involved in this regulation were not identified in these studies (Ouchi et al. 2005).

In adrenocortical cells, the pituitary hormone ACTH induces a cAMP-dependent and transcription-independent increase in VEGF mRNA expression (Chinn et al. 2002). It was further established that the early ACTH-induced nucleocytoplasmic translocation of HuR triggers VEGF mRNA stabilization, whereas TIS11b, which is induced later by ACTH, participates in the downregulation of VEGF mRNA (Cherradi et al. 2006). The increase in TIS11b mRNA was accompanied by an increase in multi-phosphorylated forms of TIS11b protein (Cherradi et al. 2006). At least three products of different mobility are apparent in adrenocortical cells. These products correspond to phosphorylated forms, as evidenced by their conversion to a single, more mobile product after phosphatase treatment of the cell extracts (Duan et al. 2009). Protein kinase A is predicted to phosphorylate serine or threonine residues in TIS11b, but these potential phosphosites remain to be determined. TIS11b phosphorylations at Ser92 and Ser203 by protein kinase B and p38 MAPK have been established through removal of mobility changes by S/A substitutions (Maitra et al. 2008; Schmidlin et al. 2004). These phosphorylations attenuate TIS11b activity in part through directing sequestration by cytoplasmic 14-3-3 anchor proteins.

## 17.5 Implication of ARE-BPs in Tumor Angiogenesis

Hypoxia in the tumor environment controls the expression of the transcription factor HIF1- $\alpha$  through both transcriptional and posttranscriptional mechanisms. Whereas hypoxia stabilizes the HIF1- $\alpha$  protein through enzymatic inhibition of its prolyl-hydroxylation and its proteasomal degradation, it also destabilizes the HIF1- $\alpha$  mRNA in a delayed manner through TTP binding onto its ARE-containing 3'-UTR (Chamboredon et al. 2011). This accounts for the transient induction of HIF1- $\alpha$  protein that peaks 3 h after the onset of hypoxia and decreases thereafter. Interestingly, a number of HIF1- $\alpha$  transcriptional targets are also ARE-mRNAs and are thereby also regulated at the posttranscriptional level. These include VEGF-A, carbonic anhydrase IX, the glucose transporter GLUT-1, and plasminogen activator inhibitor-1, indicating that transcriptional and posttranscriptional mechanisms coordinately control the time frame of expression of the angiogenic gene repertoire. In renal carcinomas, the loss of von Hippel-Lindau (VHL) tumor suppressor gene expression is directly responsible for the accumulation of HIF1- $\alpha$  protein. VHL has an E3 ubiquitin-ligase activity that binds to the hydroxyproline residues of HIF1- $\alpha$  and earmarks it with an ubiquitin tag for targeting to and degradation by the proteasome. Interestingly, under normoxia, VHL also decreases TIS11b mRNA levels through the action of the micro-RNA miR-29b (Sinha et al. 2009). In contrast, under hypoxia, VHL does not affect miR-29b expression but increases TIS11b mRNA stability and TIS11b protein expression. There is thus a clear interplay between the VHL status and the hypoxic status of renal carcinoma cells to control the levels of TIS11b target gene products, including VEGF.

From a more general point of view, it appears that mutations in AREs have been very rarely described in cancer, whereas dysregulation of ARE-binding protein expression is common (Kanies et al. 2008; Mendell and Dietz 2001). Several recent studies have reported a decreased expression of TTP family members in human cancer cell lines and tumors (Brennan et al. 2009; Rounbehler et al. 2012; Young et al. 2009), and this appears to be an early event in tumor progression. Concomitant elevation of the expression level of the mRNA-stabilizing protein HuR is also observed in early stages of colorectal cancer progression (Young et al. 2009). In Myc-expressing B cell lymphomas, Myc was shown to directly suppress transcription of TTP, and restoring TTP was able to reverse Myc-induced lymphomagenesis (Rounbehler et al. 2012). This clearly indicates that TTP functions as a tumor suppressor.

