From The Department of Microbiology, Tumor and Cell Biology
Karolinska Institutet, Stockholm, Sweden

EXPLORING THE ROLE OF P73-ISOFORMS DURING TUMOR DEVELOPMENT

Habib Abdullah Mohammad Sakil

Karolinska Institutet

Stockholm 2017

Published by Karolinska Institutet.
Printed by E-Print

ISBN 978-91-7676-830-3

Exploring the role of p73-isoforms during tumor development
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Habib Abdullah Mohammad Sakil

Principal Supervisor:
Associate Professor Margareta Wilhelm
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology

Co-supervisor(s):
Professor Marie Arsenian Henriksson
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology

Opponent:
Professor Claire Lewis
University of Sheffield
Department of Oncology & Metabolism

Examination Board:
Associate Professor Guillem Genové
Karolinska Institutet
Department of Medicine, Huddinge (MedH)

Associate Professor Mikael Lindström
Karolinska Institutet
Department of Medical Biochemistry and Biophysics (MBB)

Associate Professor Anna Dimberg
Uppsala University
Department of Immunology, Genetics, and Pathology

To all those who died of cancer…

ABSTRACT

The p73 gene was first discovered in 1997 as a family member of p53, and since then this gene has been studied extensively in cancer biology. Among its two main classes of isoforms, TAp73 acts as a tumor suppressor, while in contrast ΔNp73 was shown to act in a dominant negative fashion as an oncogene which opposes the functions of TAp73 and p53. This thesis focuses on further deciphering the role of p73 isoforms in tumor angiogenesis, tumor microenvironment and drug resistance which promote tumor development. Tumor initiation heavily relies on the loss of tumor suppressors or gain of function of oncogenes in tumor cells, subsequently resulting in the production of factors that modulate the tumor microenvironment in favor of tumor progression. Solid tumors commonly have a hypoxic core due to extensive growth in size, thus limiting oxygen levels for tumor cell survival. Tumor cells overcome this via producing angiogenic factors to recruit more blood vessels into the tumor for meeting the oxygen and nutrition demands. In **Paper I**, we discovered that absence of the tumor suppressor TAp73 leads to increased production of angiogenic factors and tumor angiogenesis. Moreover, this angiogenic response was driven via HIF1α. On the other hand, ΔNp73 showed positive regulation of angiogenic gene expression and angiogenic events. In **Paper II**, we further verified the role of TAp73 in regulation of the angiogenic chemokine CCL2, where TAp73 suppresses CCL2 expression via regulation of NFκB activity. Additionally, TAp73 deficient tumors favored increased recruitment of tumor supporting macrophages. A negative correlation of *TP73* with macrophage markers was also confirmed in several human cancer datasets. In **Paper III**, we further investigated the role of ΔNp73-mediated regulation of HIF1α stability in normoxia. ΔNp73 was found to positively regulate HIF1α stability via regulation of ECV genes, which are important components of the proteasome mediated HIF1α degradation pathway. Finally, in **Paper IV**, we addressed ΔNp73's involvement in drug resistance. Oncogenic ΔNp73 upregulated the expression of multidrug resistance related ABC transporters. Additionally, ΔNp73 deficiency reduced drug efflux capacity of breast cancer cells. The positive correlation of ABC transporters and ΔNp73 was also confirmed in melanoma.

In summary, the findings in this thesis further enlighten the role of p73 isoforms in different aspects of tumor development that support chemoresistance and tumor progression.

LIST OF SCIENTIFIC PAPERS

I. Marina Stantic, **Habib A. M. Sakil**, Hanna Zirath, Trixy Fang, Gema Sanz, Alejandro Fernandez-Woodbridge, Ana Marin, Evelyn Susanto, Tak W. Mak, Marie Arsenian Henriksson, and Margareta T. Wilhelm
TAp73 suppresses tumor angiogenesis through repression of proangiogenic cytokines and HIF-1α activity.
PNAS, 2015, 112, p220-225

II. **Habib A. M. Sakil**, Johanna Wolfsberger, Marina Stantic, Trixy Fang, and Margareta T. Wilhelm
TAp73 deficiency enhances CCL2 expression and increase intra-tumoral infiltration of tumor-associated macrophages.
Manuscript, 2017

III. Marina Stantic, Johanna Wolfsberger, **Habib A. M. Sakil**, and Margareta T. Wilhelm
ΔNp73 enhances HIF-1α protein stability through repression of the ECV complex.
Submitted, 2017

IV. **Habib A. M. Sakil**, Marina Stantic, Johanna Wolfsberger, Suzanne Egyhazi Brage, Johan Hansson and Margareta T. Wilhelm
ΔNp73 regulates the expression of the multidrug-resistance genes ABCB1 and ABCB5 in breast cancer and melanoma cells.
Cellular Oncology, 2017, doi: 10.1007/s13402-017-0340-x

PUBLICATION NOT INCLUDED IN THE THESIS

A Kostecka, A Sznarkowska, K Meller, P Acedo, Y Shi, **Habib A. M. Sakil**, A Kawiak, M Lion, A Królicka, M Wilhelm, A Inga, and J Zawacka-Pankau
JNK–NQO1 axis drives TAp73-mediated tumor suppression upon oxidative and proteasomal stress.
Cell Death Dis. 2014; 5(10): e1484. doi: 10.1038/cddis.2014.408

CONTENTS

LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
ABCB1	ATP-binding cassette sub-family B member 1
ABCB5	ATP-binding cassette sub-family B member 5
ABCA2	ATP-binding cassette sub-family A member 2
ABCA5	ATP-binding cassette sub-family A member 5
ABCA6	ATP-binding cassette sub-family A member 6
ABCA8	ATP-binding cassette sub-family A member 8
ABCA9	ATP-binding cassette sub-family A member 9
ABCA10	ATP-binding cassette sub-family A member 10
ABCD2	ATP-binding cassette sub-family D member 2
ABCG2	ATP-binding cassette sub-family G member 2
ABL	Abelson murine leukemia viral oncogene homolog 1
AD	Atopic dermatitis
ALL	Acute lymphoblastic leukemia
APCs	Antigen presenting cells
APL	Acute promyelocytic leukemia
ATF3	Activating transcription factor 3
ATM	Ataxia-telangiectasia mutated
Ang-1	Angiopoietin 1
α-SMA	Alpha smooth muscle actin
BAD	Bcl-2-associated death promoter
Bai1	Brain-specific angiogenesis inhibitor 1
BAX	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
BCL3	B-cell lymphoma 3-encoded protein
bHLH	Basic helix–loop–helix
BIK	Bcl-2-interacting killer
BRCA1	BReast Cancer genes 1
Breg	B regulatory cell
Bub1	Budding uninhibited by benzimidazoles 1

Bub3	Budding uninhibited by benzimidazoles
BubR1	Budding uninhibited by benzimidazoles receptor 1
CAD	Caspase activated DNAse
CAFs	Cancer associated fibroblasts
CBP	CREB-binding protein
CCL2	Chemokine (C-C motif) ligand 2
CCL3	Chemokine (C-C motif) ligand 3
CCL4	Chemokine (C-C motif) ligand 4
CCL5	Chemokine (C-C motif) ligand 5
CCL20	Chemokine (C-C motif) ligand 20
CCR2	C-C chemokine receptor type 2
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
CD25	Cluster of differentiation 25
CD31	Cluster of differentiation 31
CD94	Cluster of differentiation 94
CD95	Cluster of differentiation 95
CD146	Cluster of differentiation 146
CD206	Cluster of differentiation 206
cDC2s	Classical dendritic cells
CDKs	Cyclin-dependent kinases
Chk1	Checkpoint kinase 1
COX2	Cyclooxygenase 2
Cox4i1	Cytochrome C oxidase subunit IV
CSF1	Colony Stimulating Factor 1
CSF3	Colony Stimulating Factor 3
CSF1R	Colony Stimulating Factor receptor 1
Cxcl1	Chemokine (C-X-C motif) ligand 1
CXCL8	Chemokine (C-X-C motif) ligand 8
CXCL9	Chemokine (C-X-C motif) ligand 9
CXCL10	Chemokine (C-X-C motif) ligand 10

CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
Cul2	Cullin 2
DDR2	Discoidin domain receptor family, member 2
DNAM-1	DNAX Accessory Molecule-1
DRAM	DNA damage regulated autophagy modulator
ECM	Extracellular matrix
EMT	Epithelial to mesenchymal transition
Ereg	Epiregulin
EGFR	Epidermal growth factor receptor
Egr-1	Early growth response protein 1
FBXO45	F-box protein 45
FGF	Fibroblast Growth Factor
FGF2	Fibroblast growth factor 2
FSP1	Fibroblast-specific protein 1
GPCRs	G-protein-coupled receptors
GLUT1	Glucose transporter 1
GLUT3	Glucose transporter 3
GM-CSF	Granulocyte Colony Stimulating Factor
HCK	Hematopoietic cell kinase
HDM2	Human double minute 2 homolog
HIF	Hypoxia-inducible factor
HIF1α	Hypoxia-inducible factor 1-alpha
HIF2α	Hypoxia-inducible factor 2-alpha
HIF3α	Hypoxia-inducible factor 3-alpha
HRE	Hypoxia Response Elements
IBS	Irritable bowel syndrome
ICAD	inhibitor of caspase activated DNAse
ICAM-2	Intercellular Adhesion Molecule 2
IFNγ	Interferon gamma
IGF1R	insulin-like growth factor receptor 1
IgG	Immunoglobulin G

IKBkA	Inhibitor of NFκB kinase subunit alpha
IKBkB	Inhibitor of NFκB kinase subunit beta
IKBkG	Inhibitor of NFκB kinase subunit gamma
IKBkE	Inhibitor of NFκB kinase subunit epsilon
IKK2	Inhibitor of nuclear factor kappa-B kinase subunit beta
iMCs	Immature myeloid cells
iNOS	Inducible nitric oxide synthase
IL1β	Interleukin 1 beta
IL-4	Interleukin 4
IL6	Interleukin 6
IL8	Interleukin 8
IL10	Interleukin 10
IL13	Interleukin 13
IL-17	Interleukin 17
IL21	Interleukin 21
IL22	Interleukin 22
JNK	c-Jun N-terminal kinase
KEGG	Kyoto Encyclopedia of Genes and Genomes
KIR	Killer-cell immunoglobulin-like receptors
LDHA	Lactate dehydrogenase A
MDR1	Multidrug resistance gene 1
MDM2	Mouse double minute 2 homolog
MDSC	Myeloid-derived suppressor cells
MET	Mesenchymal to Epithelial Transition
MMP7	Matrix metalloproteinase 7
MMP9	Matrix metalloproteinase 9
MMP12	Matrix metalloproteinase 12
Mrc1	Mannose receptor 1
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NFκB1	Nuclear factor κB 1

NFκB2	Nuclear factor κB 2
NFκBIA	NFκB inhibitor alpha
NFκBIB	NFκB inhibitor beta
NFκBIE	NFκB inhibitor epsilon
NKG2A	Natural-killer group 2, member A
NKG2D	Natural-killer group 2, member D
NO	Nitric oxide
p53AIP1	p53-regulated apoptosis-inducing protein 1
PARP1	Poly [ADP-Ribose] Polymerase 1
PD-1	Programmed cell death-1
PDGF	Platelet-derived growth factor
PDGF-β	Platelet-derived growth factor beta
PDGFC	platelet-derived growth factor c
PDGFRα	Platelet-derived growth factor receptor alpha
PDGFRβ	Platelet-derived growth factor receptor beta
PDK1	Pyruvate Dehydrogenase Kinase 1
PD-L1	Programmed death-ligand 1
PERP	p53 apoptosis effector related to PMP-22
PHD	Prolyl hydroxylase
PHD1	Prolyl hydroxylase 1
PHD2	Prolyl hydroxylase 2
PHD3	Prolyl hydroxylase 3
PIAS-1	Protein inhibitor of activated STAT
PIR2	p73 induced ring finger protein 2
PlGF	Placental growth factor
PUMA	p53 upregulated modulator of apoptosis
pVHL	Von Hippel-Lindau
PLK3	Polo-like kinase 3
RanBP9	RAN Binding Protein 9
ROS	Reactive oxygen species
RNPC1	RNA-binding region-containing protein 1

SAC	Spindle assembly checkpoint
SAM	Sterile alpha motif
SEMA3A	Semaphorin 3A
Sema4D	Semaphorin 4D
Spl	Squamous promoter binding protein like
STATs	Signal transducer and activator of transcription
SUMO-1	Small ubiquitin-related modifier 1
TAMs	Tumor associated macrophages
TCGA	The Cancer Genome Atlas
TGFβ	Human double minute 2 homolog
TID	Transcription inhibitory domain
TNF-R1	Tumor necrosis factor receptor 1
TNF-α	Tumor necrosis factor alpha
TNF	Tumor necrosis factor
Tnfaip2	Tumor necrosis factor alpha induced protein 2
TRAIL-R2	TNF-related apoptosis-inducing ligand receptor 2
TRAIL-R1	TNF-related apoptosis-inducing ligand receptor 1
Treg	T regulatory cells
TRK	Tropomyosin receptor kinase
VE-cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
VEGFR1	Vascular endothelial growth factor receptor 1
VEGFR2	Vascular endothelial growth factor receptor 2
VEGFR3	Vascular endothelial growth factor receptor 3
VEGFA	Vascular endothelial growth factor A
VEGFC	Vascular endothelial growth factor B
VEGFD	Vascular endothelial growth factor D
WHO	World Health Organization
ZEB1	Zinc Finger protein 1

1 INTRODUCTION

1.1 CANCER AND TUMOR

Tumor is a generic term given for a mass or lump of cells. In normal physiology, cell division is a regular and tightly controlled event, however, under unfavorable circumstances when cells undergo abnormal cell division it leads to the formation of tumors or neoplasms. Tumors can be benign or malignant. Benign tumors are usually confined to a specific location and cannot invade neighboring tissue or spread to other parts of the body. Malignant tumors not only invade but also acquire the ability to migrate to distant sites through the circulatory or lymphatic system, resulting in cancer. According to WHO, cancer was the second major cause of global death claiming nearly 8.8 million lives worldwide in 2015. In males, the most common types of cancers include lung, prostate, colorectal, gastric and liver cancer whereas in females it includes breast, colorectal, lung, cervix and gastric cancer[1]. The term cancer was first coined from the Greek word ´´karkinos´´ for crab because similar to a crab 'once it gets hold of you it never lets go'[2]. The reason behind the use of this terminology is that even after a cancer patient is treated with currently available therapies, there may still be dormant or resistant cells that later undergo clonal expansion to form new tumors. This still holds cancer as an unresolved mystery.

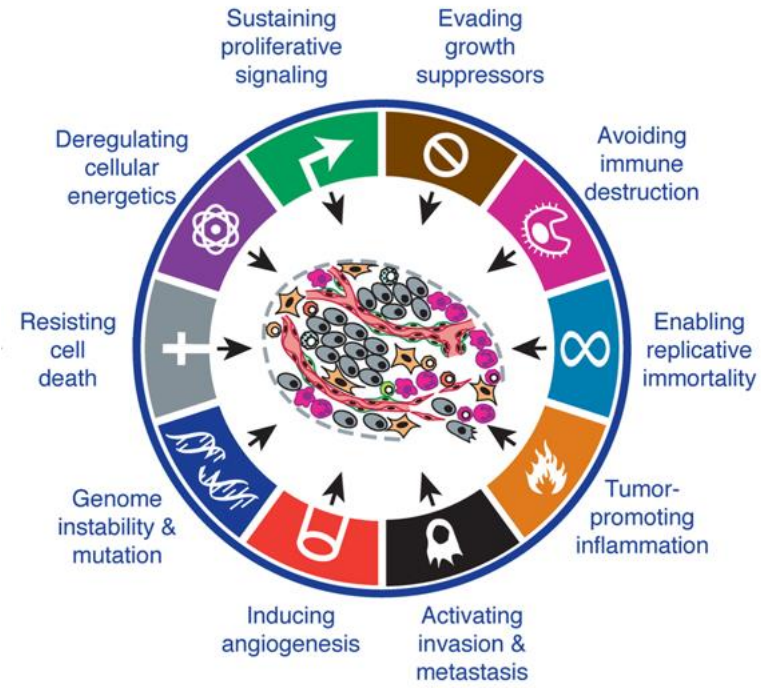

Figure 1. The hallmarks of cancer. Adapted from *Hanahan and Weinberg, Cell 2011* in compliance with the conditions of the Elsevier user license.

1.1.1 The hallmarks of cancer

Hanahan and Weinberg primarily listed six important traits that normal cells need to acquire starting from the initiation of cancer to cancer development, progression and metastatic dissemination[3]. In an updated version, they included four new features that are referred to as the emerging hallmarks of cancer[4]. Together, these ten prime characteristics are: (1) continuous proliferative signaling causing uncontrolled clonal expansion (2) insensitivity to growth inhibitory signals (3) resistance to cellular apoptosis or cell death (4) limitless clonal expansion (5) tumor angiogenesis (6) invasion and metastasis to secondary site in distal part of the body (7) altered cell metabolism (8) recurrent mutation and genomic instability (9) altered phenotypic characteristics to avoid immune cell mediated clearance and (10) sustained inflammatory microenvironment[4]. All these features have been depicted in Figure 1.

1.1.2 Oncogenes and tumor suppressors

Tumor cells are very distinct from their normal cellular counterparts in many aspects including; growth regulation, morphology, cellular interaction, membrane proteins, and differential gene and protein expression. These unique features are mainly supported by two family of genes, namely proto-oncogenes and tumor suppressor genes, which function to control cell growth, survival and differentiation; nonfunctional mutation or deletion of tumor suppressor genes may result in cancer progression[5]. In most cases, cancer results due to gain of function of proto-oncogenes/oncogenes and/or loss of function of tumor suppressor genes and proteins.

Proto-oncogenes are a class of genes, which play a significant role in different checkpoints of cell division, differentiation, proliferation, cell survival and cell death (apoptosis); over-activation of these genes results in malignancy. Proto-oncogenes mostly remain active during embryonic development and are generally turned off during adulthood. Mutations or genetic alterations in the DNA, like deletions or amplifications, may activate proto-oncogenes. This could ultimately increase expression or alter proto-oncogene function to provide survival advantage to non-tumoral cells. The most common proto-oncogenes are growth factors, receptor/non-receptor tyrosine kinases, serine threonine kinases, G-protein-coupled receptors (GPCRs) and transcription factors.

Oncogenes are the mutated form of proto-oncogenes that become activated by genetic changes including; chromosomal translocations, deletions, insertions, amplifications, point mutations, or enhanced transcription of their genes. All these events can be instrumental in altering the protein structure, function, or regulation of the oncoprotein. Additionally, different viruses and viral proteins can transform cells via conversion of proto-oncogenes to oncogenes that later

fuels cell growth through controlling key cell division factors and inhibiting cellular apoptosis, thus allowing cells to multiply in an uncontrolled manner resulting in tumor development. Oncogenes or oncoproteins can be broadly categorized into several groups including non-receptor TKs (SRC, ABL), serine/threonine kinases (AKT, RAF1), growth factors (PDGF), growth factor receptors (EGFR, MET, RET, TRK), small G proteins (H-RAS, K-RAS), transcription factors (ATF3, MYC), anti-apoptotic proteins (BCL-2, MDM2) and many more[6].

Tumor suppressor genes and their encoded proteins are involved in regulating cell growth, cell cycle, and proliferation; additionally, they play an essential role in apoptosis and DNA repair. They mainly function to guard and check the presence of any cellular abnormalities to halt tumor formation. In cancer, tumor suppressors are classified into five different categories as shown in Table 1[5].

Table 1. Category of Tumor suppressors proteins and their targeted function

Tumor suppressor type	Example
Intracellular proteins controlling or suppressing different cell cycle stages	p16 and Rb
Receptors or signal transducers that control cell proliferation	TGFβ, hedgehog receptor patched
Checkpoint-control proteins to stop cell division in case of any anomalies in DNA or chromosome	p53
Apoptosis inducers	p53
DNA repair enzymes	PARP1, ATM and BRCA1

Some tumor suppressor proteins may fall into several groups because of their different tumor suppressive functions, such as the transcription factor p53. In addition, p53 has been referred to as "the guardian of the genome" as it controls nearly all the steps before a cell can be transformed to a tumor cell[7].

1.1.3 Tumor microenvironment

A solid tumor resembles an organ in which tumor cells and stromal cells create the tumor microenvironment that plays a key role in tumor formation, progression and metastasis. The stromal compartment may also contribute to drug resistance or unresponsiveness to chemotherapy. In the tumor microenvironment, apart from the stromal ECM, cells in the stromal compartment can be broadly categorized into two groups based on their cell of origin;

these include (a) tissue resident cells found before and after tumor formation and (b) tumor infiltrated cells that travel to the tumor site from other parts or organs of the body (Figure 2).