Griseri et al. recently reported that TTP mRNA levels were quite variable among breast cancer cell lines and did not correlate with protein levels (Griseri et al. 2011). Interestingly, they identified a synonymous polymorphism (rs3746083) in one allele of the TTP gene that is more frequent in patients with HER2-positive breast cancer and appears significantly correlated with a lack of response to Herceptin/trastuzumab treatment. Although this polymorphism did not modify the encoded protein sequence, it appeared to strongly decrease the mRNA translation efficiency of the variant allele. Similarly, a monoallelic frameshift mutation (I373fsX91) in the TIS11d gene was observed in leukemic cells from a patient with acute myeloid

leukemia (Hurwitz et al. 2004). The mutant TIS11d protein showed a reduced inhibitory effect on HeLa cell proliferation that seemed to correlate with p21 downregulation.

In addition to these mechanisms, it has been shown that the loss of TTP expression may also occur in breast carcinomas as a consequence of variations in micro-RNA expression. MiR-29a was shown to suppress TTP expression in breast epithelial cells and to be inversely correlated with TTP levels in human breast carcinoma specimens (Gebeshuber et al. 2009). Evidence of increased DNA methylation of the TTP gene promoter has also recently been shown in hepatocellular carcinoma, where TTP expression appeared to be dependent upon a single CpG methylation site (Sohn et al. 2010).

HuR has been linked to carcinogenesis through its ability to stabilize mRNAs like VEGF, IL-8, TNF- $\alpha$ ,  $\beta$ -catenin, c-myc, and cyclooxygenase-2 (Cherradi et al. 2006; Dixon et al. 2001; Levy et al. 1998; Nabors et al. 2001; Szabo et al. 1991). AUF1 has been shown to bind transcripts encoding immune regulators such as the interleukins IL-1 $\beta$ , IL-2, IL-3, and IL-6, TNF- $\alpha$ , and many other mRNAs which might indirectly affect angiogenesis (Gratacos and Brewer 2010).

## 17.6 Therapeutic Use of AUBP Functions in Cancer Treatment

The observation that ARE-binding proteins target and regulate the levels of a number of short-lived mRNAs involved in angiogenesis, inflammation, and tumorigenesis has incited some research teams to exploit these properties for anticancer therapy. A first attempt was published in 1998 in which the authors immunized mice against the mRNA-stabilizing protein HuD (ELAVL4), which is highly expressed in non-small cell lung carcinoma and neuroblastoma tumors (Carpentier et al. 1998). When compared to controls, mice immunized against HuD showed significant neuroblastoma growth inhibition and increased intratumoral CD3+ lymphocytic infiltrates. Although no transcriptomic analysis was performed in these tumors, this was the first demonstration that the neutralization of an mRNA-stabilizing protein could have beneficial antitumoral activity. Later on, Stoecklin et al. tested an opposite strategy by overexpressing the mRNA-destabilizing protein TTP in a v-H-Ras-dependent mast cell tumor model (Stoecklin et al. 2003). These cells express abnormally stable IL-3 mRNA as part of an oncogenic autocrine loop. When TTP-expressing cells were transplanted in mice, tumor growth was delayed by 4 weeks, and late-appearing tumors appeared to escape tumor suppression by loss of TTP. Decreased IL-3 mRNA levels were observed in TTP-expressing tumors, but, unfortunately, the expression levels of other candidate target mRNAs were not analyzed in this study.

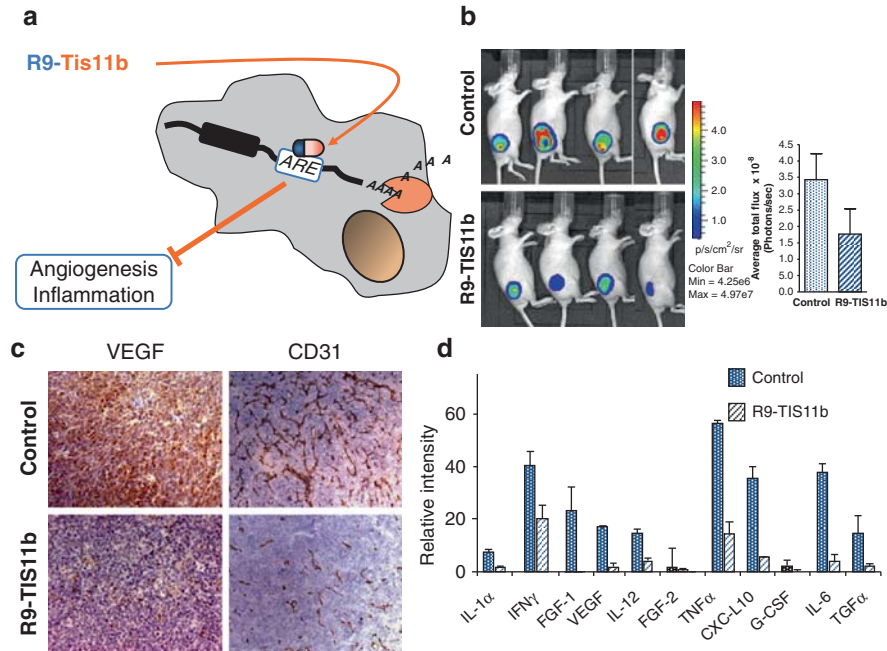
Essafi-Benkhadir et al. contributed complementary information using Raf1-ER-transformed Chinese hamster fibroblasts (Essafi-Benkhadir et al. 2007).

They observed that activation of the MAP kinase pathway by Raf results in upregulation of TTP expression and that overexpression of TTP in these cells reduced the half-life of VEGF-A mRNA. They transformed these cells by overexpression of Ras-val12 and generated inducible TTP expression using a tetracycline-dependent expression vector. These cells were then implanted subcutaneously into nude mice, and their growth and vascularization were analyzed. Doxycycline-induced expression of TTP resulted in a marked reduction of tumor growth, tumor VEGF levels, and tumor vascularization. Interestingly, this effect lasted for at least 35 days without tumor escape to doxycycline treatment. Whether other TTP target mRNAs were modified simultaneously to VEGF mRNA was not evaluated in this study.

More recently, we attempted to develop a therapeutic strategy based on the use of a cell-permeant variant of TIS11b/ZFP36L1 (Planel et al. 2010). Since TIS11b interacts intracellularly with VEGF mRNA 3'-UTR and favors its degradation by the exosome machinery (Ciais et al. 2004), we reasoned that addition of a protein transduction domain (a short peptidic tag that allows proteins to translocate through the plasma membrane of eukaryotic cells) to this protein would make it possible to inject it in pre-established tumors with reasonable chances that it reaches and acts on its intracellular target mRNAs. Among several possibilities tested, we found that an (Arg)<sub>9</sub>N-terminal tag was most efficient for favoring intracellular translocation of TIS11b. When we injected the recombinant R9-TIS11b protein into subcutaneous LLC (Lewis lung carcinoma) tumors, we observed a significant decrease in the tumor growth rate, in the tumor vascular density, and in the tumor VEGF expression level (Fig. 17.2). We also evaluated the effects of this treatment on the expression of a dozen growth factors and cytokines using antibody arrays. Very interestingly, not only VEGF but also FGF-1, EGF, IL-1 $\alpha$ , IL-6, IL-12, and TNF- $\alpha$  protein levels were significantly decreased in R9-TTP-treated tumors. This work was the first to establish that treatment of cancerous tumors with an ARE-binding protein can be a novel multitarget therapy that simultaneously targets several important factors involved in angiogenesis and inflammation.

## 17.7 Perspectives

As an important step of tumor progression, tumor angiogenesis has been identified as a valuable target for cancer therapies. Several anti-angiogenic drugs have been developed during the last decade, and some of them, including anti-VEGF antibodies (bevacizumab) and tyrosine-kinase inhibitors (sunitinib, sorafenib, pazopanib, etc.), have reached the pharmaceutical market. However, none of these drugs cures cancer, and, despite initial enthusiasm, it is now well established that patients develop resistance to anti-angiogenic treatments over time, which limits their beneficial effect. Second-line treatments targeting tumor angiogenesis through different