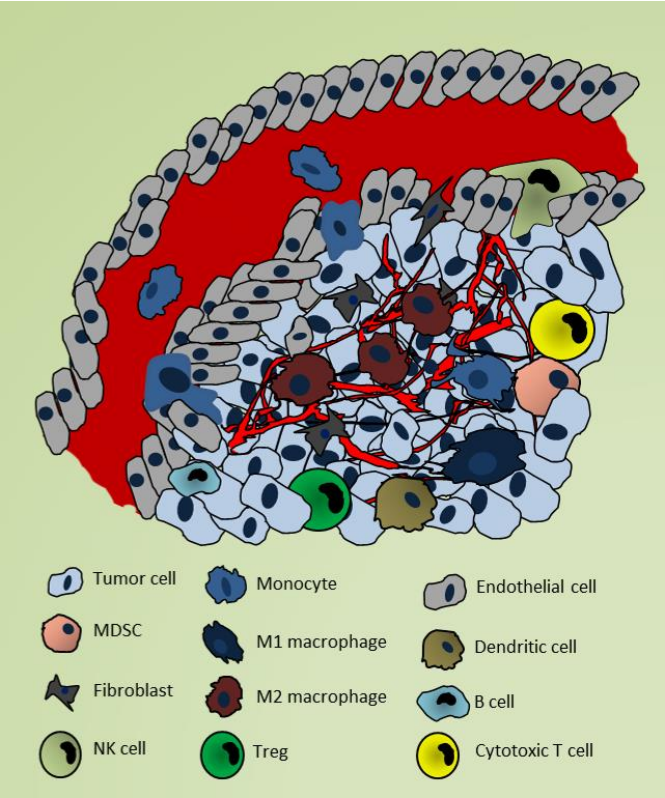

Figure 2. Heterogeneous population of cells in a tumor microenvironment.

Resident cells are mostly endothelial cells, pericytes, fibroblasts and occasionally neighboring tissue macrophages. The infiltrated cells that invade the tumor are mostly immunomodulatory cells that primarily respond to an immune reaction or inflammation elicited by the growing tumor. In most cases, tumor infiltrating immune cells includes T cells, B cells, macrophages, Myeloid-derived suppressor cells (MDSC), dendritic cells, and other cells from the hematopoietic lineage[8,9]. The presence of a multitude of cells in the tumor microenvironment initiates autocrine and paracrine signaling between the different cell types, which ultimately determine the fate of tumor development.

1.1.4 Angiogenesis

When a tumor mass increases and the diameter exceeds 1-2 mm in size, the tumor microenvironment experiences low oxygen pressure or hypoxia. Moreover, tumor cells go through a deprivation of nutrients and also accumulate cellular waste[10]. To avoid these unfavorable conditions tumor cells initiates expression of pro-angiogenic factors, which subsequently stimulate endothelial cells to proliferate and extend newly formed blood vessels

towards the tumor. A well-known angiogenic factor VEGF (vascular endothelial growth factor) elicits its effects on endothelial cells through binding to its cognate tyrosine kinase receptors (VEGFR1, VEGFR2 and VEGFR3). Activation of VEGF receptors ultimately results in initiation of multiple downstream signaling pathways that stimulate endothelial cell sprouting; endothelial cells start losing their cell-cell contacts in preparation to form an endothelial tip cell that can direct vascular growth through sensing pro-angiogenic factors. The leading Tip cell is followed by proliferating endothelial stalk cells to form a vascular sprout that ultimately form new blood vessels[11]. The newly formed vascular network supports tumor survival through providing oxygen and nutrients, as well as transporting waste products away from the tumor. Physiological angiogenesis or vasculogenesis is a phenomenon in which new blood vessels develop from progenitor cells which is a very unique characteristic found in embryonic development, tissue regeneration and the female reproductive system[12]. In contrast, in pathological angiogenesis, blood vessels develop from the existing vasculature and is a common feature seen in different human diseases like Irritable bowel syndrome (IBS), endometriosis, atherosclerosis, arthritis, diabetic retinopathy, obesity, asthma, and cancer[13]. Unlike the normal vasculature, tumor blood vessels have distinctive features; they are irregular and very loosely connected, making them very dilated and leaky. The leakiness of the vessels is associated with poor pericyte coverage on the surface of the tumor vessels causing excess drainage of fluid and other proteins[14]. Such a feature may serve good for prognosis as it may help better delivery of chemotherapeutic drugs to the tumor site, however, it may also enable tumor cells to escape the primary site and form a secondary tumor in other parts of the body[9]. Moreover, tumor cells can downregulate expression of angiogenic inhibitors (thrombospondins, angiostatin and endostatin) in order to favor tumor angiogenic cascades. Angiogenic inhibitors are known to suppress endothelial cell proliferation, migration, invasion, and cell adhesion molecules, thus limiting the formation of new blood vessels[15]. The relationship between angiogenesis and tumor development was first proposed by Judah Folkman; he described that tumor growth depends on the mutual interaction between the tumor and endothelial cells including the pro-angiogenic factors produced by them[16]. Based on this theory, many therapies have been developed to inhibit tumor angiogenesis or new vessel formation. Currently, the most common angiogenesis inhibitor found in the clinic is VEGF inhibitors that includes both targeted antibody (Bevacizumab) and small molecule inhibitors (Sunitinib and Sorafenib). However, such therapies have not been successful for cancer treatment due to their adverse side effects. These inhibitors not only cause microvascular regression in tumors but also in other organs such as thyroid, liver, kidney and GI tract resulting in hypothyroidism, internal bleeding, proteinuria and GI perforation[17]. Moreover, in a study it

was reported that inhibition of VEGF resulted in a pro-invasive phenotype in glioblastoma[18]. Thus, alternative treatment strategies are required to prevent tumor progression.

Endothelial cells

Endothelial cells play an important role in tumor development starting from tumor initiation, progression, invasion, dissemination and resistance to drug therapy. Endothelial cells form blood vessels and thus act as a medium of delivering nutrients and oxygen and eliminating waste products from the tumor[19]. In the tumor microenvironment, endothelial cells are activated by diverse cytokines and factors produced by tumor cells and immune cells. These stimulate endothelial cell proliferation and subsequently direct endothelial sprouting towards the tumor favoring angiogenesis[20]. Tumor endothelial cells are characterized by their expression of CD31, CD146 and ICAM-2[21]. In the tumor, pro-inflammatory cytokines (like TNFα and IL1β) produced by immune cells can stimulate endothelial cells to increase the expression of adhesion molecules that can, in turn, support continuous inflammation and favor the tumor microenvironment[21,22]. Adhesion molecules on endothelial cells can increase the 'stickiness' of the endothelium to adhere circulating immune cells and can thus promote inflammation[23]. Additionally, tumor endothelial cells possess a regulatory role in metastasis and have also been shown to suppress T cell function in lung cancer through the production of an immune suppressive factor, prostaglandin E_2 [24,25].

Pericytes

Pericytes are mesenchymal originated mural cells commonly found covering the microvascular endothelial walls to stabilize the blood vessels. Additionally, they also promote proliferation of endothelial cells via providing them with trophic factors[9]. Pericytes expresses α-SMA, PDGFRβ, CD146 and/or NG2 depending on the type of microenvironment they are located in[26,27]. Based on marker expression and function, pericytes can be categorized into two types. Both Type 1 and Type 2 express PDGFRβ, CD146 and NG2 but only Type 2 pericytes express Nestin. In the tumor microenvironment, Type 2 pericytes are recruited to the tumor blood vessels to support angiogenesis[28,29]. Apart from vessel coverage, they also have immunosuppressive roles such as inhibition of cytolytic T cell function[30]. In cancer, increased pericytic coverage is linked to worse prognosis in skin cancer and renal carcinoma; moreover, it is also associated with chemoresistance and poor patient outcome[31]. Activated endothelial cells are known to express PDGF-β which attract the PDGFRβ expressing pericytes to stabilize vessel integrity and to produce angiogenic VEGF and Ang-1[32]. Limiting pericyte recruitment

to the vessel wall via PDGF-β inhibition reduces tumor angiogenesis and metastasis[33-35]. However, although ablation of pericytes results in disorganized blood vessels leading to suppression of tumor growth, pericyte depletion may also open the door for metastatic spreading of cancer cells as is seen in breast cancer[36]. Therefore, targeting of pericytes for cancer treatment may pose to be a complicated strategy.

Proangiogenic chemokines and cytokines

Cytokines are small proteins that are secreted by cells to influence the behavior of neighboring cells or the releasing cell itself, whereas chemokines are cytokines that can attract or recruit other cells from distal sites/organs to the site of production. Tumor cells are known to produce both cytokines and chemokines to support continuous tumor cell proliferation, recruit caner-associated fibroblasts and endothelial cells and also initiate the migration of immune cells to the tumor site; subsequently, these cells produce more growth factors and/or proangiogenic factors to drive tumor progression (Table 2)[9,37]. To prevent tumor development, recruited immune cells produce inflammatory cytokines and chemokines, of which a majority are also known to have pro-angiogenic properties. Additionally, infiltrated immune cells can be polarized by the tumor cells to produce increased levels of pro-angiogenic factors to fuel the formation of new blood capillaries to the tumor[9]. In breast cancer, it has been shown that CCL2 chemokine produced by the tumor cells can recruit inflammatory macrophages, which supports tumor angiogenesis and metastasis to the lung[38]. Apart from immune cells, the hypoxic environment in the tumor is another important regulator that instigates the expression of these chemokines and cytokines[9]. The role of hypoxia in tumor angiogenesis will be discussed in section 1.1.8.

Table 2. Angiogenic chemokines and cytokines expressed in the tumor microenvironment

Cell type	Angiogenic factors
Macrophage/TAM	VEGFA, VEGFC, VEGFD, FGF2, TNF-α, IL6, IL-1β, CXCL8, CXCL12, COX2, PlGF, PDGF, MMP7, MMP9, MMP12, Sema4D
MDSC	VEGFA, FGF2, MMP9, CCL2 and IL-1β
CAFs	VEGFA, PDGFC, FGF2, CXCL12, osteopontin and CSF3
Dendritic cells	VEGF, TNF-α and CXCL8
B cells	VEGFA, FGF2, MMP9 and IgG
NK cells	VEGFA
Pericytes	VEGFA, ANGPT1 and ECM components
T cells	
T_H2 cells	IL-4
$T_H17$cells	IL-17
T_{reg} cells	VEGFA

1.1.5 Inflammation and immune cells

Inflammation has recently been included as an emerging hallmark of cancer[4]. In most solid tumors, the lack of oxygen and nutrients, and the recruitment of different immune cells create an inflammatory milieu. The inflammatory signal mainly comes from inflammatory chemokines and cytokines produced by immune cells recruited to the tumor as an immune response. Primarily, the function of the immune cells is to resolve anomalies found in the tumor site, clear out dead cells, and aid tissue remodeling and regeneration; however, tumor cells tend to overtake this process and misuse it for tumor progression. In fact, inflammatory cells including macrophages, dendritic cells, NK cells, T cells and B cells form an integral component of the tumor stromal compartment. Dvorak first reported that a wound and the tumor stromal compartment are similar in the sense that both contain numerous blood vessels, ECM producing fibroblasts, and inflammatory cells. He proposed the idea that a tumor is like a wound that never heals[22]. The link between inflammation and tumorigenesis was first proposed by Rudolf Virchow in 1863 after the observation that infiltrating leukocytes are a hallmark feature of tumors[39]. Inflammatory signals induced by infections has also been linked to cancer progression with poor prognosis[40]; infection causing agents include helicobacter pylori in gastric cancer, human papillomavirus in cervical cancer, and Epstein–Barr virus in Burkitt's lymphoma. Additionally, inflammation induced by oncogene activation (Ras, Myc) has also been linked to different types of cancer[41]. Furthermore, inflammatory cells produce reactive oxygen species (ROS) and nitrogen that act as mutagens and cause more mutations in the tumor cells, in contrast to cells in the non-inflamed site[42]. Immune cells recruited to the tumor site are known to produce pro-inflammatory factors, many of which are proangiogenic in nature (see Table 2). The NFκB family of transcription factors significantly regulates the inflammatory response or inflammatory cytokine expression by immune cells, and their central function linked to inflammation is further discussed in section 1.1.7.

Macrophages

During an inflammatory response, innate immune cells are the first to act against foreign pathogens. These consist of different myeloid lineage cells like macrophages, neutrophils and mast cells. Immune cells are also known to facilitate tissue remodeling and regeneration, however, in tumors they support tumor vascularization and metastatic spreading. Tumor recruited myelogenic immune cells are very heterogeneous and they act differently depending on the site where they are localized, i.e. whether they are at the invading front, next to blood vessels, or in the hypoxic area of the tumor microenvironment[43-45]. Monocyte derived

macrophages are known to function during different stages of development, including bone and mammary gland formation. They are also reported to control angiogenesis in wound healing and tumor development[46]. Macrophages recruited to the tumor can portray dual function as pro- or anti-tumorigenic effectors. A large number of studies suggest that macrophages assist in tumor development; chemokines produced in the tumor microenvironment such as CSF1, CCL2, CXCL12, VEGFA and SEMA3A, signal macrophage recruitment and polarize tumor infiltrated macrophages towards a tumor promoting type[47]. Once recruited to the tumor or infection site macrophages become activated to their classically activated macrophage (M1) or alternatively activated macrophage (M2) phenotypes (Figure 3). Macrophages are activated to their M1 phenotype mainly by the T_H1 cytokines (IFNγ, TNFα and GM-CSF) produced by the T_H1 cells and products from pathogens. Characteristically, classical M1 macrophages are known to produce ROS and nitrogen metabolites as well as different inflammatory cytokines that portray cytotoxic function on intracellular pathogens and tumor cells[48]. In the chronic skin condition psoriasis, there is an enrichment of M1 macrophages or T_H1 response, which reduces the burden of any chance of tumor development[49]. Alternatively, M2 macrophages are polarized mainly by the T_H2 cytokines (IL4, IL13 and TGFβ) expressed by the T_H2 cells.

Figure 3. Common markers expressed by M1 and M2 like macrophages[50,51].

M2 macrophages are known for their major function in parasite killing and tissue remodeling[48]. Additionally, M2 macrophages are commonly found in the tumor microenvironment due to the enrichment of T_H2 cytokines in tumors; thus, tumors educate macrophages to polarize into M2-like macrophages. The presence of M2-like macrophages in tumors has been inversely correlated with disease free survival in breast cancer[46,52]. In addition, high levels of M2-like tumor-associated macrophages (TAMs) have been linked with tumor development, invasion, metastatic spreading and angiogenic sprouting. TAMs are known to promote angiogenesis via increased production of growth factors, proangiogenic cytokines and ECM degrading enzymes

as well as by dampening the T-cell mediated cell death response[42,46,53,54]. Interestingly, the tumor produced chemokine CCL2 has been shown to recruit $CCR2^+$ inflammatory monocytes whereas inhibition of CCL2 in turn reduces TAMs resulting in suppression of tumor angiogenesis and human melanoma xenograft growth[38,55]. Hence, TAM polarization can actively remodel and reprogram the tumor microenvironment; this reprogramming may involve increased tumor angiogenesis that promotes immune cell recruitment and activation, and in turn their secretome can produce an immune suppressive tumor milieu, allowing tumor cells to evade the adaptive immune system.

T cells are the major effector cells that act in frontline defense against any anomalies, infection, microbes, viral pathogens or transformed malignant cells. The infiltration of T cells has been associated with either good or poor prognosis in cancer. T cells can be categorized into different subtypes that can play different and opposing functions in tumor development. Amongst these subtypes include $CD4^+$ and $CD8^+$ T cells. $CD8^+$ T cells show tumoricidal function; after migrating and invading the tumor they become activated and produce granzyme B and perforin, which together mediate cytotoxicity towards the tumors cells and induces apoptosis[56,57]. $CD4^+$ T cells can be further classified according to their different cytokine expression profiles; these include T_H (T helper)1, T_H2, T_H17 and Treg (T regulatory) cells. T_H1 cytokines (IFN-γ, TNF-α, and GM-CSF) produced by T_H1 cells can reprogram macrophages to their anti-tumorigenic M1 phenotype. In contrast, T_H2 cytokines (IL4 and IL13) produced by T_H2 cells can polarize macrophages towards the M2 phenotype, which is known to support tumorigenesis, invasion and metastasis in breast cancer[48,58]. T_H17 cells secrete a wide range of pro-inflammatory cytokines including IL17, IL21, IL22 and CCL20. Their role in tumor immunology is quite controversial and their function in tumor microenvironment may rely on the surrounding milieu in which they reside[59]. However, they have been reported to enhance tumor development via producing immunosuppressant IL17[60]. In contrary, T_H17 cells can be polarized into different subsets, which help regression of melanoma[61]. Treg cells are $CD4^+$ $CD25^+$ T cells that can inhibit the anti-tumorigenic immune response of $CD8^+$ T cells via production of TGFβ and IL10, thus favoring tumor development[59,62-64]. Taken together, the function of T cells and their polarization depend on exposure to different cytokines and the microenvironment into which they are recruited. Tumor cells tend to reprogram the T cells towards the more tumor-supporting Treg cells to promote tumor progression, angiogenesis and dissemination[63]. Increased Treg cell signatures have been found to be associated with different types of cancers and with worse prognosis in patients[65]. Adoptive transfer of tumor infiltrated lymphocytes/$CD8^+$ T cells (TILs) is a very promising therapeutic approach where after

isolation and immunomodulation TILs are infused back into cancer patients. TIL therapy has recently been adopted in many clinical settings for cancer treatment[66]. Antibody targeted therapy against immune-checkpoint molecules such as PD-1, PD-L1, and CTLA-4 is another approach currently used in the clinic to block immune-escape of cancer cells together with enhanced cancer cell killing via cytotoxic T cell activation[67].

Natural killer (NK) cells arise from lymphoid progenitors and are active effector cells of the innate immune system. As killer cells they exert cytolysis via factors like perforin and granzyme B and the production of cytokines that in turn impede tumor progression. Increased NK cell recruitment and activation is associated with a good prognosis in different cancers[68,69]. In a tumor setting, malignant cells tend to maintain a balance between NK cell inhibition or activation. NK cells express multiple receptors including NKG2D, DNAM-1 and cytotoxicity receptors (NKp30, NKp44 and NKp46), which aid in NK cell activation via binding to specific ligands expressed by malignant cells to mediate cytolytic function[70]. DNA damage, viral infections, or oncogene activation induces expression of NKG2D and DNAM-1 ligands that result in increased NK cell recognition of the affected cell. Inhibition of these ligands leads to impaired NK cell mediated cytolysis[71,72]. In the tumor microenvironment, signals or markers that activate NK cells get masked and thus tumors can bypass NK cell mediated killing. Immune cells such as Tregs and dendritic cells are known to produce TGFβ that inhibits NK cell proliferation and impairs their cytolytic function[73-76]. Cancer associated fibroblasts (CAFs) are known to similarly inhibit NK cells via TGFβ which can also reduce the expression of perforin and granzyme B[77]. NK cells can express HLA-I, KIR, CD94/NKG2A and PD-1 receptors that can block NK cell mediated killing of normal autologous cells[70]. They are also known to secret several chemokines and cytokines such as IFNγ, TNFα, GMCSF, CCL3, CCL4 and CCL5 to maintain interaction with the heterogeneous population of immune cells found in the tumor microenvironment[78-80]. Interestingly, tumor cells tend to reprogram the tumor infiltrated NK cells to produce several pro-angiogenic factors (like VEGF and PDGF) to support tumor development[71]. Melanoma cells suppress expression of NK cell activation receptors NKp30, NKp44 and NKG2D that impair the cytotoxic efficiency of NK cells[81]. Multiple myeloma has higher expression of PD-1, which is known to inhibit NK cells and block its instigated tumoricidal events[82]. Inhibitors of CTLA4/CD28 and PD-1/PD-L1 pathway can promote T cell and NK cell mediated anti-tumor activity[77].

Myeloid-derived suppressor cells (MDSCs) are a diverse group of cells that arise from the myeloid lineage. They are mainly immature myeloid precursor cells that include granulocytes, macrophages and dendritic cells. Interestingly, they become activated, recruited and expanded

to high numbers in response to any sustained inflammation or tumor mediated signaling, however, unlike other immune cells, MDSCs show immunosuppression rather than immunostimulation. In mice, phenotypically they are known to express CD11b and Gr1 (Ly6C and Ly6G). MDSC monocytic lineage have high Ly6C expression whereas MDSC granulocytic lineage have both Ly6C and Ly6G expression[83]. Solid tumors commonly produce GM-CSF, VEGF and TGFβ, which are known to influence polarization of myeloid cells towards an MDSC type and concurrently support expansion of MDSCs[84,85]. CCL2, CXCL8 and CXCL12 are produced by the tumor microenvironment to increase MDSC signature or accumulation; this has been linked with poor survival and worse prognosis in a variety of cancers[83]. Tumor supportive MDSCs suppress the tumoricidal function of NK cells, B cells and cytotoxic T cells. Additionally, they produce nitrate intermediate NO and ROS, which results in nitration of receptors necessary for infiltration and function of T cells and NK cells[83]. Furthermore, MDSCs also favor the generation of Treg cells, promote endothelial cell sprouting to form new blood vessels towards the tumor and additionally produce MMPs to assist in invading surrounding tissue for metastatic spreading[42,86-88]. They can also secrete IL10 and TGFβ proteins that can impair tumor-inhibiting immune response. At the same time, they overexpress PD-L1 ligand that bind to PD-1 receptor of T cells and thus mask T cell mediated cytotoxic response[83]. Depletion of MDSC via CSF1R inhibition have been shown to enhance the radiotherapy response in prostate cancer patients[89].

B cells are a category of lymphoid cells that primarily function by secreting cytokines, producing antibodies, and presenting antigen-antibody complex in response to immune signals[90]. Because of its antigen recognizing, processing, presenting, and antibody generating capabilities, it forms an integral part of the immune system. It can affect other major immune cells including NK cells, T cells and macrophages as well as the function of other cells which can determine the fate of tumor development[91]. Similar to other immune cells, B cells are also found in different subtypes, which allow interactions with multiple cell types. Increased B cell presence along with $CD8^+$ T cells has been linked with good prognosis in ovarian cancer[92]. However, the role of B cells in cancer is quite contradictory as different subtypes can show both tumor promoting and tumor suppressing activities[93,94]. It is now clear that similar to immunosuppressive T cell subtypes (Treg), B cells also have a Breg cell subtype that mainly exerts pro-tumorigenic effect[91]. Increased infiltration of Breg cells in tumors is associated with poor prognosis in lung, gastric and other cancers[95-97]. Breg cells are also known to produce TGFβ and IL10 that creates an immunosuppressive milieu ultimately favoring the conversion of $CD4^+$ T cells into Treg cells thus supporting metastasis[98]. B cells recruited to prostate tumors

have been shown to express lymphotoxin that promotes tumor progression[99]. Additionally, Breg cells also interact with tumor-infiltrated macrophages and polarize them towards tumor supporting macrophages[100].

Dendritic cells are the antigen presenting cells (APCs) commonly found in different cancers and inflammatory sites. The role of dendritic cells in cancer is controversial as it is proposed that they may change their functional abilities over the course of tumoral disease progression. In the early stages of cancer, they may portray immunostimulatory functions including antigen presentation, T cell activation and differentiation. But in the late stages of cancer they may be reprogramed to exert tumor supporting immunosuppressive functions[101]. As dendritic cells are APCs, they communicate with other immune cells such as NK cells and B cells[102]. In the tumor microenvironment, tumor cells can polarize classical dendritic cells into tumor supporting subtypes (regulatory dendritic cell), which express TGFβ to fuel immunosuppressive Treg cell proliferation[103,104]. In different cancers, distinct subsets of dendritic cells have been linked with disease outcome; these mostly include immature dendritic cells which lack immunostimulatory function[105]. Vaccination with distinct dendritic subtypes (cDC2s) results in tumor regression[106] and inhibition of PD-1 ligation on dendritic cells restores T cell killing capacities, opening up new promising paths for developing new therapeutics against cancer[102].

1.1.6 Fibroblasts in cancer

Fibroblasts were first identified in the tumor stroma and wounds by Dvorak[22]. From that time onwards, the importance of fibroblasts in the tumor stroma and tumor microenvironment came into light. Fibroblasts are multipotent cells; resident quiescent fibroblasts easily get recruited and activated by wound healing signals, as well as by tumor produced TGFβ and CXCL12 [107,108]. Fibroblasts commonly found in tumors are referred to as cancer associated fibroblasts (CAFs), and they constitute a major component of the tumor stromal compartment[108]. CAFs can be identified by their marked expression of αSMA, vimentin, FSP1, PDGFRα, PDGFRβ and DDR2[109]. They are known to produce important glycoproteins (laminin and fibronectin) and collagen I-IV that make up the ECM architecture of the tumor stroma[108]. On the other hand, they also produce ECM degrading MMPs that may allow epithelial to mesenchymal transition (EMT) events and enhance tumor invasion properties[110]. EMT is a process by which cancer cells can undergo multiple biochemical changes to acquire a mesenchymal cell phenotype to allow them to gain enhanced migratory and invasive characteristics[111]. Altered activation and pro-tumorigenic function of CAFs has been described in stomach cancer and breast cancer[109,112]. Moreover, proangiogenic VEGFA and VEGFC production by fibroblasts has

been associated to enhanced breast cancer cell dissemination to the lungs[113]. CAFs are known to suppress immune responses via expression of PDL1 and PDL2 to limit T cell function[114]. Moreover, the induction of several proangiogenic chemokines and cytokines such as CCL2, CCL5, CXCL9, CXCL10, TGFβ, TNF, IL4, IL6 and IL10 by CAFs also show its immunomodulatory capacities in the tumor microenvironment[108]. Exosomes derived from CAFs have been shown to promote tumor growth and drug resistance in colon cancer[115]. Furthermore, CAFs actively enhance chemoresistance in both breast cancer and prostate cancer[116,117].

1.1.7 Nuclear factor-kappa B

NFκB (Nuclear factor-kappa B) is a family of transcription factors that play a significant role in inflammation-facilitated immune response and tumor development[118]. In presence of parasites, microbes, pathogenic particles, tissue injury, or hypoxic environment NFκB gets activated via pro-inflammatory cytokines TNFα and IL1β. The NFκB family is composed of five group members (NFκB1, NFκB2, RelA (p65), c-Rel, and RelB) that can form homo- and heterodimers. Unlike other members, NFκB1 and NFκB2 are proteolytically modified and matured into p50 and p52 respectively. These two proteins form dimers and translocate to the nucleus, however, since they lack a transactivation (TA) domain they cannot contribute to NFκB target gene transcription, but rather act as transcriptional repressors of NFκB target genes[119,120].

NFκB activation is regulated by a family of inhibitors known as IκB (NFκBIA, NFκBIB, NFκBIE and BCL3). IκB can sequester NFκB dimers in the cytoplasm and prevent NFκB activation and translocation into the nucleus. A group of kinases referred to as IKK or IκB kinase (IKBkA/Chuck, IKBkB, IKBkG and IKBkE) indirectly regulate NFκB activation through phosphorylation of IκB, thus preventing it to bind NFκB[120]. In quiescent cells, NFκB proteins remain bound to inhibitory molecule IκB and reside in the cytoplasm. However, any signal leading to ubiquitination of IκB releases NFκB, which allows NFκB complex translocation into the nucleus to initiate transcription of target genes (IL6 and IL8)[121]. The binding of ligands like TNFα and CD40L to their cognate cell surface receptors leads to the activation of NFκB that is mainly mediated via two signaling pathways, known as the canonical (Classical) and non-canonical (Alternative) pathway (Figure 4). In the canonical pathway, NFκB activation involves phosphorylation of IκB by IKK complex, which results in ubiquitination followed by proteasomal degradation of IκB and allows p50/p65 nuclear translocation. Non-canonical NFκB activation signaling involves phosphorylation and

proteolytic modification of the larger precursor p100, which ultimately matures into the smaller precursor p52 and leads to nuclear translocation of the RelB/p52 heterodimer[122].

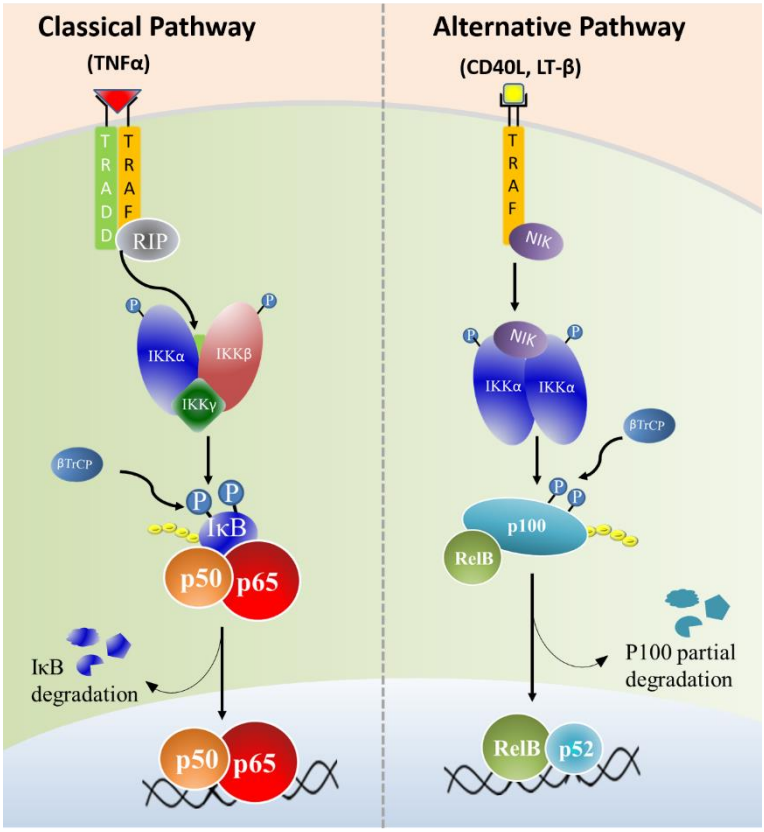

Figure 4. Classical and alternative NFκB signaling pathway.

NFκB inhibition confers reduced number of mammary stem cells, vascularization, macrophage recruitment and thus decreased progression of HER2-neu–induced breast cancer[123]. In the tumor microenvironment, NFκB inhibition not only impedes anti-apoptosis activities but also shifts the macrophage ratio towards M1 phenotype via suppressing M2 macrophage polarization and thus aids tumor regression[124,125]. Constitutive activation of NFκB is commonly found in many solid cancers where it supports tumorigenesis via transcription of anti-apoptotic genes, regulation of EMT, upregulation of MMPs, degradation of ECM, immune evasion, and metastatic spreading. Activation of NFκB as a result of mutations in the upstream IKK-NFκB pathway has also been reported to support growth of myeloma, skin cancer, lung cancer and additionally can reverse the macrophage polarization towards M2 type which later supports cancer progression[124,126-128]. NFκB activation may support cell proliferation and inhibit apoptosis of cancer stem cells through supporting a pro-inflammatory milieu[129,130]. Furthermore, NFκB has also been reported to control tumor angiogenesis via enhanced expression of VEGF and its relevant receptor[131-133]. Although NFκB activation has been

associated with tumorigenesis, in response to stress signals, NFκB activation can also inhibit tumor development through cancer cell killing via cytotoxic immune cell response[134].

1.1.8 Hypoxia and HIF1α

Hypoxia can be defined as a state in which the tissue is deprived of sufficient oxygen. In cancer, it is known to play an important role in tumor development and tumor angiogenesis. In most tumors, the oxygen diffusion limit is approximately 1-2 mm[135]; when the tumor grows beyond this limit, the central region of the tumor usually experiences a drop in oxygen levels. The tumor cells in this hypoxic core then adapt to express proangiogenic factors that promote angiogenesis by stimulating the proliferation and migration of endothelial cells to this region. Moreover, it has been shown to affect the infiltration of immune cells to the tumor, the hypoxic environment prevents NK cell recruitment to the tumor site and allows tumor cells to escape a cytolytic fate, whereas, TAMs are recruited to support tumor progression[135-137]. The hypoxic microenvironment also leads to the stabilization and activation of hypoxia inducible factor (HIF). HIF is a family of transcription factors that includes two basic helix–loop–helix-PAS (bHLH) proteins, HIFα and HIFβ. HIFα contains an alpha subunit (HIF1α, HIF2α and HIF3α) and HIFβ contains a beta subunit (HIF1β). In hypoxic conditions, members of these two subunits can form heterodimeric complexes that can translocate into the nucleus and transcribe genes containing a hypoxia response element in their promoter. Oxygen level is a determining factor for stability of HIFα subunits but not for HIF1β, which is constitutively expressed and more stable[138]. In general, HIFs are well known to regulate cell cycle, apoptosis, proliferation and expression of proangiogenic genes[139-141]. Under low oxygen condition, HIF1α can switch the metabolic pathway of cells from oxidative to glycolytic. Notably, HIF1α supports glycolysis via upregulating glycolytic enzymes and increasing the expression of glucose transporters (GLUT1 and GLUT3) to increase glucose uptake, and thus can support cancer cell survival[138]. HIF1α is the most well studied HIF family member, and has been shown to promote EMT whereas inhibition of HIF1α reduces migratory and invasive properties of cancer cells[135,142]. In breast cancer, HIF1α activation has been reported to be associated with increased metastasis and poor patient survival[143]. Additionally, loss of HIF1α in $CD4^+$/$CD8^+$ cells favors increased clonal expansion and expression of anti-tumorigenic IFNγ production[144].

The stability of HIF1α (Figure 5) mainly depends on its hydroxylation mediated by PHD (prolyl hydroxylase), which is a family of enzymes consisting of PHD1, PHD2 and PHD3. In normoxia, PHDs hydroxylates the proline residues P402 and P564 on HIF1α[145]. Following hydroxylation, a tumor suppressor adaptor protein pVHL (von Hippel-Lindau), that acts as a

substrate recognition component of an E3 ubiquitin ligase complex, binds to HIF1α. The E3 ubiquitin ligase complex includes other binding partners Elongin B and C, RING-box protein 1 (Rbx1) and Cullin 2 (Cul2) and together form the ECV complex, which catalyzes the polyubiquitination of HIF1α to allow for proteasomal degradation.

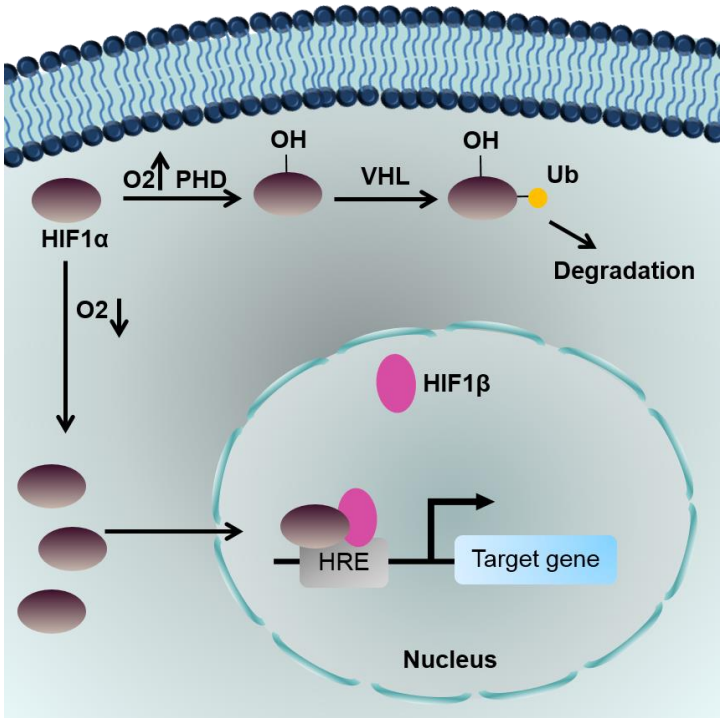

Figure 5. Schematic pathway of HIF1α activation and transcription of target gene.

Ubiquitination involves the collective action of three enzymes E1, E2 and E3. E1 activates ubiquitin and transfers it to E2. E3 conjugates with the ubiquitin bound E2 and finally transfers ubiquitin to target proteins[146,147]. Since pVHL plays a pivotal role in mediating HIF1α degradation in normoxic conditions, the loss or mutation of pVHL has been shown to lead to HIF1α activation and associated tumor development, most often in renal carcinoma[145].

PHD enzymes use molecular oxygen to hydroxylate the proline residue on HIF1α; low oxygen conditions prevent PHD's catalyzing activity, resulting in HIF1α stabilization, accumulation and translocation to the nucleus (Figure 5). After nuclear translocation, HIF1α forms a heterodimer with HIF1β that binds to the hypoxia response elements (HRE) and induces subsequent transcription of target genes related to apoptosis, metabolism, survival and angiogenic factors such as VEGF, FGF2, TNFα and CXCL8[137,148] (see Table 3 for additional target genes). Similar to HIF1α, HIF2α is also regulated in an oxygen dependent manner whereas HIF3α is known to be a repressor of both HIF1α and HIF2α[77]. Interestingly, HIF1α

may even be activated in absence of hypoxia through inflammatory NFκB signaling; moreover, NFκB has also been reported to be activated by HIF1α[149,150].

Table 3. HIF1α target genes regulate different biological pathways

HIF1α target genes	Biological pathway	HIF1α target genes	Biological pathway
VEGF	Angiogenesis	KRT 14, 18, 19	Invasion and metastasis
VEGFR		VIM	
ENG		MIC2	
LEP		MMP2	
ADM		PAI1	
LRP1		CATHD	
NOS2		FN1	
TGF-β3		Collagen type V (α_1)	
Cyclin G2	Growth and survival	GAPDH	Metabolism
TGF-α		GLUT1, 3	
EPO		HK1	
IGF2		HK2	
NIP3		ENO1	
NIX		LDHA	
ET1		PKM	
ADM		PGK1	
WAF-1		LEP	

1.2 P53 FAMILY OF TRANSCRIPTION FACTORS

The p53 family of transcription factors consists of the tumor suppressor p53 and its paralogs p63 and p73. All family members share structural homology in the transactivation domain (TAD), oligomerization domain (OD), and highest degree of homology in the DNA binding domain (DBD), suggesting that all p53 paralogs can bind to the same target gene promoters.

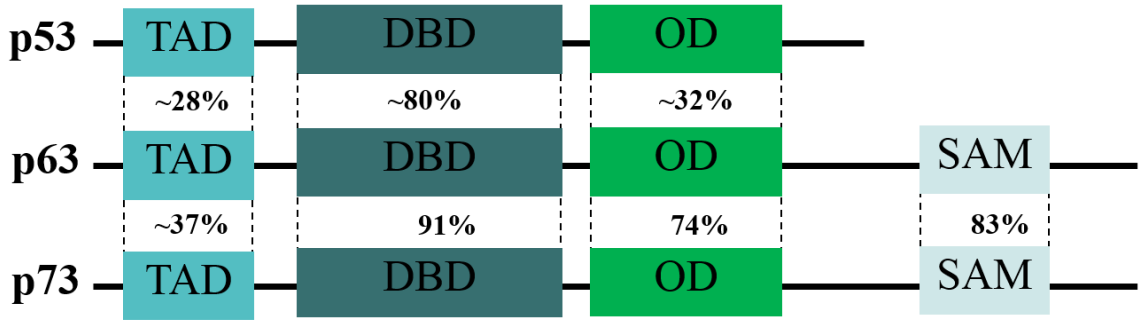

Figure 6. Structural similarities among p53 family members (percentage refers to amino acid sequence identity).

Additionally, p63 and p73 also have a sterile alpha motif (SAM) domain, which is absent in p53 (Figure 6). Nine different mRNA isoforms are known to encode p53, as a result of alternative splicing, alternative promoter usage and additionally alternate translation initiation sites which lead to the translation of around 12 distinct protein isoforms[151]. Unlike p53, p63

and p73 are mainly expressed in different isoforms using two different promoters and alternative splicing at the c-terminal domain. Considering p53's tumor suppressive role and structural homology, other family members have been speculated to have similar function.

In a vast majority of cancers, p53 is frequently found to be mutated whereas mutation in p63 and p73 is quite rare. Notably, p53 has been referred to as 'the guardian of the genome' due to its ability to prevent cancer development by protecting the genome from genotoxic stress[152]. As a tumor suppressor, wild type p53 regulates genomic integrity, cell cycle checkpoints, proliferation, differentiation, senescence and cell death mechanisms including apoptosis, whereas mutant p53 exerts a tumor promoting function[153]. Intriguingly, p53 knockout (KO) mice predispose to spontaneous tumor development whereas mice deleted for the p63 gene show abnormalities in limb and skin development. Mice that lack all p73 isoforms, mainly show developmental problems related to the brain[154-156]. The phenotypes of p73 isoform specific knockout mice are discussed in section 1.3.3 'Role of p73 in development and other disease'.

1.3 THE P73 GENE

1.3.1 Discovery of p73

Mourad Kaghad et al. first discovered TP73 gene in 1997 and reported it to be located on chromosome 1p36, a region commonly deleted or silenced in pancreatic cancer, neuroblastoma, melanoma, breast carcinoma and hepatocellular carcinoma, suggesting this gene's potential role as a tumor suppressor[157]. The TP73 gene spans 65 kb composed of 14 exons; being a p53 homolog, p73 can transcribe similar genes including those related to cell cycle checkpoints and apoptosis, thus demonstrating tumor suppressive function when p53 is not functional or absent in cells[158,159]. Unlike p53, p73 is rarely mutated in cancer suggesting an 'alternative backup' by the cell to avoid malignancy[159]. Despite having tumor suppressive function, p73 also possesses other functions in metabolism, fertility and neuronal biology[160-162].

1.3.2 P73 and its isoforms

The role of p73 as a tumor suppressor is more complicated than originally thought due to the opposing functions of its isoforms. The p73 gene expresses two different categories of proteins; full-length isoforms (TAp73) that act as transcription factors and N-terminal truncated variants (ΔNp73) that lack the TAD resulting in transcriptionally inactive isoforms which instead function in a dominant negative manner like oncogenes. These isoforms mainly arise due to

the usage of two alternate promoters; the upstream P1 promoter generates TAp73 isoforms, whereas the downstream P2 promoter yields the ΔNp73 isoforms. To add more complexity, mRNA splicing at exon 2 and/or 3 gives rise to more isoforms including ΔEx2p73 and ΔEx2/3p73, which exert functions similar to the ΔNp73 isoforms, whereas, alternative splicing at the C terminal end results in p73 isoforms denoted as α, β, γ, δ, ε, ζ and η (Figure 7). The full length α isoform is the longest with a SAM domain while the δ isoform is the shortest[163]. In-silico analysis predicted the presence of even more isoforms, however whether they are transcribed as proteins is still unclear[164].