**Fig. 17.2** Antitumoral therapy using the mRNA-destabilizing properties of TIS11b. **(a)** Fusing a polyarginine (R9) tag to the tristetraprolin family member TIS11b allows it to pass the plasma membrane and bind to AU-rich sequences (ARE) located in the 3'-untranslated region of target mRNAs. This allows deadenylases and exosome RNases to degrade the mRNA. As many ARE-containing genes contribute to angiogenesis and inflammation, the expected effect is an inhibition of these two processes. **(b)** Nude mice were injected subcutaneously with luciferase expressing-Lewis lung carcinoma cells. On day 6, once the tumors had reached a size of 50 mm<sup>3</sup>, R9-TIS11b protein was injected into the tumors. Bioluminescence imaging of the mice performed at day 20 reveals a twofold reduction in tumor size in the R9-TIS11b-treated mice. **(c)** Immunohistochemical analysis of the control and R9-TIS11b-treated tumors reveals a decreased VEGF expression and a decreased vascularization (assessed by CD31 staining). **(d)** Quantification of the protein levels of various angiogenic and inflammatory cytokines in control and R9-TIS11b-treated tumors using antibody arrays reveals a multitarget action of R9-TIS11b (The data shown in **(b-d)** were published in Planel et al. (2010))

mechanisms are therefore urgently needed to bypass these resistance problems and to maintain therapeutic pressure on the development of the tumoral vasculature. As reviewed in this article, exploiting the biological mechanisms that control mRNA stability/decay of the master genes of angiogenesis and inflammation represents an original strategy that fits these requirements and certainly deserves further investigation.

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## Nouvelle thérapie anti-tumorale multi-cibles basée sur la dégradation des ARNm à demi-vie courte

La formation de nouveaux vaisseaux sanguins ou angiogenèse soutient la croissance tumorale en fournissant l'oxygène et les nutriments qui lui sont nécessaires. Le rôle clé du facteur de croissance de l'endothélium vasculaire VEGF dans ce processus a suscité le développement de stratégies anti-angiogéniques pour le traitement du cancer. Cependant, des travaux précliniques et des données cliniques suggèrent l'émergence de résistances aux anti-angiogéniques, en raison notamment de la redondance des facteurs de croissance pro-angiogéniques. Il est donc nécessaire de développer des stratégies alternatives plus efficaces. En 2010, notre laboratoire a apporté la preuve de concept d'une thérapie anti-tumorale et anti-angiogénique innovante basée la dégradation des ARNm à demi-vie courte par la protéine à doigts de zinc TIS11b. Néanmoins, l'instabilité de la protéine thérapeutique a entravé la caractérisation plus détaillée de cette stratégie. Dans ce contexte, l'objectif majeur de ma thèse était l'optimisation de la stabilité et de l'activité de TIS11b et l'évaluation de son efficacité thérapeutique. Pour cela, nous avons généré une nouvelle protéine TIS11b génétiquement modifiée sur la base d'études biochimiques et moléculaires. Notamment, nous avons observé que la phosphorylation de la sérine 334 située dans le domaine C-terminal de TIS11b augmente de façon très significative la stabilité de la protéine et potentialise son activité déstabilisatrice de l'ARNm du VEGF. De plus, la délétion du domaine N-terminal augmente également la stabilité de TIS11b sans altérer son activité. Nous avons alors généré deux nouvelles protéines thérapeutiques, la protéine ZnC et la protéine ZnC<sup>S334D</sup> pour laquelle la troncation du domaine N-terminal et la substitution de la sérine S334 par un aspartate mimant une phosphorylation ont été combinées. Les nouvelles protéines ont été fusionnées à une étiquette polyarginine R9 leur permettant de traverser les membranes cellulaires (R9-ZnC et R9-ZnC<sup>S334D</sup>). Nous avons montré que R9-ZnC et R9-ZnC<sup>S334D</sup> inhibent l'expression de VEGF *in vitro* dans la lignée de cancer du sein murin 4T1. De plus, R9-ZnC<sup>S334D</sup> exerce une activité anti-proliférative, anti-migratoire et anti-invasive dans ces cellules. *In vivo*, l'injection intra-tumorale de R9-ZnC<sup>S334D</sup> dans des tumeurs 4T1 préétablies inhibe significativement l'expression du VEGF, la croissance et la vascularisation tumorales. De façon remarquable, l'analyse des extraits tumoraux indique que le traitement diminue l'expression de chimiokines clés dans les processus d'angiogenèse, d'inflammation et d'invasion (Fractalkine, MCP-1, NOV, SDF-1, Pentraxin...). Enfin, R9-ZnC et R9-ZnC<sup>S334D</sup> inhibent l'expression de marqueurs de la transition épithélio-mésenchymateuse, un processus impliqué dans la dissémination métastatique. L'ensemble de ces travaux indique que R9-ZnC et R9-ZnC<sup>S334D</sup> sont des molécules anti-tumorales multi-cibles, qui inhibent plusieurs étapes clés de la progression tumorale. Cette étude confirme que le ciblage de la stabilité des ARNm est une stratégie prometteuse et novatrice pour le développement de nouvelles thérapies anti-cancéreuses.