TAp73 is often silenced due to promoter hyper-methylation in many hematological cancers, whereas oncogenic ΔNp73 is dominantly overexpressed in many types of solid cancers and is significantly associated with poor patient prognosis. In line with this, full-length TAp73 isoforms are reported to portray tumor suppressive function whereas ΔNp73 exerts tumor promoting function[165,166]. Therefore, a balance of these isoforms is very crucial in tumorigenesis as is portrayed in acute promyelocytic leukemia (APL), where a high ΔNp73/TAp73 ratio in favor of ΔNp73 correlates with shorter overall survival[167].

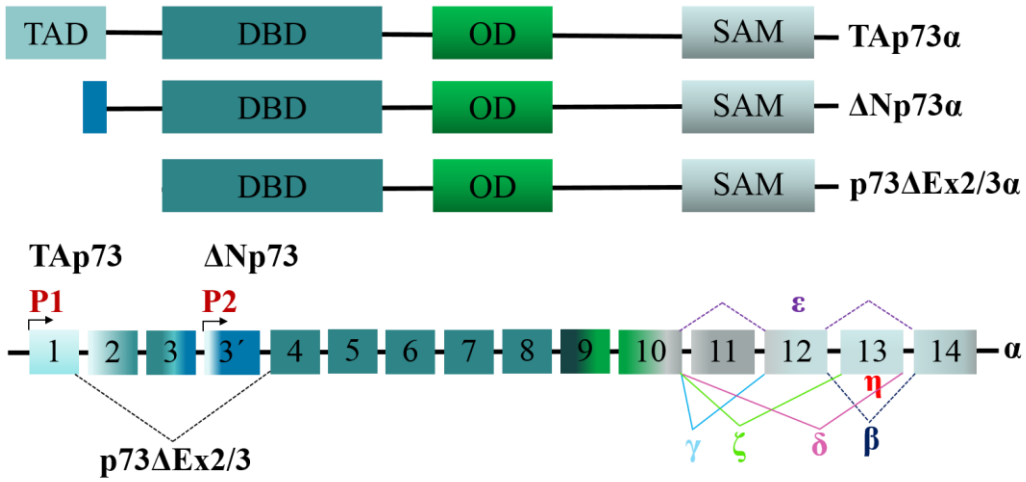

Figure 7. Schematic map to denote different p73 isoforms and distinct promoters.

1.3.3 Functional role of p73 isoforms

In cell cycle and checkpoints

Cell cycle regulatory proteins are actively involved in monitoring different checkpoints during cell division. In cancer or cell transforming events, these regulatory proteins, or in other words tumor suppressor proteins, are lost, silenced, or mutated, resulting in uncontrolled cell division and proliferation. Under normal conditions, cell cycle progression is strictly controlled and advanced through four different (G1, S, G2 and M) phases under the regulation of a group of

serine/threonine kinases known as cyclin-dependent kinases (CDKs) and their activation modulatory subunit cyclins[168]. In this regard, CDKs and cyclins form complexes that phosphorylate target proteins in order to allow cell cycle to proceed; in case of TAp73, cyclin A/CDK1/2, cyclin B/CDK1/2 and cyclin E/CDK2 complexes phosphorylate TAp73 and in turn represses p73's target gene expression[169]. Upon genotoxic stress signals, TAp73 actively facilitates cell cycle arrest in G1 and G2/M checkpoints via transactivation of the p53 target gene p21 and GADD45[170,171]. Moreover, TAp73 has also been shown to accumulate in genomic stress or DNA damage and induce upregulation of its pro-apoptotic target gene Bim, which in turn induces mitotic cell death to prevent any anomalies during mitosis[172,173]. In addition, TAp73 also regulates the mitotic phase of cell division via controlling spindle assembly checkpoint (SAC) complex proteins Bub1, Bub3 and BubR1. Conversely, loss of TAp73 leads to genomic instability resulting in aneuploid cells and lung carcinoma in mice[174].

Transformation / oncogenesis

N-terminal truncated p73 isoform ΔNp73 and ΔNp73-like ΔEx2/3p73, favor oncogenic transformation in mice. Tumorigenicity is promoted via upregulation of ΔN isoforms or via coordinating together with other oncogenes (E1A, Ras and Myc)[175,176]. On the contrary, TAp73 prevents anchorage independent growth whereas oncogenic Ras downregulates TAp73 to favor cellular transformation[175]. In support of proliferative advantage, ΔNp73 may also facilitate phosphorylation-mediated Retinoblastoma (Rb) protein deactivation, which in turn results in increased E2F signals and proliferation of fibroblasts[177].

Apoptosis and cell death

Apoptosis or programmed cell death is a tightly regulated process and crucial for embryonic development and to maintain cellular homeostasis. Apoptotic cells acquire distinctive features including cell shrinkage, blebbing, chromatin condensation, mitochondrial swelling and fragmentation of nuclear DNA. Apoptosis can be mediated through two pathways, the extrinsic and intrinsic pathways. The extrinsic or death receptor-mediated pathway gets activated upon ligation of extracellular proteins to the death receptor, which subsequently leads to the activation of initiator caspase 8. The intrinsic or mitochondrial mediated death pathway is accompanied by defective mitochondrial membrane potential which instigates cytochrome c release and subsequently initiator caspase 9 activation. Activation of the initiator caspases leads to activation of a cascade of effector caspases (caspase 3, 6 and 7). Effector caspases can cause cleavage of a wide variety of cellular targets including cytoskeletal scaffold proteins, DNA repair proteins and housekeeping enzymes. They can also activate the DNAse activity of CAD

(caspase activated DNAse) by cleaving ICAD (inhibitor of caspase activated DNAse) that can fragment DNA to cause cell death[178,179].

The p73 isoform, TAp73 is known to regulate apoptosis via transcription of several pro-apoptotic genes similar to p53. In contrast, ΔNp73 competes with TAp73 and/or p53 for binding to the same target genes resulting in impaired TAp73 and p53-mediated transcription and ensuing anti-apoptotic function. TAp73 can facilitate cell death through both the extrinsic and intrinsic apoptotic pathways. Similar to p53, p73 activation upregulates death receptors CD95, TNF-R1, TRAIL-R1 and TRAIL-R2 along with caspases-3, -6 and -8 activation, which demonstrates TAp73's role in the extrinsic apoptosis pathway. In contrast, ΔNp73 can block the transcriptional activity of TAp73 and p53, thus inhibiting their pro-apoptotic function and attenuating cell death[180-185]. TAp73 can also actively drive mitochondrial-mediated apoptosis via transactivating pro-apoptotic BH3-only proteins (BAX, NOXA, PUMA, BAD and BIK) and other apoptotic cofactors (p53AIP1, PERP and RanBP9)[158,186].

Autophagy and senescence

Autophagy is a stress-induced mechanism of non-apoptotic cell death via lysosomal degradation of the intracellular organelles and other cellular contents. However, it is known to be engaged in both tumor promotion and suppression depending on the environment of resident cells[187]. TAp73 regulates expression of DNA damage regulated autophagy modulator 1 (DRAM), but surprisingly, TAp73-mediated formation of autophagosome resulting in autophagy was found to be independent of DRAM activation. In the same study, there was no effect of ΔNp73 on starvation-induced autophagy[188]. In another study, TAp73 was shown to transactivate autophagy related gene and protein ATG5 expression independent of p53[189]. In addition, knockdown of p73β significantly reduces expression of autophagy markers in breast cancer cells[190].

TAp73 has been shown to have contradictory roles in senescence. As a tumor suppressor, TAp73 and its target gene p21 cooperates with RNPC1 to regulate premature cellular senescence in absence of p53; however, Du et al. showed that TAp73 depletion confers cellular senescence[162,191]. In agreement with the latter study, another report showed that TAp73 negatively regulates cellular senescence through Cytochrome C oxidase subunit IV (Cox4i1) activity and ROS generation[192].

In metabolism

p73 and its isoforms play a vital role in regulating cellular metabolism. In a study investigating mouse primary cortical neurons, it was shown that p73 isoforms differentially affect the glycolysis pathway, which is essential for the survival of neuronal cells; loss of TAp73 enhanced glycolysis with increases in intracellular glucose uptake, whereas ΔNp73 deficiency decreased glucose metabolism[193]. Loss of TAp73 can also affect cellular bioenergetics through mitochondrial dysfunction. A study has shown that TAp73 knockout cells have lower basal ATP levels as a result of impaired oxidative phosphorylation; in support of the previous study, decreased oxidative phosphorylation is thought to be linked to glucose addicted cells with increased glycolysis activity. The mechanism by which TAp73 is thought to control mitochondrial function is through the transcription and expression of Cox4i1, and loss of TAp73 leads to reduced expression of Cox4i1 which in turn compromises the activity of complex IV of the electron transport chain leading to oxidative imbalance, ROS generation and consequential mitochondrial dysfunction[192].

In angiogenesis

Angiogenesis is one of the most preliminary steps tumor cells adopt to feed the tumor niche with oxygen and nutrients and to aid evasion from the primary tumor site. To promote angiogenesis, tumor cells produce chemokines and cytokines that induce blood vessel sprouting towards the tumor site. Studies in mouse models have shown that TAp73 suppresses carcinogen-induced tumors and tumor vascularization[194] whereas hypoxia induced ΔNp73 inversely promotes tumor angiogenesis via regulation of VEGF-A[195]. Additionally, ΔNp73 was shown to control the migration and vascular network forming capacities of endothelial cells through the regulation of VEGF and TGFβ signaling[196].

In metastasis

Metastasis can be defined as the dissemination of cancer cells to distant organs. Prior to metastasis, tumor cells acquire invading or migratory phenotypes through reprogramming from epithelial to mesenchymal state that aid cancer cells to propagate more easily to distant sites. The p73 isoform TAp73 can inhibit the migratory property of tumor cells through the regulation of a number of factors. TAp73 can transactivate the microRNAs miR-34a and miR-3158, which in turn downregulate the EMT associated targets vimentin, β-catenin and lef1[197]. In a study conducted by Rodhe et. al., it was shown that TAp73β can induce p57 (Kip2) expression which in turn inhibits the actin cytoskeleton dynamics of tumor cells thereby reducing their motility[198]. The inhibitory role of TAp73 in cell migration and invasion has also

been portrayed via its induction of forkhead transcription factor (FOXF1), which in turn regulates the transcriptional activity of E-cadherin. E-cadherin is known to maintain cell polarity and epithelial structure, and its decrease may promote tumor cell motility and invasion[199]. In pancreatic carcinoma, TAp73 was shown to control TGF-β signaling to block epithelial to mesenchymal transition whereas its loss led to increase in EMT events[200]. Selectively TAp73 loss, but not ΔNp73, exhibits increased migratory capacities and EMT phenotypes with increased expression of Snail-1, Slug and Twist coupled with downregulation of E-cadherin by MCF10A cells[201,202]. In contrary to TAp73 function, ΔNp73 has been shown to support tumor invasion and metastasis cascades through EPLIN-mediated IGF1R regulation[203]. In addition, ΔNp73 like splice variants ΔEx2/3p73 was also shown to correlate with lymph node metastasis in colon tumors[204].

Role of p73 in drug resistance

Among the p73 isoforms oncogenic ΔNp73 is frequently involved in cancer drug resistance. High expression of ΔNp73 has been linked to anti-apoptotic function, which drives daunorubicin resistance in childhood acute lymphoblastic leukemia (ALL)[205]. ΔNp73 can selectively block miR-205 activity and limit its inhibitory function on anti-apoptotic Bcl-2 and ABC transporters including ABCA2 and ABCA5, which together lead to multi-drug resistance by malignant melanoma cells[206]. ABC (ATP-binding cassette) transporters are a group of 49 ABC genes categorized into seven subfamilies designated as A to G based on structure and sequence homology. They are transmembrane proteins that regulate influx and efflux of different molecules, including drugs and other small molecules, through the cell's cytoplasmic membrane[207]. Interestingly, cancer cells take advantage of overexpressing these transporters to facilitate increased efflux of drug molecules. Among the ABC transporters, ABCB1/MDR1 and ABCB5 have been well studied for exerting multidrug resistance in different types of cancer[208,209]. ΔNp73α can upregulate ABCB1/MDR1 expression by inhibiting p53 function to potentiate the efflux capacity of gastric cancer cells[210,211].

Role of p73 in development and other disease

Apart from its role in cancer, the function of p73 has also been explored in developmental processes as well as in other pathologies. p73 plays a major part in neurobiology in which case it maintains neuronal stemness and CNS neurogenesis[212]. Impairment of p73 function has been linked with neurodegeneration in association to Alzheimer's disease[213]. Apart from this, p73 can also actively regulate keratinocyte differentiation factor as well as osteoblast and myoblast differentiation[214-216]. It is also known to regulate liver metabolism and proliferation of cardio-

myocytes[217,218]. Loss of p73 has been reported to abrogate self-renewal and differentiation capacity of mouse neuro-progenitor cells[216]. In disease, p73 is involved in inflammatory disorders such as atopic dermatitis (AD) and gastritis[219]. In the study of p73 knockout mouse models, it has been shown that lack of expression of all p73 isoforms display severe developmental abnormalities including immunological, pheromone sensing, hippocampal dysgenesis, enlarged ventricles, and sympathetic neuronal loss. On the other hand, TAp73 and ΔNp73 knockout mice exhibit fewer developmental anomalies; similar to p73 KO mice, ΔNp73 KO mice display enlarged ventricles and loss of neurons, whereas TAp73 KO mice display hippocampal dysgenesis. Interestingly, TAp73 KO mice also develop spontaneous lung cancer suggesting TAp73's role as a bona fide tumor suppressor[166,220]. Additionally, TAp73 distinctly controls both male and female reproduction capacity[166,221], and its deficiency may accelerate aging and irregularities in respiratory airway multiciliogenesis[192,222].

1.3.4 P73 regulation

Transcriptional regulators of p73 that influences its activity

Epigenetic regulation has an important contribution in the expression of different p73 isoforms and their respective functions. The P1 promoter contains three CpG methylation islands whereas the P2 promoter only has one; this suggests that methylation greatly influences the transcription and expression of TAp73[223]. Methylation patterns may shift the balance of p73 isoforms leading to the repression or accumulation of particular isoforms. P1 promoter hypermethylation results in silencing of TAp73 expression in chondrosarcoma[224]. Notably, such events are frequently observed in hematological cancers[225-227]. However, the causal role or interlinked relationship of methylation pattern of P1 promoter and hematological cancers is yet to be understood. Interestingly, hypermethylation of the promoter can prevent binding of transcriptional repressors allowing constitutive expression of a particular gene. As such, hypermethylation of P1 promoter blocks binding of transcriptional repressor ZEB1 to the P1 promoter allowing enhanced TAp73-mediated target gene expression in ovarian cancer[228]. In contrast, P2 promoter hypomethylation leads to increased ΔNp73 expression, which primarily exhibits oncogenic function, and such events are commonly seen in solid cancers[229,230].

Important functions and expression of TP73 gene depends on upstream pathways or effectors that can induce or repress distinct promoters that subsequently result in the differential expression of certain isoforms. The upstream region of the human p73 gene promoter is known to have regulatory binding sites for Egr-1,2,3, Spl, and AP-2[231]. Additionally, it also contains three putative binding sites for apoptosis inducer E2F1 transcription factor[232]. Egr-1 has been

reported to bind to five different binding sites in the upstream P1 promoter of the p73 gene. Binding to the P1 promoter leads to the transcription of TAp73 isoform, and this in turn can enhance apoptosis and repress tumor cell growth. However, such effects have not been observed for the P2 promoter or P2 promoter derived ΔNp73[232,233]. Similarly, E1A also selectively transactivates P1 promoter driven TAp73 expression but not ΔNp73[234]. Sp1 binding sites have been shown to be present in both the P1 and P2 promotors; although Sp1 binding to P1 promoter positively regulates TAp73 expression, no such direct interaction was found for P2[235].

The Zinc Finger protein (ZEB) can directly bind to p73 genes and transcriptionally repress p73 expression[214]. ΔNp73 promoter contains a putative binding site for the tumor suppressor HIC1 (Hypermethylated In Cancer) and binding of HIC1 to the P2 promoter portrays transcriptional repression of ΔNp73 isoform in gastric cancer[236]. To add more, oncogenic c-MYC physically interacts with TAp73, which indirectly prevents TAp73-mediated target gene BAX activation[237]. Interestingly, both TAp73 and p53 are found to transactivate transcription of P2 derived ΔNp73 isoform which in turn inhibits their activity, suggesting a negative feedback loop or dominant negative property of oncogenic ΔNp73[238-241] .

Regulators that increase p73 stability

Post-translational modification plays a very crucial part in determining p73 stability, degradation and biological function. In DNA damage stress responses, physical interaction of p73α with Pin1 and acetylation of p73α by p300 increases p73α's stability followed by increased apoptotic functions[242,243]. c-Jun N-terminal kinase (JNK) and checkpoint kinase 1 (Chk1) driven phosphorylation enhances p73α target gene transcription to mediate cell cycle arrest or cell death in response to any genotoxicity[244,245]. In parallel, c-Abl, a non-receptor tyrosine kinase, also phosphorylates p73 proteins to increase stability of both TAp73 and ΔNp73 isoforms[246]. Moreover, PIAS-1 sumoylates p73α to potentiate p73 stability, but surprisingly blocks its transcriptional activity[247]. p73 has also been shown to induce the Mdm2 promoter to increase Mdm2 expression which later interacts with p73α. However, Mdm2 does not cause p73 degradation, but rather blocks its transcriptional activity[170,248,249]. Inhibition of MDM2 and its human homolog HDM2 via Nutlin-3 can release TAp73 from Mdm2 and induce TAp73 mediated transcription[250,251]. Phosphorylation of TAp73 on threonine residue 86 by Cyclin/CDK complex and HCK (Src family kinase) leads to repression of p73 transcriptional activity[169,252].

Regulators that influence p73 degradation

The degradation of p73 is usually regulated by ubiquitin proteasome dependent and independent mechanisms[253]. ΔNp73 selectively undergoes proteolytic degradation induced by PIR2 (p73 induced ring finger protein 2), a ubiquitin protein ligase and target gene of p73[254]. In line with this, PML (promyelocytic leukemia protein) catalyzes p300-associated p73 acetylation, which decreases p73 ubiquitination and proteasomal degradation[255]. In cancer, the FBXO45 ubiquitin ligase is upregulated in cancer cells to facilitate proteasomal mediated TAp73 degradation leading to impaired cell death response[256], while SUMO-1, a ubiquitin modifier, can accelerate p73-targeted proteasomal degradation processes[257]. In this regard, chemotherapy induced stress increases pro-survival kinase PLK3 expression to abrogate apoptosis events via facilitating phosphorylation of TAp73 resulting in reduced TAp73 protein stability[258]. Apart from ubiquitin ligases, both TAp73 and ΔNp73 can also be cleaved and degraded by calpains[253].

1.3.5 Clinical importance of p73 as a target for cancer therapy

Isoform specific knockout studies show differential roles of p73 isoforms in tumor development, where TAp73 loss promotes tumor development and ΔNp73 depletion shows impaired tumor development[166,174,220]. In a dominant negative fashion, ΔNp73 can form heteromeric complexes with TAp73 and also compete with p53 for binding to the target gene promoter, thus suppressing their transcriptional activity. On the other hand, ablation of ΔNp73 releases both p53 and TAp73 to exert their subsequent apoptotic function[259]. Thus, maintaining a balance between these two p73 isoforms predicts tumor development and response to chemotherapy. In this regard, increasing the ratio of TAp73/ΔNp73 favors apoptotic signals, chemosensitivity and good prognosis[183]. In contrast, increasing the ratio of ΔNp73/TAp73 correlates with reduced survival, increased chemoresistance and increased events of disease relapse[167,183]. Interestingly, TAp73 loss is shown to correlate with EBV-mediated gastric cancer; and in pancreatic cancer TAp73 loss activates TGF-β signaling to promote EMT functions[200,260]. ΔNp73 has been shown to favor cancer stemness via IGF1R signaling in melanoma and lung cancer[261]. Additionally, ΔNp73 upregulation is associated with poor prognosis in neuroblastoma, breast cancer, cervical cancer and lung cancer patients[230,262-264]. Moreover, higher expression of ΔNp73 together with mutant p53, is linked to poor survival and decreased clinical responsiveness to platinum-based therapy in ovarian cancer[265]. Overexpression of ΔNp73-like p73ΔEx2/3 variants are also aberrantly expressed in cancer and portray drug resistance and poor prognosis in different malignancies including breast cancer, colon cancer and in metastatic melanoma[204,266-268].

As mentioned above, ΔNp73 is shown to be associated with therapeutic failure in different cancers where it mainly contributes to prevent apoptosis. Inhibition of overexpressed ΔNp73 indirectly favors the balance towards TAp73 that in turn induces expression of apoptotic genes followed by apoptosis events. In line with this, inhibition of p73ΔEx2/3 isoform results in regression of melanoma growth, suggesting its tumor-promoting function[269]. Additionally, ΔNp73 and ΔNp73 like p73ΔEx2/3 variant upregulation benefits cancer cells with multi-drug resistance characteristics via inducing expression of multi-drug resistance genes (ABCA2 and ABCA5), thus protecting cancer cells from drug induced cell death[206]. Importantly, chemotherapeutic treatment frequently results in activation of TAp73 to induce apoptosis, however, cancer cells tend to harbor increased anti-apoptotic p73 counterparts to block TAp73 and p53 function to support ongoing oncogenesis[270].

2 AIMS

The overall purpose of this thesis was to decipher novel roles of p73 isoforms in different processes and/or components of tumor development involving tumor angiogenesis, multi-drug resistance and regulation of tumor microenvironment.

Specific aims of the thesis:

Paper I - To investigate the role of p73 isoforms in regulation of pro-angiogenic factors and tumor angiogenesis.

Paper II - To study the role of TAp73 in regulating the chemokine CCL2 and its impact on tumor microenvironment.

Paper III - To uncover the molecular mechanisms by which ΔNp73 affects the protein stability of HIF1α.

Paper IV - To elucidate the involvement of ΔNp73 in conferring multi-drug resistance in cancer.

3 RESULTS AND DISCUSSION

3.1 TAP73 SUPPRESSES TUMOR ANGIOGENESIS THROUGH REPRESSION OF PROANGIOGENIC CYTOKINES AND HIF-1A ACTIVITY (PAPER I)

P73 encodes two main class of proteins of which TAp73 is the full-length isoform that contains transactivation (TA) domain and the NH2-terminal truncated shorter isoform ΔNp73 that lacks the TA domain. ΔNp73 possesses oncogenic properties whereas TAp73 functions similar to archetypical p53 by showing cell cycle arrest, apoptosis, tumor suppression and maintaining genomic stability. Among the p53 family members, tumor suppressor p53 is reported to regulate tumor angiogenesis via transcriptional repression of proangiogenic factors[152]. However, the role of p73 and its isoforms in tumor angiogenesis was not well studied at the time we conducted this investigation.

To address the role of p73 isoforms in tumor development, we took advantage of using $TAp73^{+/+}$ (WT), $\Delta Np73^{+/+}$ (WT), $TAp73^{-/-}$ (KO) and $\Delta Np73^{-/-}$ (KO) mouse models from which we isolated primary mouse embryonic fibroblast (MEFs). We transformed the MEFs with E1A/H-Ras^{V12} and used these cell lines in the current study as well as in **Paper II** and **III**. We found no difference in cell proliferation between TAp73 WT and KO MEFs, but upon TAp73 loss cells acquired increased anchorage-independent growing capacity, suggesting their enhanced potential to grow as tumors. The knockdown of TAp73 in BJ-TE fibroblasts was also reported to show similar accelerated anchorage-independent growth capacity[175]. We next injected TAp73 WT and KO MEFs in immunocompromised nude mice and found that TAp73 KO MEFs grow bigger tumors with shorter latency compared to TAp73 WT tumors. This is in agreement with the findings shown by Tomasini et al. where TAp73 deficient mice experience spontaneous lung cancer[166]. Interestingly, TAp73 KO MEFs tumors were very reddish in appearance, suggesting increased blood vessels or vascularization; this was confirmed by an endothelial cell marker, endomucin, staining. In contrast, N-terminal truncated ΔNp73 KO tumors showed decreased endomucin staining indicating ΔNp73 as a positive modulator of tumor angiogenesis. Moreover, studying B-cell lymphoma tumors arising from transgenic $TAp73^{-/-}$/EμMyc and $\Delta Np73^{-/-}$/EμMyc mice again confirmed similar increases in endomucin positive blood vessels upon TAp73 loss whereas ΔNp73 deficiency reduced tumor vasculature. Similar to our study, Amelio et al. showed that TAp73 deficiency enhances carcinogen-induced tumor progression and tumor angiogenesis[194], whereas Dullo et al. reported that ΔNp73 enhances tumor angiogenesis[195].

A common trait of all tumor cells is the ability to produce proangiogenic factors that in turn increase blood vessel formation and promote tumor angiogenesis. To investigate whether the effect of p73 isoforms in tumor angiogenesis is indeed due to factors produced by the tumor cells and not merely tumor size, we used a short term Tg(fli:EGFP) transgenic zebrafish xenograft model where we injected TAp73 WT and KO MEFs in zebrafish embryo. Due to the transparency of the model and ease of visualization of the developing vasculature that expresses GFP (green fluorescent protein), the interactions between tumor cells and the endogenous endothelium can be easily followed *in vivo*. TAp73$^{-/-}$ cells induced increased endothelial sprouting towards the injected cells, whereas ΔNp73$^{-/-}$ cells caused significant reduction in endothelial sprouting compared to WT. This data highlighted the differential role of these two isoforms in regulation of tumor angiogenesis.

Morphologically, tumor induced blood vessels are very irregularly branched and markedly lack pericytic coverage that allows high vascular permeability or leaky tumor vessels[271]. To check whether the MEF generated tumors also portrayed vascular permeability we injected high molecular dextran prior to tumor isolation. We observed extravascular dextran leakage, suggesting increased vascular permeability, in TAp73 KO tumors compared to WT. Abnormal tumor blood vessels are also characterized by reduced cell-cell contacts and junction molecules like VE-cadherin[272]. Tumor produced angiogenic factors like VEGF, FGF and angiopoietin have been reported to portray similar reduction of VE-cadherin junctions and leaky vessels which are known to support tumor cell extravasation and metastasis[272,273]. Therefore, we checked whether TAp73 KO MEFs produced factors that could impair VE-cadherin junctions in endothelial cells by adding conditioned medium (CM) from MEFs exposed to hypoxia on primary human dermal endothelial cells (HuDMEC). Interestingly, we found prominent disruption of cell-cell contact together with reduction of VE-cadherin at endothelial cell junctions when the HuDMECs were exposed to TAp73 KO CM. Taken together, this indicated to us that TAp73 deficiency stimulates tumor cells to produce proangiogenic secretomes that induce endothelial cell sprouting and vessel permeability.

To determine which angiogenic factors are differentially expressed to support enhanced tumor angiogenesis in TAp73 KO tumors, we performed a PCR based angiogenic gene expression array using TAp73 WT and KO tumors. Notably, we found that 12 proangiogenic genes (Col18a1, Fgfr3, Cxcl1, Cxcl2, Ccl2, IL1b, IL6, Vegfc, Ereg, Tnfaip2, MMP19, and Thbs2) were significantly upregulated upon TAp73 loss and one gene, brain specific angiogenesis inhibitor (Bai1), was significantly downregulated. Some angiogenic genes (Cxcl1, Cxcl2,

Ccl2) that we found upregulated in TAp73 KO tumors are also known to have pro-inflammatory function via recruiting immune cells. The effect of Ccl2 in promoting immune cell infiltration in TAp73 deficient tumors has been investigated in detail in **Paper II**. We also found upregulation of Vegfc in TAp73 KO tumors, which may explain how TAp73 deficiency results in increased vascular permeability and reduces VE-cadherin junction molecules. VEGF-C is a classical lymph-angiogenic factor that drives endothelial sprouting or angiogenesis via binding to the angiogenic receptor VEGFR-2 expressed by endothelial cells and is known to mediate increased permeability by reducing the junction protein VE-cadherin[274,275]. Interestingly, we also show that TAp73β and p53 directly upregulates Bai1, whereas ΔNp73 inhibits its expression. Bai1 is a transmembrane protein that is cleaved into vaculostatin, a well-known angiogenic inhibitor[276].

The regulation of 9 genes (Cxcl1, Cxcl2, Ccl2, IL1β, IL6, Vegfc, Ereg, Tnfaip2 and Bai1) was further validated via qRT-PCR in TAp73 WT and KO tumors, which confirmed similar regulation; moreover, these genes were oppositely expressed in $\Delta Np73^{-/-}$ tumors. This once again suggested that TAp73 and ΔNp73 might differentially regulate expression of angiogenic genes. Isoform specific regulation of angiogenic genes was also confirmed *in vitro* using TAp73 KO MEFs and ΔNp73 KO MEFs compared to their WT counterpart, and interestingly the regulation of the genes was further potentiated when cells were exposed to hypoxia. This result suggested that expression of angiogenic genes is indeed from the tumor cells and not merely from the stroma and that p73 is involved in regulation of hypoxia-induced angiogenic gene expression.

Next, we validated our findings in human breast cancer and lung cancer cell lines in which knockdown of TAp73 led to increased expression of angiogenic genes, which again was further elevated in hypoxia. Considering the involvement of hypoxia in regulation of proangiogenic genes that we observed in our results, we looked into the TCGA breast cancer patient sample dataset to identify whether we could recapitulate similar gene signatures linked to the p73 isoform. However, we focused our dataset analyses only for ΔNp73 and not TAp73, considering that ΔNp73 is the most abundantly expressed isoform in cancer[164]. Moreover, higher expression of ΔNp73 has also been correlated with worse prognosis in breast cancer[204]. Therefore, we separated the patient groups based on high expression or no expression of ΔNp73 and performed GSEA. Interestingly, we found that hypoxia and angiogenesis genes were highly enriched in patients who had high expression of ΔNp73.

Most solid tumors experience hypoxic environments after the tumor size reaches a certain limit, and this hypoxic event induces the expression of HIF1α, a central regulator that controls distinct biological pathways including angiogenic gene signature. Upon hypoxia, HIF1α is stabilized and translocated into the nucleus where it binds to Hypoxia Response Elements (HRE) and confers transcription of target genes. Therefore, we looked in depth to see whether the upregulated proangiogenic genes in TAp73$^{-/-}$ cells have a HRE in their gene promoter; indeed, we found that all the genes had HRE's in their promoters. By performing ChIP for HIF1α with TAp73$^{-/-}$ MEFs grown in hypoxia, we discovered that HIF1α actively binds to these promoters, except for Cxl2, to activate transcription of the proangiogenic genes. This was further confirmed upon siRNA-mediated HIF1α knockdown in TAp73$^{-/-}$ MEFs grown in hypoxia, which resulted in decreased expression of proangiogenic genes. Together, our data demonstrated that TAp73 represses HIF1α dependent expression of proangiogenic genes. Additionally, we investigated whether TAp73 knockdown or overexpression could influence HIF1α levels. We found that TAp73$^{-/-}$ MEFs had increased HIF1α protein accumulation, whereas the opposite was observed in ΔNp73$^{-/-}$ MEFs. Knockdown of TAp73 in MCF7 breast cancer cells showed similar upregulation of HIF1α, whereas overexpression of TAp73β downregulated HIF1α protein levels. Similar to our findings, Amelio et al. reported that TAp73 suppresses tumor angiogenesis via promoting HIF1α degradation. Herein, TAp73 functions as a scaffold protein for HIF1α and facilitates mouse-double-minute-2 (MDM2)-mediated polyubiquitination and subsequent proteasomal degradation of HIF1α, similar to the mechanism of HIF1α degradation shown by p53[194,277]. However, in contradiction to our findings, Dullo et al. reported that hypoxia induced HIF1α blocks Siah1 which in turn leads to TAp73 stabilization and subsequent increased angiogenic target gene activation and angiogenesis, thus concluding that TAp73 promotes tumor development and angiogenesis[278]. A possible explanation for the differing results may be due to the use of different mouse models that are characterized by different immune components. In their study, they used a SCID mouse model that lacks T cells, B cells and NK cells whereas in our studies we used Nude mice which lack T cells but still have functional B cells and NK cells. Additionally, we verified our results in an immunecompetent spontaneous B cell lymphoma mouse model, where we again observed increased vascularization in support of our TAp73$^{-/-}$ MEFs tumor studies in Nude mice.

Taken together, we demonstrated that TAp73 loss promotes tumor cells to produce increased pro-angiogenic factors that lead to tumor angiogenesis. This enhancement of angiogenesis signature is mainly conferred via increased HIF1α activity. In contrast, ΔNp73 loss leads to reduced tumor angiogenesis and downregulation of pro-angiogenic factors in concomitance

with decreased HIF1α activity. To conclude, we have discovered that TAp73 and ΔNp73 isoforms play opposite roles in tumor angiogenesis through regulation of HIF1α activity.

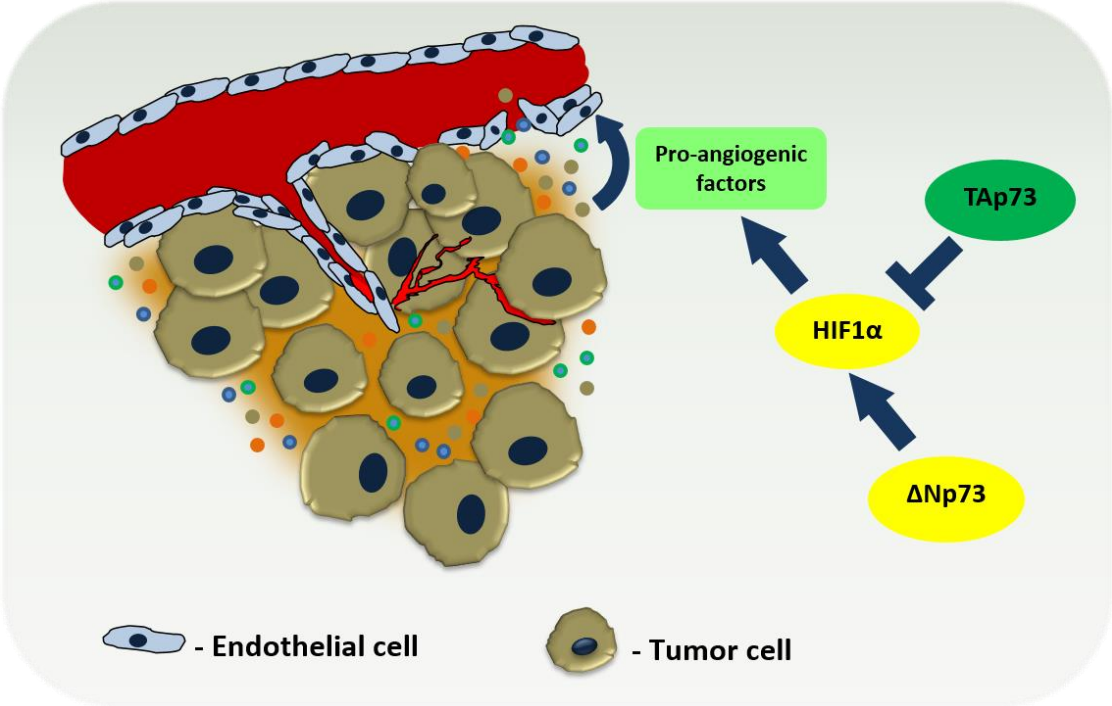

Figure 8. Graphical abstract showing how TAp73 and ΔNp73 are involved with HIF1α to regulate the expression of proangiogenic genes and subsequent tumor angiogenesis.

3.2 TAP73 DEFICIENCY ENHANCES CCL2 EXPRESSION AND INCREASES INTRA-TUMORAL INFILTRATION OF TUMOR-ASSOCIATED MACROPHAGES (PAPER II)

In **Paper I**, we reported that TAp73 deficient tumors, MEFs, and breast cancer cells have increased expression of proangiogenic chemokines and cytokines. Among these factors, the chemokine CCL2 was found to be highly upregulated. CCL2 belongs to a group of low molecular weight C-C chemokines that is commonly expressed by different cell types including myeloid, endothelial, epithelial, fibroblasts and most importantly different cancer cells. Apart from its proangiogenic characteristic, CCL2 is well known for its function as a macrophage chemoattractant and has the ability to recruit a variety of immune cells including T cells, NK cells and monocytes to the site of production. Tumors commonly express CCL2 to attract macrophages, which in turn mediate production of proangiogenic factors. These can then stimulate endothelial cell proliferation and migration leading to tumor angiogenesis, dampen the immune response and support tumor development[279-281]. Considering the fact that tumors favor recruitment of immune cells to the tumor microenvironment to support tumor progression and that CCL2 was upregulated in our results in **Paper I**, in this study we tried to explore how

TAp73 loss may induce CCL2 expression to promote immune cell infiltration which in turn drives tumor development. However, unlike **Paper I**, here we investigated TAp73 driven regulation of CCL2 independent of hypoxia.

We used two different pairs of TAp73$^{-/-}$ and TAp73$^{+/+}$ E1A/Ras transformed MEFs and found that TAp73 knockout cells harbor increased expression of Ccl2 mRNA, which was complemented with increased Ccl2 protein expression as shown by Western blot and ELISA.

To assess TAp73-driven Ccl2 regulation, we reintroduced TAp73 isoforms in TAp73 knockout MEFs and found a downregulation of Ccl2 mRNA expression. To see if similar regulation could be recapitulated in cancer cell lines, we overexpressed TAp73 isoforms in two different murine breast cancer cells (E0771 and 4T1) and showed similar downregulation of Ccl2 mRNA. Interestingly, for both TAp73$^{-/-}$ MEFs and breast cancer cell lines, TAp73 dependent repression of Ccl2 was mainly observed by TAp73β but not the TAp73α isoform. This may be explained by the structural difference between TAp73α and TAp73β. Unlike TAp73β, TAp73α contains an extra sterile alpha motif (SAM) and transcription inhibitory domain (TID) which limits the accessibility of binding partner p300/CBP to the p73 activation motif, for which TAp73α acquires poor transcriptional capacity compared to TAp73β[282]. This means that TAp73α and TAp73β can have differential transcriptional activities and function on the same target genes[283,284]. In agreement, a study showed that TAp73α upregulates c-Jun expression whereas TAp73β downregulates its expression[283].

To recapitulate similar regulation in human cell lines, we knocked down TAp73 in two breast cancer cell lines using two different siRNA oligos and found increased expression of CCL2 mRNA. On the other hand, overexpression of TAp73 isoforms, particularly TAp73β, shows significant downregulation of CCL2. CCL2 expression negatively correlates with patient prognosis, and supports metastatic cascade which interferes with disease free survival in a variety of cancers[281,285-288]. High expression of CCL2 is commonly found in breast cancer, lung cancer, colon cancer, liver cancer and prostate cancer[281,286,287]. Additionally, precancerous senescent hepatocytes have been shown to produce CCL2 to recruit CCR2^{+} immature myeloid cells (iMCs), which upon differentiation into macrophages are responsible for the clearance of senescent cells; tumor cells prevent differentiation of iMCs into macrophages which in turn inhibits NK cell function and promotes hepatocellular cancer growth[289]. To verify if the correlation between TP73 and CCL2 holds significance in human cancers, we evaluated our results in clinical samples by analyzing publicly available human cancer datasets (TCGA). We found a significant negative correlation between *TP73* and *CCL2* expression in a panel of 50

breast cancer cell lines and three different breast cancer patient datasets. In agreement, datasets from liver cancer, lung cancer and colon cancer also showed similar negative correlation between *TP73* and *CCL2*. Together, these findings consolidate the idea that TAp73 negatively regulates CCL2 expression.

To explore the precise mechanism of Ccl2 regulation in more depth, we used a Luc-reporter tagged Ccl2 promoter construct and performed a reporter assay along with overexpression of TAp73 and ΔNp73 isoforms. Notably, we again found that TAp73β but not TAp73α or ΔNp73 isoforms, represses Ccl2 promoter activity. To decipher whether TAp73 driven regulation of Ccl2, is conferred via binding to the Ccl2 promoter, we analyzed the Ccl2 promoter region using MatInspector transcription factor binding tool and found no binding sites for TAp73. This led us to think that TAp73 may indirectly regulate expression of Ccl2. Interestingly, the murine Ccl2 promoter is reported to contain four putative NFκB binding sites[290] . It has been reported that Ccl2 is a transcriptional target gene of NFκB and that the aberrant expression of both has been commonly observed in cancer[291,292]. To verify whether the NFκB-binding motifs found in the Ccl2 promoter have any role in TAp73 mediated Ccl2 promoter activity, we mutated each NFκB binding site and performed reporter assays with overexpression of TAp73α or TAp73β. Intriguingly, we discovered that deletion of NFκB binding site 1 or 2 results in enhanced Ccl2 promoter activity, implicating that both binding sites are important for NFκB mediated Ccl2 suppression. However, the reduced activity of the Ccl2 promoter by TAp73β was still evident even after loss of the binding sites. Additionally, deletion of either NFκB binding site 3 (NKB-3) or 4 (NKB-4) significantly reduced Ccl2 promoter activity, which was still reduced upon overexpression of TAp73β. Furthermore, a very strong reduction of Ccl2 promoter activity was seen when both binding site NKB-3 and NKB-4 were collectively removed, and no further suppression was observed upon co-expression with TAp73β. Taken together, we showed that NKB-3 and NKB-4 sites are important for Ccl2 promoter activation and TAp73β can impede NFκB mediated transcriptional activation of the Ccl2 promoter.