**Mots Clés :** thérapie multi-cible, angiogenèse tumorale, TIS11b (ZFP36L1/BRF1), dégradation des ARNm, ingénierie des protéines, phosphorylation, Tristetraprolin

### A novel multi-target cancer therapy based on destabilization of short-lived mRNAs

One of the innovative aspects of anti-cancer therapies is the possibility of preventing tumor growth by blocking blood supply. Cancer cells induce the formation of their own blood vessels from pre-existing vasculature, a process called angiogenesis. One of the most important proangiogenic factors is vascular endothelial growth factor (VEGF). The success of bevacizumab (a humanized anti-VEGF monoclonal antibody) combined to chemotherapy for the treatment of human metastatic cancers has validated VEGF as an efficient target. However, despite the initial enthusiasm, resistance to these anti-angiogenic treatments resulting from compensatory mechanisms occurs upon time. For this reason, there is a real need for new anti-angiogenic drugs that will target the angiogenic process through distinct mechanisms. In 2010, our laboratory has successfully developed an anti-angiogenic and anti-tumoral therapy based on destabilization of short-lived mRNAs by the zinc finger protein TIS11b. However, the therapeutic protein was highly unstable, thus making it difficult to further characterize the experimental therapy. In this context, the main task of my thesis was the optimization of TIS11b stability and activity followed by the evaluation of the multi-target action of our novel protein on tumor development. In a first part of this work, biochemical and molecular approaches allowed us to demonstrate that phosphorylation of the C-terminal serine S334 in TIS11b protein markedly increases its stability. In addition, deletion of the N-terminal domain of TIS11b highly increases its protein stability without affecting its activity. Therefore, we integrated N-terminal truncation (ZnC) and C-terminal substitution of S334 by an aspartate to mimic a permanent phosphorylation at S334 (ZnC<sup>S334D</sup>) as a novel TIS11b engineering strategy. Both proteins were fused subsequently to a cell-penetrating peptide polyarginine (R9). *In vitro* studies revealed that R9-ZnC and R9-ZnC<sup>S334D</sup> inhibit VEGF expression in the murine breast cancer cells 4T1. In addition, R9-ZnC<sup>S334D</sup> impaired proliferation, migration, invasion and anchorage-independent growth of 4T1 cells. *In vivo*, intra-tumoral injection of either protein significantly reduced VEGF expression and tumor vascularization. Strikingly, antibody array analyses of tumor extracts demonstrated a reduced expression of several chemokines such as Fractalkine, MCP-1, NOV, SDF-1 and Pentraxin upon R9-ZnC or R9-ZnC<sup>S334D</sup> treatment. These factors, which are produced by several cell types within tumor tissue, are key drivers of tumor angiogenesis, tumor-promoting inflammation and invasion. Furthermore, the expression of markers of the epithelial-to-mesenchymal transition was also significantly reduced, suggesting an anti-invasive effect of R9-ZnC and R9-ZnC<sup>S334D</sup>. Thus, we provide R9-ZnC and R9-ZnC<sup>S334D</sup> as potential novel multi-target agents which inhibit key hallmarks of cancer progression. This work supports the emerging link between mRNA stability and cancer and proposes novel concepts for the development of innovative anti-cancer therapies.

**Key words:** multi-target therapy, tumor angiogenesis, TIS11b (ZFP36L1/BRF1), ARE-mediated mRNA decay, phosphorylation, protein engineering, Tristetraprolin