To understand the molecular interaction between TAp73 and NFκB, we transfected NFκB consensus binding site reporter constructs into TAp73 WT and KO MEFs. We found that TAp73 KO MEFs showed increased NFκB transcriptional activity; this finding was further complemented with qRT-PCR data showing increased expression of NFκB target genes (Cxcl1, Cxcl5, and Cxcl10) in TAp73 KO MEFs compared to WT. To further assess whether the increased NFκB activity observed in the TAp73$^{-/-}$ MEFs could result in increased Ccl2 expression, we inhibited NFκB activity in MEFs using SC514 and performed qRT-PCR and ELISA for Ccl2. Mechanistically, SC514 mainly inhibits IKK2 to stabilize IκBα/NFκB binding

which ultimately blocks translocation of NFκB subunits into the nucleus and thus prevents NFκB-mediated transcription[293]. We observed higher levels of Ccl2 mRNA as well as secreted Ccl2 protein from TAp73 deficient cells, whereas inhibition of NFκB impeded this upregulation and normalized Ccl2 levels closer to wild-type MEFs. This suggested that TAp73 loss favors increased NFκB activity resulting in higher expression of Ccl2.

The NFκB family is composed of five members including NFκB1 (p105), NFκB2 (p100), RelA (p65), c-Rel, and RelB. Among them, NFκB1 (p105) and NFκB2 (p100) undergo proteasomal modification and are cleaved into p50 and p52 respectively. The cleaved subunits and other family members commonly form homodimers or heterodimers prior to nuclear translocation and transactivation of targeted genes. Notably, NFκB-mediated Ccl2 promoter activation has been reported to be induced by the p65/p65 or p65/p50 dimers[290]. Therefore, we investigated the expression of these NFκB subunits in TAp73$^{+/+}$ and TAp73$^{-/-}$ MEFs via western blot. However, we found no differences in p65 or p50 expression. Upon NFκB activation, p65 together with its dimeric partner is known to translocate into the nucleus to activate target genes. Therefore, we checked p65 expression via immunohistochemistry (IHC), but found no difference in nuclear translocation of p65 between TAp73 WT and KO MEFs. Generally, NFκB activity is determined via several positive (IKBkA/Chuck, IKBkB and IKBkE) and negative (NFκBIA, NFκBIB, NFκBIE and BCL3) regulators including transcriptional repressor (p50/p52). Therefore, we checked these regulators in the TAp73$^{+/+}$ and TAp73$^{-/-}$ MEFs via qRT-PCR and revealed no significant difference in gene expression except for NFκB2. NFκB2 is a precursor protein that is proteolytically modified and matured into p52 subunits which lack the TAD, and acts as a transcriptional repressor against NFκB activity[294,295]. In line with our qRT-PCR results for NFκB2, we observed distinct downregulation of p52 protein in TAp73 KO MEFs compared to WT. Thus, our results indicate that TAp73 deficiency reduces transcriptional repressor p52 that in turn favors increased NFκB-mediated transcriptional activity and subsequent target gene expression of Ccl2.

As mentioned previously, the tumor microenvironment can induce the expression of CCL2 to potentiate infiltration of immune cells that can in turn promote tumor progression. In breast cancer, CCL2 has been shown to recruit CD11b^{+}/Ly6C^{+}/Gr^{+} inflammatory monocytes to promote metastasis events via supporting enhanced angiogenesis[38]. CCL2 also instigates CCL3 expression by metastasis-associated macrophages that in turn support increased events of lung metastasis[285]. Since TAp73 deficiency results in enhanced expression of Ccl2, we hypothesized that TAp73 knockout tumors may have increased recruitment of macrophages into the tumors.

To verify this, we used TAp73$^{-/-}$ and TAp73$^{+/+}$ MEFs generated tumors raised in Nude mice. Tumor sections stained for F4-80 pan macrophage marker showed that TAp73$^{-/-}$ tumors harbored increased macrophage recruitment compared to WT tumors. Additionally, we also performed FACS analysis for F4-80 and CD11c macrophage markers and once again observed increased F4-80^{+}/CD11c^{+} double positive macrophages recruited to the TAp73 KO tumors compared to WT tumors. Our data demonstrated that TAp73 loss favors increased macrophage recruitment to tumors.

Different factors produced by the tumor microenvironment can reprogram macrophages towards tumor supporting TAMs rather than tumor inhibiting type. The recruited TAMs can dampen the immune response and also produce more proangiogenic factors to support tumor development[47]. In line with this, CCL2 has been shown to polarize CD11b^{+} macrophages to M2 like macrophages (CD206^{+} TAMs)[296]. Therefore, we checked tumor sections for TAM marker CD206 to determine whether the increased macrophage population observed in the TAp73 KO tumors were indeed TAMs. CD206 staining confirmed that macrophages in the TAp73$^{-/-}$ tumors were in fact mainly tumor-associated macrophages. FACS analysis for double positive CD206^{+}/F4-80^{+} populations again portrayed that TAp73$^{-/-}$ tumors were enriched with a higher proportion of TAMs compared to WT. By performing qRT-PCR for macrophage markers, we again showed that TAp73 KO tumors have increased expression of TAMs marker *Mrc1* (CD206) and *Arg1*, whereas anti-tumorigenic macrophage marker iNOS (*NOS2*) remained unchanged between both tumor types. To further validate our *in vivo* results in human clinical samples, we analyzed publicly available cancer patient datasets and confirmed a negative correlation between *TP73* and *MRC1* (CD206) expression in breast cancer and lung cancer.

In summary, our study proposed a novel mechanism by which TAp73 represses transcriptional activation of Ccl2 via interference of NFκB activity. Moreover, TAp73 loss in tumors favors upregulation of Ccl2 that in turn supports increased recruitment of tumor-associated macrophages to support tumor development.

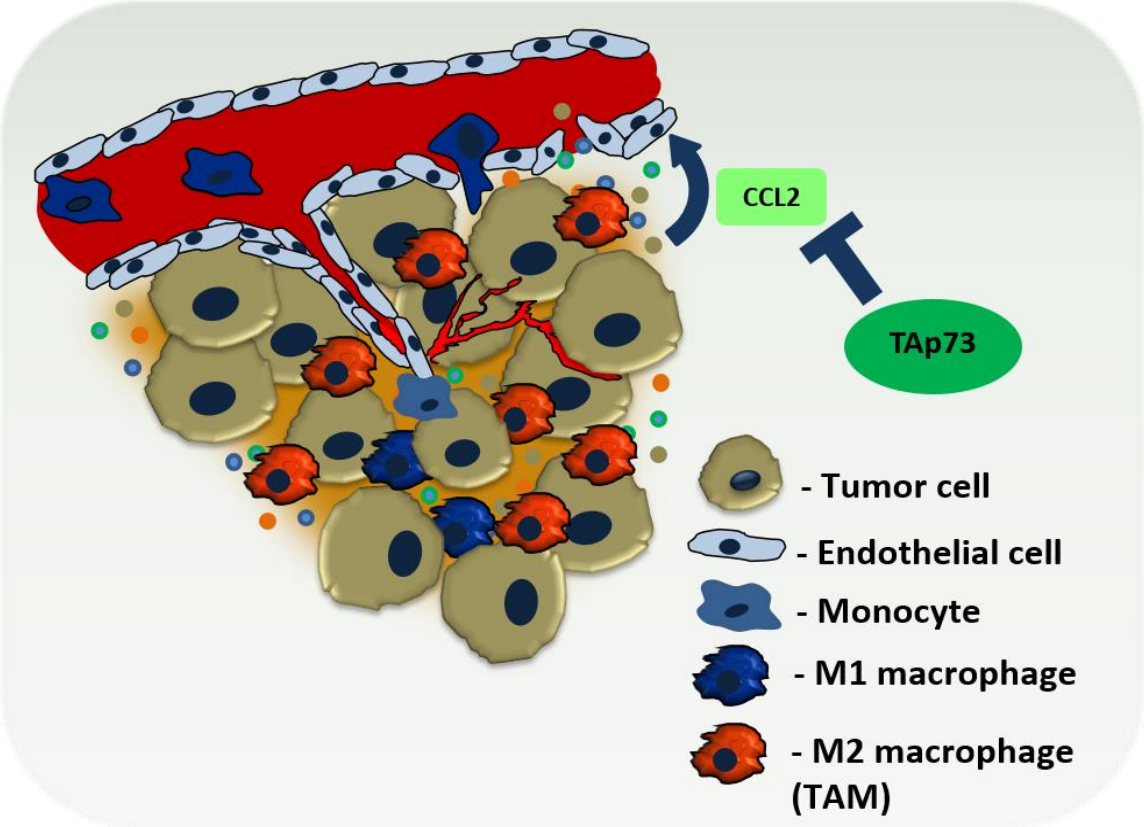

Figure 9. Graphical abstract showing CCL2 repression by TAp73 and its impact on tumor microenvironment.

3.3 ΔNP73 ENHANCES HIF-1A PROTEIN STABILITY THROUGH REPRESSION OF THE ECV COMPLEX (PAPER III)

Most solid tumors often experience low oxygen tension. The hypoxia exposed tumor cells then start to express hypoxia inducible factor (HIF), a master regulator that regulates genes essential for tumor cell survival, including those related to angiogenesis and metabolism which help to adapt to an alternate environment. In **Paper I**, we demonstrated that ΔNp73 absence conferred reduced HIF1α stability; in this paper, we continued our study to unravel the underlying molecular mechanism of how ΔNp73 is involved in regulation of HIF1α stability.

To investigate, we knocked down ΔNp73 using siRNA and shRNA targeting ΔNp73 in human breast cancer cell lines MCF7 and MDA-MB-231. Upon ΔNp73 knockdown, we observed significant downregulation of HIF1α protein compared to control cells. Intriguingly, ΔNp73 deficiency associated downregulation of HIF1α was observed in both normoxic and hypoxic conditions, suggesting that ΔNp73 ablation can affect HIF1α protein stability independent of a hypoxic environment. Additionally, in **Paper I**, we showed that ΔNp73 loss results in decreased HIF1α protein but not mRNA, and in this study, we again found no effect of ΔNp73

on HIF1α mRNA expression which suggests that ΔNp73 influences the protein stability of HIF1α but not the transcription of HIF1α. In line with this, our qRT-PCR data indicated downregulation of HIF1α target genes (*VEGFA, LDHA* and *PDK1*) concomitant to HIF1α protein downregulation. This suggested that ΔNp73 deficiency mediates reduction of HIF1α, which subsequently reduces HIF1α's transcriptional activity. To reconfirm this regulation, we overexpressed ΔNp73α to determine whether higher expression of ΔNp73 could upregulate HIF1α protein. Indeed, we found upregulation of HIF1α protein upon overexpression of ΔNp73α, suggesting that ΔNp73 can positively regulate HIF1α stability. Interestingly, during hypoxia HIF1α can stabilize ΔNp73 via suppression of E3 ligase Siah1[195]. Together with our results, this could reflect a possible feedback mechanism between ΔNp73 and HIF1α at varying oxygen conditions.

To translate our findings *in vivo*, we established tumor from ΔNp73 WT and KO E1A/Ras transformed MEFs. In line with our previous reports, we observed that ΔNp73 deficiency led to impaired tumor formation[220]. We analyzed the tumors for HIF1α and discovered similar downregulation of HIF1α protein in ΔNp73 KO tumors compared to ΔNp73 WT tumors. Considering the size difference between $\Delta Np73^{-/-}$ and $\Delta Np73^{+/+}$ tumors, we examined the expression of HIF1α within the hypoxic region by injecting HydroxyProbe-1 in the mice prior to tumor isolation. Using immunofluorescence staining for HIF1α and HydroxyProbe-1, we again observed significant reduction of HIF1α staining within the hypoxic regions in ΔNp73 KO tumors compared to WT. Together; our data demonstrated that ΔNp73 deficiency attenuates tumor induced HIF1α expression.

The stability, accumulation and activity of HIF1α protein is regulated via post-translational modification cascades[297]. In normoxia, HIF1α levels are negatively regulated via proteasomal degradation and ubiquitination pathway mediated by Von Hippel-Lindau protein (pVHL). Since ΔNp73 deficiency results in decreased HIF1α stability, we hypothesized a possible role for ΔNp73 in interfering with ubiquitination of HIF1α, which in turn affects its stability. To study this, we inhibited the proteasomal degradation pathway using MG132 in shCtrl and shΔNp73 breast cancer cells. Interestingly, we observed normalization of HIF1α protein levels in ΔNp73 knockdown cells similar to the levels seen for control cells. Additionally, performing Co-IP we showed that ΔNp73 deficient cells have increased ubiquitin bound to HIF1α, suggesting that ΔNp73 loss leads to increased ubiquitin binding to HIF1α protein, leading to its ubiquitination and proteasomal degradation. This mechanism was observed for both ΔNp73 KO MEFs and human breast cancer ΔNp73 KD MCF7 and MDA-MB-231 cells. However, in normoxic condition prior to ubiquitin-mediated degradation, HIF1α is first hydroxylated by a

group of prolyl-4-hydroxylase (PHD 1-3) enzymes which helps in recognition of HIF1α by pVHL, which in turn ubiquitinates HIF1α and targets it for proteasomal degradation. Among the PHD enzymes, PHD2 is a target gene of p53 and is well known for maintaining steady-state levels of HIF1α. ΔNp73 is known to impede p53-mediated transcription[238], suggesting that ΔNp73 may interfere with p53-mediated PHD2 transcription in order reduce HIF1α degradation and thus increase HIF1α stability. To test this theory, we checked hydroxylated HIF1α after blocking the proteasomal degradation machinery by MG132 treatment, and surprisingly found no differences in hydroxylated HIF1α protein levels between ΔNp73 control and knockdown cells. This indicated that ΔNp73 does not interfere with hydroxylation of HIF1α.

The tumor suppressor pVHL is a multipurpose adaptor protein encoded from von Hippel-Lindau gene (VHL) which functions as a substrate recognition subunit of an E3 ligase complex termed as ECV. The ECV complex comprises of pVHL, Cullin 2 (Cul2), Elongin B (Tceb1), Elongin C (Tceb2) and Rbx1; the complex primarily targets HIF1α for ubiquitin mediated proteasomal degradation in normoxic condition. During hypoxia, HIF1α does not undergo hydroxylation, which prevents pVHL mediated proteasomal degradation resulting in HIF1α accumulation, followed by increased proliferation and angiogenesis as portrayed in VHL disease[298]. Based on the fact that HIF1α stability is controlled by the adaptor protein pVHL, we sought to investigate whether pVHL drives ΔNp73 deficiency mediated downregulation of HIF1α. To confirm this concept, we performed siRNA-mediated knockdown of ΔNp73 in two different renal carcinoma cell lines, RCC4^{+VHL}(with functional VHL) and RCC4 (deficient for VHL). Interestingly, we found no differences in HIF1α levels when pVHL remained mutated or nonfunctional, but in presence of functional pVHL, ΔNp73 deficiency instigated similar downregulation of HIF1α protein as demonstrated earlier. This implied that ΔNp73 loss mediated reduction of HIF1α is mainly conferred via a pVHL dependent mechanism.

Both ΔNp73 and HIF1α are considered to be bad prognostic factors in a variety of cancers; high expression of these isoforms lead to poor response to drug therapy, disease aggressiveness and poor patient outcome in liver cancer, gastric cancer, colon cancer and breast cancer[180,299,300]. Considering their major involvement in cancer, we evaluated our results in patient samples to establish a link between ΔNp73 and HIF1α. By analyzing TCGA breast cancer dataset in paper I, we have shown that higher expression of ΔNp73 is positively associated with angiogenesis and hypoxia gene signatures in breast cancer patients. For the current study, we reanalyzed the same data and performed gene set enrichment analysis

(GSEA) on samples that had high levels of ΔNp73, in order to search for pathways most affected. We found that genes associated with ubiquitin-mediated proteolysis were downregulated in patients who had high expression of ΔNp73. When we looked in more depth for genes involved with ubiquitin-mediated proteolysis, we found that the ECV genes (RBX1, CUL2, TCEB1 and TCEB2) were significantly downregulated.

Next, to validate our patient data results we ectopically overexpressed ΔNp73α in two human breast cancer cell lines and found similar downregulation of ECV genes as observed for patients who had high ΔNp73 levels. To confirm this regulation, we also knocked down ΔNp73 in the same breast cancer cell lines and notably found upregulation of the same ECV genes. Concomitantly, we also reconfirmed similar regulation in MEFs where ΔNp73 KO MEFs exhibited higher expression of ECV genes at both mRNA and protein levels. Additionally, reintroduction of ΔNp73α in the $\Delta Np73^{-/-}$ MEFs conversely downregulated the expression of ECV genes. Based on these findings, we demonstrated that ΔNp73 negatively regulates the expression of ECV complex members (RBX1, CUL2, TCEB1 and TCEB2) in order to interfere with ubiquitination of HIF1α, as confirmed in both breast cancer cells and breast cancer patient samples.

Rbx1 is a RING-finger protein recruited by Cul2 that is able to recognize important E2 ubiquitin-conjugating enzyme, which is necessary for mediating E3 ligase function of ECV complex[298]. To explore whether ΔNp73 mediated downregulation of Rbx1 results in accumulation of HIF1α we performed IP for pVHL in ΔNp73 KO and WT MEFs followed by western blot. Interestingly, we observed increased Rbx1 bound to pVHL in ΔNp73 KO MEFs whereas no differences were seen in pVHL binding to HIF1α. This suggested that ΔNp73 deficient cells may have an active ECV complex pathway that aids HIF1α degradation. In line with these results, siRNA mediated Rbx1 knockdown showed significant upregulation of HIF1α protein, which remained unchanged when both ΔNp73 and Rbx1 were downregulated in $RCC4^{+VHL}$ cells that have functional VHL. Our data shows that ΔNp73 inhibits HIF1α ubiqutination by downregulating the ECV genes, thus impeding VHL-dependent proteasomal degradation of HIF1α, resulting in increased HIF1α stability and accumulation.

In **Paper I**, we showed that ΔNp73 loss results in decreased HIF1α protein. Herein, we further unraveled this molecular mechanism, where we demonstrated that ΔNp73 controls HIF1α stability via regulation of ECV genes.

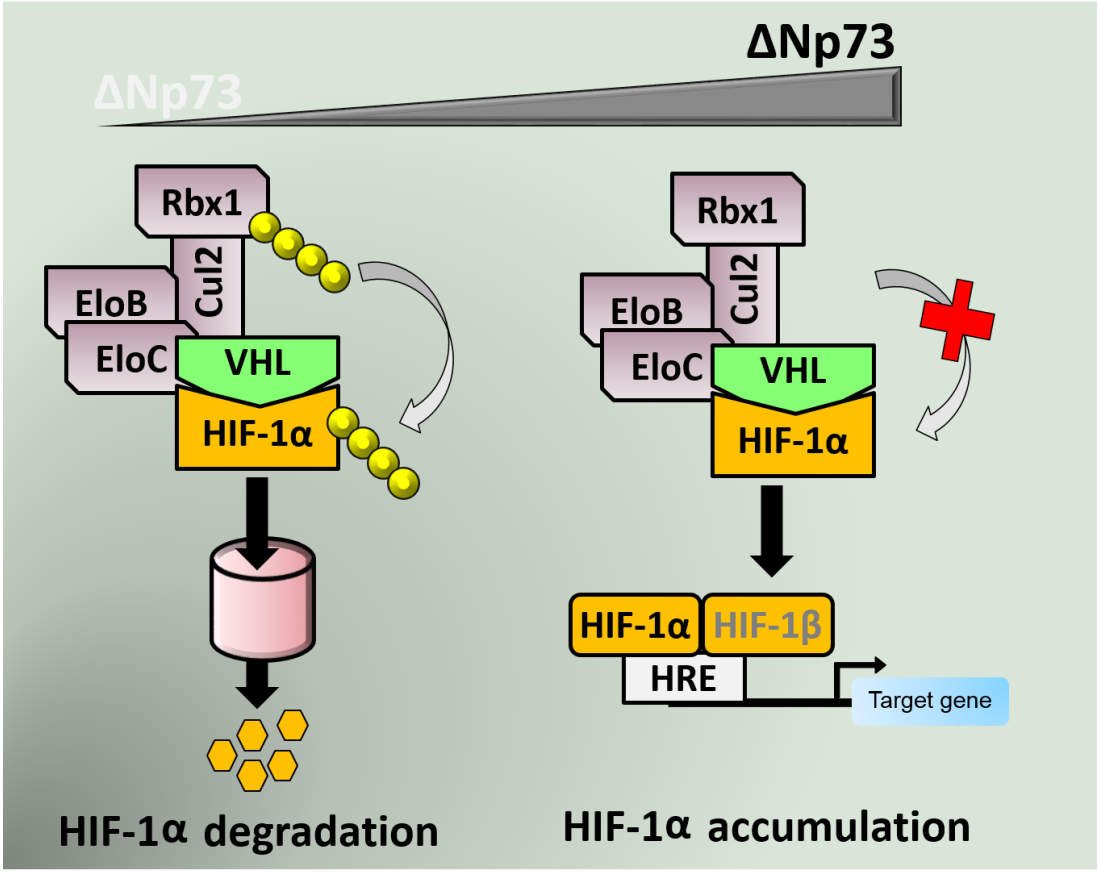

Figure 10. Graphical abstract showing ΔNp73 promoting HIF1α protein stability, resulting in transactivation of its target genes.

3.4 ΔNP73 REGULATES THE EXPRESSION OF THE MULTIDRUG-RESISTANCE GENES ABCB1 AND ABCB5 IN BREAST CANCER AND MELANOMA CELLS (PAPER IV)

In **Paper I**, we demonstrated that breast cancer samples expressing high ΔNp73 were enriched for angiogenesis and hypoxia signatures. For this study, we went back to the same dataset and scanned for pathways that were upregulated in breast cancer patient samples that had high expression of ΔNp73. We filtered the dataset based on genes that were significantly upregulated more than two-fold in samples with high ΔNp73 expression, and performed a Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis.

KEGG pathway analysis revealed that ABC pathways were most enriched; apart from higher expression of ABC genes, we also found upregulation of other pathways including tyrosine metabolism, ECM receptor interactions, cell adhesion and focal adhesion pathways. ATP binding cassettes (ABCs) are transporter proteins that primarily function by importing and exporting different molecules within the cells. Cancer cells are known to overexpress these

genes in order to facilitate increased efflux of drug molecules from the cells to avoid cytotoxicity. Moreover, ΔNp73 has been associated with impaired drug response, impeded apoptotic signals and with poor patient prognosis in a variety of cancers. Therefore, considering the involvement of both ΔNp73 and ABC transporters in drug resistance and cancer patient disease outcome, we focused our study on the ABC transporters to decipher how ΔNp73 interplays with the ABC family to exert drug resistance.

We looked into more depth to determine which ABC genes were upregulated in the high ΔNp73-expressing samples. We observed significant upregulation of several ABC members including ABC subfamily A (ABCA5, ABCA6, ABCA8, ABCA9, ABCA10), ABC subfamily B (ABCB1 and ABCB5), ABC subfamily D (ABCD2) and ABC subfamily G (ABCG2). The association of p73 isoforms with ABC transporters has been previously reported in different malignancies; ABCA5 transporter is shown to be positively regulated by splice variant ΔNp73 in malignant melanoma, whereas association of p73-isoforms with ABCB1 has been reported in colon cancer and neuroblastoma[206,211,268]. However, to the best of our knowledge, ΔNp73 driven regulation of ABC genes in breast cancer was not previously investigated. Considering the high incidence of breast cancer treatment failure as a result of drug resistance, the role of ΔNp73 in ABC transporter regulation may shed new light into the mechanisms behind chemotherapeutic failure.

To investigate the ΔNp73-driven regulation of ABC genes, we transiently overexpressed ΔNp73 isoform into two human breast cancer cell lines MCF7 (p53 WT) and MDA-MB-231 (mutant p53). Next, we performed qRT-PCR to check the expression of the top five upregulated ABC genes that were found to be enriched in the high ΔNp73-expressing breast cancer patient samples. Interestingly, similar to the dataset results, we observed upregulation of ABCB1 and ABCB5 genes upon overexpression of ΔNp73α whereas expression of ABCA8, ABCA9 and ABCB10 were undetected in both cell lines; a possible explanation could be that these transporters might be expressed by other cells present in the tumor stromal compartment. Hence, we focused our study on ABCB1 and ABCB5 genes. In a study by Vilgelm et al., it was reported that ΔNp73 could upregulate ABCB1 expression via limiting p53-driven transrepression of the ABCB1 gene in gastric cancer[211]. However, here we demonstrated upregulation of both ABCB1 and ABCB5 genes irrespectively of p53 status. This suggests that ΔNp73 may mediate regulation of these transporters through interaction with other proteins or pathways exclusive of p53. In a study by Bao et al., it has been shown that miR-298 confers doxorubicin drug resistance in metastatic breast cancer through upregulation of ABCB1[301];

whether ΔNp73 regulates miR-298 or other miRNAs to inhibit ABCB1 and ABCB5 in breast cancer would be interesting to investigate.

To further verify the regulation of ABC genes, we performed shRNA-mediated knockdown of ΔNp73 in both breast cancer cell lines to detect whether downregulation of ΔNp73 can result in downregulation of ABC genes. As expected, qRT-PCR results indicated downregulation of ABC genes upon knockdown of ΔNp73. Furthermore, siRNA-targeted knockdown of ΔNp73 in both cell lines confirmed significant downregulation of both ABCB1 and ABCB5 genes. Together, these results highlighted that ΔNp73 positively regulates *ABCB1* and *ABCB5* expression in breast cancer cells.

The high expression ΔNp73 has been reported to be associated with poor patient outcome following chemoresistance in a myriad of cancers including breast cancer[180]. Moreover, ABCB1 and ABCB5 have been associated to drug resistance in breast cancer through mediating doxorubicin efflux[301,302]. Taken together, these reports suggested a possible role that interlinked ΔNp73 and ABC transporters in developing drug resistance in breast cancer. Considering this, we checked whether ΔNp73 deficiency could influence the proliferation and efflux capacity of MCF7 and MDA-MB-231 cells upon doxorubicin treatment. We performed an efflux assay and found that ΔNp73 ablation leads to increased intracellular accumulation of doxorubicin in ΔNp73 deficient cells compared to control cells. We further confirmed the results with a WST-1 assay, where doxorubicin treated ΔNp73 deficient cells showed reduced proliferation in line with decreased efflux capacity. Collectively, our results highlighted that ΔNp73 deficiency impairs drug efflux capacity of breast cancer cells and sensitize them towards chemotherapy-induced cell death.

Finally, to evaluate our results in another cancer type and to verify the clinical relevance of our findings, we investigated the correlation of ΔNp73, ABCB1 and ABCB5 in melanoma patient samples. We did not detect the expression of ΔNp73 in the samples, but instead found the expression of the ΔNp73-like p73ΔEx2/3 isoform. p73ΔEx2/3 is the predominant isoform expressed in melanoma, and the absence of a TA domain gives it similar function as ΔNp73[267]. Hence, we hypothesized that in melanoma p73ΔEx2/3, similar ΔNp73, may be involved with the regulation of ABCB1 and ABCB5 expression. Using qRT-PCR, we confirmed that p73ΔEx2/3-like ΔNp73 is positively correlated with the expression of ABCB1 and ABCB5. Next, we overexpressed p73ΔEx2/3α and p73ΔEx2/3β isoforms in SK-MEL-28 melanoma cells to verify our previous result. Likewise, overexpression of p73ΔEx2/3 isoforms again showed upregulation of ABCB1 and ABCB5, similar to our results seen for ΔNp73 in breast

cancer cell lines. Our data implies that in melanoma the predominant ΔNp73-like p73ΔEx2/3 isoform mainly drives the expression of multidrug resistance genes ABCB1 and ABCB5.

In line with ΔNp73's positive role in tumor angiogenesis shown in Paper I, here we again demonstrate ΔNp73's active role in development of drug resistance via controlling expression of multidrug resistance genes.

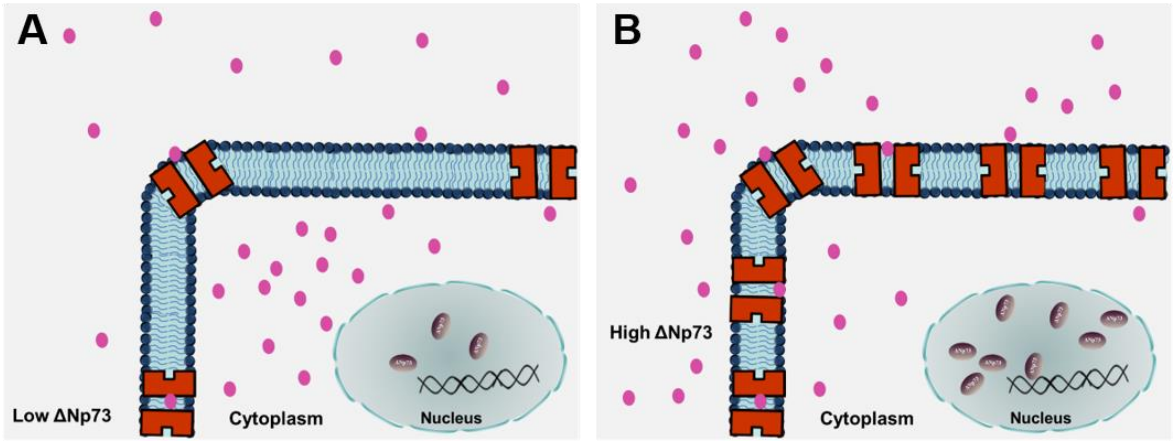

Figure 11. Graphical abstract to show how ΔNp73 mediates drug resistance. **A.** Low ΔNp73 expression scenario **B.** High ΔNp73 expression scenario. Orange color denotes ABC transporter, pink color for doxorubicin and brown color for ΔNp73.

4 CONCLUSION AND FUTURE PERSPECTIVES

Tumor development occurs through different stages including avoidance of cell cycle arrest and apoptosis, adaptation to harsh hypoxic environment, bypassing immune surveillance, forming a niche and reinforcing new blood vessels. Additionally, tumor cells can take advantage of cellular efflux machinery and enhance the expression of multi-drug resistance proteins to avoid drug mediated cytotoxicity. In this thesis, some of these points were addressed to decipher the role of p73 isoforms in tumor development.

In **Paper I**, we have shown that TAp73 suppresses expression of several pro-angiogenic factors whereas TAp73 loss favors tumor cells with increased expression of these factors, which in turn results in increased tumor vascularization. On the other hand, oncogenic ΔNp73 loss results in decreased tumor vascularization and decreased expression of pro-angiogenic genes. This demonstrates a novel mechanism by which different p73 isoforms show opposite functions in regulating tumor angiogenesis. Additionally, we showed that TAp73 regulates proangiogenic factors via inhibition of HIF1α activity and that in absence of TAp73, HIF1α is upregulated. In contrast, ΔNp73 enhances HIF1α activity. In **Paper III**, we studied the regulation of HIF1α by ΔNp73 in more detail.

In **Paper II**, we continued to explore how TAp73 regulates one of the pro-angiogenic chemokines found in **Paper I**. Here we reported that TAp73 represses tumor-induced production of macrophage chemotactic protein 1(MCP1/CCL2). We also confirmed similar negative correlation between *TP73* and *CCL2* in six different cancer datasets. Importantly, we showed that TAp73-driven CCL2 repression is mainly conferred by inhibiting NFκB-mediated activation of the CCL2 promoter. We also found that TAp73 KO tumors that had higher expression of CCL2, also harbored increased tumor infiltrated macrophages. In addition, these macrophages were found to be mostly M2-like tumor associated macrophages (TAMs). We also reported similar negative correlation of TP73 and M2-like TAMs marker CD206 expression in human breast cancer and lung cancer.

In **Paper III**, we continued the study following a lead from **Paper I**. Here, we demonstrated that ΔNp73 positively regulates HIF1α stability. In absence of ΔNp73, HIF1α is rapidly ubiquitinated and degraded via proteasomal machinery and this degradation is dependent on pVHL. Similar downregulation of HIF1α in absence of ΔNp73 was further confirmed in *in vivo* tumors. Additionally, we revealed that ΔNp73 downregulates members of the ECV complex, which results in increased HIF1α stability and accumulation. Here we showed a novel mechanism through which ΔNp73 can control HIF1α stability by regulating the ECV genes.

In P**aper IV**, we focused our study on deciphering the role of p73 in cancer drug resistance, which is a very important factor in deciding patient drug therapy. Since, ΔNp73 is known to be aberrantly expressed in variety of cancers and is involved in chemoresistance, we focused our study on this isoform. We reported that oncogenic ΔNp73 supports drug resistance via promoting expression of ABC transporters. Additionally, we showed that ΔNp73 overexpression promotes expression of ABCB1 and ABCB5 gene. On the other hand, downregulation of ΔNp73 results in decreased expression of the same ABC transporters that leads to decreased drug efflux capacity by breast cancer cells. In line with this, similar regulation was also confirmed in melanoma patient samples. Together our data demonstrated ΔNp73's positive role in drug resistance that could hold significance in cancer patient therapy.

In **Paper I** and **II**, we found that p73 regulates several pro-angiogenic factors. Some of these factors are known to have function in metastasis. Hence, it would be interesting to study p73 isoforms involvement in tumor metastasis. In contrary to our **Paper I**, there are existing contradictory reports about TAp73 promoting tumor angiogenesis, this may imply that c-terminal splice variants i.e. α, β, γ, δ, ε, ζ and η are also needed to be considered for drawing conclusions about different p73 isoforms's function. Since CCL2 is known to promote tumor angiogenesis and recruitment of TAMs in TAp73 KO tumors, it would be interesting to see if these events could be reversed upon blocking of CCL2 expression *in-vivo*. Additionally, macrophages are also known to produce CCL2, as such, it would be interesting to study whether the absence or presence of TAp73 within macrophages may have any effect on macrophage function in tumor development. In **Paper I** and **III**, we showed the regulation of HIF1α by p73 isoforms. Therefore, regulation of other HIF family members by p73 isoforms would be an interesting area for further investigation. Moreover, p73ΔEx2/3 isoform that acts like ΔNp73 should also be investigated for its effect in regulation of HIF1α stability and tumor angiogenesis. **In Paper IV** we deciphered the role of ΔNp73 in drug resistance and regulation of ABC transporters, similarly, the involvement of TAp73 in the regulation of ABC transporters could be an exciting area to consider for future direction.

In conclusion, this thesis delineates a novel mechanism by which p73 isoforms can influence the tumor microenvironment through differential regulation of angiogenic factors and subsequent angiogenic events. Furthermore, we confirm the oncogenic role of ΔNp73 in supporting multi-drug resistance through regulation of ABC transporters. By providing further knowledge on how p73 can affect tumor development and resistance, we highlight the significance of p73 as an important target for the design of new cancer therapies.

5 ACKNOWLEDGEMENTS

First of all, I would like to thank my supervisor **Margareta Wilhelm** for having me as a PhD student. It has been a long journey starting from my Master's thesis till now. I admire your great love for research. You are surely one of the most dedicated and enthusiastic researchers I met. Coming from a pharmacy background, it was very hard for me to cope with new molecular techniques but with your help and positive encouragement I managed to succeed. I have learned a lot from you and hope to carry on all the knowledge in my future endeavors.

My cosupervisor, **Marie Arsenian Henriksson**, what can I say! The moment when you told me that I am brave to ask questions in a large audience at the Uppsala Stratcan meeting, I was really overwhelmed by your positive encouragement. I am very thankful for your continuous support and optimism. Thank you for believing in me and your great discussions. Not to mention, asking me repeatedly when is my thesis going to finish!!! ☺

Marina, I cannot express how grateful I am to you. Thank you very much for sharing your experiences and all your support throughout my PhD studies. Your management skills are *top notch* when it comes to finishing tasks!

Evelyn, even though we did not share a common project the discussion and suggestions you put forward were always amazing. The way you dealt with projects was very inspiring to me. Thank you for all your help, especially with troubleshooting of different molecular techniques in the lab.

Ana Marin, your dedication level is more than 100% ☺. Thanks for all your help! You are the next one in line so good luck with your PhD studies. If I ever needed to find you at MTC, all I had to do was follow the sound of your giggling ☺!

Johanna (jojo or yoyo in Swedish ☺) you are the lifesaver of the group for understanding Swedish and making appointments ☺! We did not have much time together but still we did a lot of work in collaboration. You are very hardworking and enthusiastic about science, and thanks for your help! I wish you good luck with your PhD studies.

Ganna Oliynyk (G), I am grateful for all your support and really appreciate the time we spent together to study The Biology of Cancer in preparation for the book exam ☺. You have always been really kind and generous when it came to helping out your fellow colleagues!

Johanna Dzieran, you are truly a perfectionist! One of the things out of many that I really admire about you. I really appreciate your help in the lab and in managing issues with Agresso. Thank you for being patient and dealing with my Agresso issues repeatedly ☺.

Maríavi and Lourdes Co., you are not only good at producing scientific results but also very good at making cake ☺. Thank you for your support during my PhD studies.

Áine, **Aida** and **Elena**, it has been a great pleasure knowing you. Thanks for all the good times in the lab and sharing FIKA!

Also, many thanks to former members from Marie's group, **Diogo** and **Ulrica**.

Much appreciation goes out to **Galina Selivanova** and her group for always helping with reagents and suggestions. Thanks **Gema** for your bioinformatics support and to my table tennis buddies **Sylvain**, **Ali**, **Madhu** and **Jiawei**, for the great games and conversations ☺. Many thanks to **Joannaa Zawacka-pankau** and **Ana** for our collaboration and having good discussions about the p73 field.

My sincere gratitude to **Lars-Gunnar Larsson** and his group members (**Fan**, **Marcela**, **Alina**, **Mariam** and **Qinzi**) for helping with reagents and sharing thoughts. Not to mention **Wesam,** the fishing guy (you know what I mean ☺), thanks man I really enjoyed your company and our coffee breaks.

Many thanks to **Sonia lain** and her group members **Inge, Marcus**, **Gergana**, **Andres** and **Suhas** for sharing reagents and technical support. Not to mention, I really enjoyed our conversations over coffee.

A very big thanks to **Yihai Cao** and his former and current group members for sharing thoughts, ideas, reagents and lots of fun facts! **Taka, Hideki, Masaki, Kayoko, Carina** and **Dr. Patrik**, I am very thankful to all of you for your kind help. **Patrik,** thanks buddy for all the conversations and sharing moments of frustration ☺.

My heartfelt gratitude to **Charlotte Rolny** and her group from CCK for their help with FACS. **Tatjana,** thank you very much for your help and also sharing your interesting thoughts on different food habits!

Teresa Frisan and **Lisa,** it was amazing to meet you and collaborate on an exciting project. I really had a good time working with you.

Alejandro, the bioinformatics expert! I really admire the way you treat life and science together.

Hamid and **Krister,** thanks for sharing good stories on exciting projects and of course sweets from Iran ☺.

Thanks to everyone at Administration who provided constant help and support through my years spent as a PhD student. My sincere appreciation to **Gesan**, **Åsa**, **Pontus**, **Magnus**, **Torbjörn**, **Per**, **Mia** and **Vivian** for their kind support.

I would also like to acknowledge a long list of friends from MTC: **Sadia**, **Mushtak**, **Mobassher**, **Stefan**, **Magdalena, Twana** and **Shawon,** and former researchers **Atiq** and **Eahsan Rasul**. Thank you for your company. I am also grateful to my friends in other departments at KI: **Shafiq**, **Shahjahan**, **Sabbir**, **Risul Amin**, **Mehedi Parag**, **Anwar, Sadia**, **Rokeya Sultana, Sultan Ahmed** and **Mushfiqur Rahman.**

Many thanks to all former and current MSA buddies (**Samer, Agata, Soazig, Arnika, Paola, Silke, Néstor, Vanessa, Farzaneh, Sunitha, Joanna, Shady, Ganesh, Afrouz and Benedek**). I really enjoyed working together with you people. Keep up the good work!

Last but not least, this PhD study would not have been possible without the standing support from my wife **Tanzina Mollick** and continuous encouragement from my **family**. My best friends **Rahat**, **Mohit** and **Rubel,** thank you guys for all the inspiring thoughts and believing in me.

6 REFERENCES

1 World Health Organization (WHO). *Cancer*, <http://www.who.int/cancer/en/> (2017, February).

2 Hajdu, S. I. A note from history: landmarks in history of cancer, part 1. *Cancer* **117**, 1097-1102, doi:10.1002/cncr.25553 (2011).

3 Hanahan, D. & Weinberg, R. A. The hallmarks of cancer. *Cell* **100**, 57-70 (2000).

4 Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* **144**, 646-674, doi:10.1016/j.cell.2011.02.013 (2011).

5 Lodish H, B. A., Zipursky SL, et al. in *Molecular Cell Biology* (ed New York: W. H. Freeman) (2000).

6 Croce, C. M. Oncogenes and cancer. *The New England journal of medicine* **358**, 502-511, doi:10.1056/NEJMra072367 (2008).

7 Lane, D. P. Cancer. p53, guardian of the genome. *Nature* **358**, 15-16, doi:10.1038/358015a0 (1992).

8 Watnick, R. S. The role of the tumor microenvironment in regulating angiogenesis. *Cold Spring Harbor perspectives in medicine* **2**, a006676, doi:10.1101/cshperspect.a006676 (2012).

9 De Palma, M., Biziato, D. & Petrova, T. V. Microenvironmental regulation of tumour angiogenesis. *Nature reviews. Cancer* **17**, 457-474, doi:10.1038/nrc.2017.51 (2017).

10 Hoff, P. M. & Machado, K. K. Role of angiogenesis in the pathogenesis of cancer. *Cancer treatment reviews* **38**, 825-833, doi:10.1016/j.ctrv.2012.04.006 (2012).

11 Ribatti, D. & Crivellato, E. "Sprouting angiogenesis", a reappraisal. *Developmental biology* **372**, 157-165, doi:10.1016/j.ydbio.2012.09.018 (2012).

12 Kubis, N. & Levy, B. I. Vasculogenesis and angiogenesis: molecular and cellular controls. Part 1: growth factors. *Interventional neuroradiology : journal of peritherapeutic neuroradiology, surgical procedures and related neurosciences* **9**, 227-237, doi:10.1177/159101990300900301 (2003).

13 Carmeliet, P. Angiogenesis in health and disease. *Nature medicine* **9**, 653-660, doi:10.1038/nm0603-653 (2003).

14 Tammela, T. & Alitalo, K. Lymphangiogenesis: Molecular mechanisms and future promise. *Cell* **140**, 460-476, doi:10.1016/j.cell.2010.01.045 (2010).

15 Huang, Z. & Bao, S. D. Roles of main pro- and anti-angiogenic factors in tumor angiogenesis. *World journal of gastroenterology* **10**, 463-470 (2004).

16 Folkman, J. Tumor angiogenesis: therapeutic implications. *The New England journal of medicine* **285**, 1182-1186, doi:10.1056/NEJM197111182852108 (1971).

17 Cao, Y. Future options of anti-angiogenic cancer therapy. *Chinese journal of cancer* **35**, 21, doi:10.1186/s40880-016-0084-4 (2016).

18 Keunen, O. *et al.* Anti-VEGF treatment reduces blood supply and increases tumor cell invasion in glioblastoma. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 3749-3754, doi:10.1073/pnas.1014480108 (2011).

19 Lopes-Bastos, B. M., Jiang, W. G. & Cai, J. Tumour-Endothelial Cell Communications: Important and Indispensable Mediators of Tumour Angiogenesis. *Anticancer research* **36**, 1119-1126 (2016).

20 Shekhar, M. P., Werdell, J., Santner, S. J., Pauley, R. J. & Tait, L. Breast stroma plays a dominant regulatory role in breast epithelial growth and differentiation: implications for tumor development and progression. *Cancer research* **61**, 1320-1326 (2001).

21 Dudley, A. C. Tumor endothelial cells. *Cold Spring Harbor perspectives in medicine* **2**, a006536, doi:10.1101/cshperspect.a006536 (2012).

22 Dvorak, H. F. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *The New England journal of medicine* **315**, 1650-1659, doi:10.1056/NEJM198612253152606 (1986).

23 Cronstein, B. N. & Weissmann, G. The adhesion molecules of inflammation. *Arthritis and rheumatism* **36**, 147-157 (1993).

24 Mulligan, J. K., Rosenzweig, S. A. & Young, M. R. Tumor secretion of VEGF induces endothelial cells to suppress T cell functions through the production of PGE2. *Journal of immunotherapy* **33**, 126-135, doi:10.1097/CJI.0b013e3181b91c9c (2010).

25 Yadav, A. *et al.* Tumor-Associated Endothelial Cells Promote Tumor Metastasis by Chaperoning Circulating Tumor Cells and Protecting Them from Anoikis. *PloS one* **10**, e0141602, doi:10.1371/journal.pone.0141602 (2015).

26 Armulik, A., Genove, G. & Betsholtz, C. Pericytes: developmental, physiological, and pathological perspectives, problems, and promises. *Developmental cell* **21**, 193-215, doi:10.1016/j.devcel.2011.07.001 (2011).

27 Diaz-Flores, L. *et al.* Pericytes. Morphofunction, interactions and pathology in a quiescent and activated mesenchymal cell niche. *Histology and histopathology* **24**, 909-969, doi:10.14670/HH-24.909 (2009).

28 Birbrair, A. *et al.* Type-1 pericytes accumulate after tissue injury and produce collagen in an organ-dependent manner. *Stem cell research & therapy* **5**, 122, doi:10.1186/scrt512 (2014).

29 Birbrair, A. *et al.* Type-2 pericytes participate in normal and tumoral angiogenesis. *American journal of physiology. Cell physiology* **307**, C25-38, doi:10.1152/ajpcell.00084.2014 (2014).

30 Ochs, K. *et al.* Immature mesenchymal stem cell-like pericytes as mediators of immunosuppression in human malignant glioma. *Journal of neuroimmunology* **265**, 106-116, doi:10.1016/j.jneuroim.2013.09.011 (2013).

31 Gee, M. S. *et al.* Tumor vessel development and maturation impose limits on the effectiveness of anti-vascular therapy. *The American journal of pathology* **162**, 183-193, doi:10.1016/S0002-9440(10)63809-6 (2003).

32 Ribeiro, A. L. & Okamoto, O. K. Combined effects of pericytes in the tumor microenvironment. *Stem cells international* **2015**, 868475, doi:10.1155/2015/868475 (2015).

33 Hellstrom, M. *et al.* Lack of pericytes leads to endothelial hyperplasia and abnormal vascular morphogenesis. *The Journal of cell biology* **153**, 543-553 (2001).

34 Lindahl, P., Johansson, B. R., Leveen, P. & Betsholtz, C. Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. *Science* **277**, 242-245 (1997).

35 Xian, X. *et al.* Pericytes limit tumor cell metastasis. *The Journal of clinical investigation* **116**, 642-651, doi:10.1172/JCI25705 (2006).

36 Cooke, V. G. *et al.* Pericyte depletion results in hypoxia-associated epithelial-to-mesenchymal transition and metastasis mediated by met signaling pathway. *Cancer cell* **21**, 66-81, doi:10.1016/j.ccr.2011.11.024 (2012).

37 Dimberg, A. Chemokines in angiogenesis. *Current topics in microbiology and immunology* **341**, 59-80, doi:10.1007/82_2010_21 (2010).

38 Bonapace, L. *et al.* Cessation of CCL2 inhibition accelerates breast cancer metastasis by promoting angiogenesis. *Nature* **515**, 130-133, doi:10.1038/nature13862 (2014).

39 Balkwill, F. & Mantovani, A. Inflammation and cancer: back to Virchow? *Lancet* **357**, 539-545, doi:10.1016/S0140-6736(00)04046-0 (2001).

40 Grivennikov, S. I., Greten, F. R. & Karin, M. Immunity, inflammation, and cancer. *Cell* **140**, 883-899, doi:10.1016/j.cell.2010.01.025 (2010).

41 Egeblad, M., Nakasone, E. S. & Werb, Z. Tumors as organs: complex tissues that interface with the entire organism. *Developmental cell* **18**, 884-901, doi:10.1016/j.devcel.2010.05.012 (2010).

42 Colotta, F., Allavena, P., Sica, A., Garlanda, C. & Mantovani, A. Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. *Carcinogenesis* **30**, 1073-1081, doi:10.1093/carcin/bgp127 (2009).

43 Egeblad, M. *et al.* Visualizing stromal cell dynamics in different tumor microenvironments by spinning disk confocal microscopy. *Disease models & mechanisms* **1**, 155-167; discussion 165, doi:10.1242/dmm.000596 (2008).

44 Lewis, C. E. & Pollard, J. W. Distinct role of macrophages in different tumor microenvironments. *Cancer research* **66**, 605-612, doi:10.1158/0008-5472.CAN-05-4005 (2006).

45 Wyckoff, J. B. *et al.* Direct visualization of macrophage-assisted tumor cell intravasation in mammary tumors. *Cancer research* **67**, 2649-2656, doi:10.1158/0008-5472.CAN-06-1823 (2007).

46 Pollard, J. W. Trophic macrophages in development and disease. *Nature reviews. Immunology* **9**, 259-270, doi:10.1038/nri2528 (2009).

47 Williams, C. B., Yeh, E. S. & Soloff, A. C. Tumor-associated macrophages: unwitting accomplices in breast cancer malignancy. *NPJ breast cancer* **2**, doi:10.1038/npjbcancer.2015.25 (2016).

48 Mantovani, A., Romero, P., Palucka, A. K. & Marincola, F. M. Tumour immunity: effector response to tumour and role of the microenvironment. *Lancet* **371**, 771-783, doi:10.1016/S0140-6736(08)60241-X (2008).

49 Mantovani, A., Allavena, P., Sica, A. & Balkwill, F. Cancer-related inflammation. *Nature* **454**, 436-444, doi:10.1038/nature07205 (2008).

50 Mantovani, A., Garlanda, C. & Locati, M. Macrophage diversity and polarization in atherosclerosis: a question of balance. *Arteriosclerosis, thrombosis, and vascular biology* **29**, 1419-1423, doi:10.1161/ATVBAHA.108.180497 (2009).

51 Quatromoni, J. G. & Eruslanov, E. Tumor-associated macrophages: function, phenotype, and link to prognosis in human lung cancer. *American journal of translational research* **4**, 376-389 (2012).

52 Sousa, S. *et al.* Human breast cancer cells educate macrophages toward the M2 activation status. *Breast cancer research : BCR* **17**, 101, doi:10.1186/s13058-015-0621-0 (2015).

53 Condeelis, J. & Pollard, J. W. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. *Cell* **124**, 263-266, doi:10.1016/j.cell.2006.01.007 (2006).

54 Murdoch, C., Muthana, M., Coffelt, S. B. & Lewis, C. E. The role of myeloid cells in the promotion of tumour angiogenesis. *Nature reviews. Cancer* **8**, 618-631, doi:10.1038/nrc2444 (2008).

55 Gazzaniga, S. *et al.* Targeting tumor-associated macrophages and inhibition of MCP-1 reduce angiogenesis and tumor growth in a human melanoma xenograft. *The Journal of investigative dermatology* **127**, 2031-2041, doi:10.1038/sj.jid.5700827 (2007).

56 Vesely, M. D., Kershaw, M. H., Schreiber, R. D. & Smyth, M. J. Natural innate and adaptive immunity to cancer. *Annual review of immunology* **29**, 235-271, doi:10.1146/annurev-immunol-031210-101324 (2011).

57 Schiavoni, G., Gabriele, L. & Mattei, F. The tumor microenvironment: a pitch for multiple players. *Frontiers in oncology* **3**, 90, doi:10.3389/fonc.2013.00090 (2013).

58 DeNardo, D. G. *et al.* CD4(+) T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages. *Cancer cell* **16**, 91-102, doi:10.1016/j.ccr.2009.06.018 (2009).

59 Bailey, S. R. *et al.* Th17 cells in cancer: the ultimate identity crisis. *Frontiers in immunology* **5**, 276, doi:10.3389/fimmu.2014.00276 (2014).

60 He, D. *et al.* IL-17 mediated inflammation promotes tumor growth and progression in the skin. *PloS one* **7**, e32126, doi:10.1371/journal.pone.0032126 (2012).

61 Muranski, P. *et al.* Tumor-specific Th17-polarized cells eradicate large established melanoma. *Blood* **112**, 362-373, doi:10.1182/blood-2007-11-120998 (2008).

62 Yang, Z. Z. & Ansell, S. M. The tumor microenvironment in follicular lymphoma. *Clinical advances in hematology & oncology : H&O* **10**, 810-818 (2012).

63 Yu, P. *et al.* Intratumor depletion of CD4+ cells unmasks tumor immunogenicity leading to the rejection of late-stage tumors. *The Journal of experimental medicine* **201**, 779-791, doi:10.1084/jem.20041684 (2005).

64 Zou, C. M. W. W. W. K. F. W. *Cancer Immunotherapy*. 17-44 (Springer Science, 2013).

65 Finotello, F. & Trajanoski, Z. New strategies for cancer immunotherapy: targeting regulatory T cells. *Genome medicine* **9**, 10, doi:10.1186/s13073-017-0402-8 (2017).

66 Geukes Foppen, M. H., Donia, M., Svane, I. M. & Haanen, J. B. Tumor-infiltrating lymphocytes for the treatment of metastatic cancer. *Molecular oncology* **9**, 1918-1935, doi:10.1016/j.molonc.2015.10.018 (2015).

67 Postow, M. A., Callahan, M. K. & Wolchok, J. D. Immune Checkpoint Blockade in Cancer Therapy. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **33**, 1974-1982, doi:10.1200/JCO.2014.59.4358 (2015).

68 Rusakiewicz, S. *et al.* Immune infiltrates are prognostic factors in localized gastrointestinal stromal tumors. *Cancer research* **73**, 3499-3510, doi:10.1158/0008-5472.CAN-13-0371 (2013).

69 Le Maux Chansac, B. *et al.* NK cells infiltrating a MHC class I-deficient lung adenocarcinoma display impaired cytotoxic activity toward autologous tumor cells associated with altered NK cell-triggering receptors. *Journal of immunology* **175**, 5790-5798 (2005).

70 Cantoni, C. *et al.* NK Cells, Tumor Cell Transition, and Tumor Progression in Solid Malignancies: New Hints for NK-Based Immunotherapy? *Journal of immunology research* **2016**, 4684268, doi:10.1155/2016/4684268 (2016).

71 Stojanovic, A., Correia, M. P. & Cerwenka, A. Shaping of NK cell responses by the tumor microenvironment. *Cancer microenvironment : official journal of the International Cancer Microenvironment Society* **6**, 135-146, doi:10.1007/s12307-012-0125-8 (2013).

72 Bryceson, Y. T., March, M. E., Ljunggren, H. G. & Long, E. O. Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. *Blood* **107**, 159-166, doi:10.1182/blood-2005-04-1351 (2006).

73 Terme, M. *et al.* Regulatory T cells control dendritic cell/NK cell cross-talk in lymph nodes at the steady state by inhibiting CD4+ self-reactive T cells. *Journal of immunology* **180**, 4679-4686 (2008).

74 Smyth, M. J. *et al.* CD4+CD25+ T regulatory cells suppress NK cell-mediated immunotherapy of cancer. *Journal of immunology* **176**, 1582-1587 (2006).

75 Sarhan, D. *et al.* Dendritic cell regulation of NK-cell responses involves lymphotoxin-alpha, IL-12, and TGF-beta. *European journal of immunology* **45**, 1783-1793, doi:10.1002/eji.201444885 (2015).

76 Xu, L. *et al.* Cord blood CD4(+)CD25(+) regulatory T cells fail to inhibit cord blood NK cell functions due to insufficient production and expression of TGF-beta1. *Cellular immunology* **290**, 89-95, doi:10.1016/j.cellimm.2014.05.007 (2014).

77 Hasmim, M. *et al.* Critical Role of Tumor Microenvironment in Shaping NK Cell Functions: Implication of Hypoxic Stress. *Frontiers in immunology* **6**, 482, doi:10.3389/fimmu.2015.00482 (2015).

78 Vivier, E. *et al.* Innate or adaptive immunity? The example of natural killer cells. *Science* **331**, 44-49, doi:10.1126/science.1198687 (2011).

79 Fauriat, C., Long, E. O., Ljunggren, H. G. & Bryceson, Y. T. Regulation of human NK-cell cytokine and chemokine production by target cell recognition. *Blood* **115**, 2167-2176, doi:10.1182/blood-2009-08-238469 (2010).

80 Maghazachi, A. A. Role of chemokines in the biology of natural killer cells. *Current topics in microbiology and immunology* **341**, 37-58, doi:10.1007/82_2010_20 (2010).

81 Pietra, G. *et al.* Melanoma cells inhibit natural killer cell function by modulating the expression of activating receptors and cytolytic activity. *Cancer research* **72**, 1407-1415, doi:10.1158/0008-5472.CAN-11-2544 (2012).

82 Benson, D. M., Jr. *et al.* The PD-1/PD-L1 axis modulates the natural killer cell versus multiple myeloma effect: a therapeutic target for CT-011, a novel monoclonal anti-PD-1 antibody. *Blood* **116**, 2286-2294, doi:10.1182/blood-2010-02-271874 (2010).

83 Umansky, V., Blattner, C., Gebhardt, C. & Utikal, J. The Role of Myeloid-Derived Suppressor Cells (MDSC) in Cancer Progression. *Vaccines* **4**, doi:10.3390/vaccines4040036 (2016).

84 Morales, J. K., Kmieciak, M., Knutson, K. L., Bear, H. D. & Manjili, M. H. GM-CSF is one of the main breast tumor-derived soluble factors involved in the differentiation of CD11b-Gr1- bone marrow progenitor cells into myeloid-derived suppressor cells. *Breast cancer research and treatment* **123**, 39-49, doi:10.1007/s10549-009-0622-8 (2010).

85 Flavell, R. A., Sanjabi, S., Wrzesinski, S. H. & Licona-Limon, P. The polarization of immune cells in the tumour environment by TGFbeta. *Nature reviews. Immunology* **10**, 554-567, doi:10.1038/nri2808 (2010).

86 Gabrilovich, D. I. & Nagaraj, S. Myeloid-derived suppressor cells as regulators of the immune system. *Nature reviews. Immunology* **9**, 162-174, doi:10.1038/nri2506 (2009).

87 Yang, L. *et al.* Expansion of myeloid immune suppressor Gr+CD11b+ cells in tumor-bearing host directly promotes tumor angiogenesis. *Cancer cell* **6**, 409-421, doi:10.1016/j.ccr.2004.08.031 (2004).

88 Yang, L. *et al.* Abrogation of TGF beta signaling in mammary carcinomas recruits Gr-1+CD11b+ myeloid cells that promote metastasis. *Cancer cell* **13**, 23-35, doi:10.1016/j.ccr.2007.12.004 (2008).

89 Xu, J. *et al.* CSF1R signaling blockade stanches tumor-infiltrating myeloid cells and improves the efficacy of radiotherapy in prostate cancer. *Cancer research* **73**, 2782-2794, doi:10.1158/0008-5472.CAN-12-3981 (2013).

90 Martin, F. & Chan, A. C. B cell immunobiology in disease: evolving concepts from the clinic. *Annual review of immunology* **24**, 467-496, doi:10.1146/annurev.immunol.24.021605.090517 (2006).

91 Tsou, P., Katayama, H., Ostrin, E. J. & Hanash, S. M. The Emerging Role of B Cells in Tumor Immunity. *Cancer research* **76**, 5597-5601, doi:10.1158/0008-5472.CAN-16-0431 (2016).

92 Nielsen, J. S. *et al.* CD20+ tumor-infiltrating lymphocytes have an atypical CD27- memory phenotype and together with CD8+ T cells promote favorable prognosis in ovarian cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* **18**, 3281-3292, doi:10.1158/1078-0432.CCR-12-0234 (2012).

93 Fremd, C., Schuetz, F., Sohn, C., Beckhove, P. & Domschke, C. B cell-regulated immune responses in tumor models and cancer patients. *Oncoimmunology* **2**, e25443, doi:10.4161/onci.25443 (2013).

94 Nelson, B. H. CD20+ B cells: the other tumor-infiltrating lymphocytes. *Journal of immunology* **185**, 4977-4982, doi:10.4049/jimmunol.1001323 (2010).

95 Zhou, J. *et al.* Enhanced frequency and potential mechanism of B regulatory cells in patients with lung cancer. *Journal of translational medicine* **12**, 304, doi:10.1186/s12967-014-0304-0 (2014).

96 Wang, W. W. *et al.* CD19+CD24hiCD38hiBregs involved in downregulate helper T cells and upregulate regulatory T cells in gastric cancer. *Oncotarget* **6**, 33486-33499, doi:10.18632/oncotarget.5588 (2015).

97 Schwartz, M., Zhang, Y. & Rosenblatt, J. D. B cell regulation of the anti-tumor response and role in carcinogenesis. *Journal for immunotherapy of cancer* **4**, 40, doi:10.1186/s40425-016-0145-x (2016).

98 Mizoguchi, A., Mizoguchi, E., Takedatsu, H., Blumberg, R. S. & Bhan, A. K. Chronic intestinal inflammatory condition generates IL-10-producing regulatory B cell subset characterized by CD1d upregulation. *Immunity* **16**, 219-230 (2002).

99 Ammirante, M., Luo, J. L., Grivennikov, S., Nedospasov, S. & Karin, M. B-cell-derived lymphotoxin promotes castration-resistant prostate cancer. *Nature* **464**, 302-305, doi:10.1038/nature08782 (2010).

100 Gunderson, A. J. *et al.* Bruton Tyrosine Kinase-Dependent Immune Cell Cross-talk Drives Pancreas Cancer. *Cancer discovery* **6**, 270-285, doi:10.1158/2159-8290.CD-15-0827 (2016).

101 Engelhardt, J. J. *et al.* Marginating dendritic cells of the tumor microenvironment cross-present tumor antigens and stably engage tumor-specific T cells. *Cancer cell* **21**, 402-417, doi:10.1016/j.ccr.2012.01.008 (2012).

102 Tran Janco, J. M., Lamichhane, P., Karyampudi, L. & Knutson, K. L. Tumor-infiltrating dendritic cells in cancer pathogenesis. *Journal of immunology* **194**, 2985-2991, doi:10.4049/jimmunol.1403134 (2015).

103 Ghiringhelli, F. *et al.* Tumor cells convert immature myeloid dendritic cells into TGF-beta-secreting cells inducing CD4+CD25+ regulatory T cell proliferation. *The Journal of experimental medicine* **202**, 919-929, doi:10.1084/jem.20050463 (2005).

104 Shurin, G. V., Ouellette, C. E. & Shurin, M. R. Regulatory dendritic cells in the tumor immunoenvironment. *Cancer immunology, immunotherapy : CII* **61**, 223-230, doi:10.1007/s00262-011-1138-8 (2012).

105 Ma, Y., Shurin, G. V., Peiyuan, Z. & Shurin, M. R. Dendritic cells in the cancer microenvironment. *Journal of Cancer* **4**, 36-44, doi:10.7150/jca.5046 (2013).

106 Laoui, D. *et al.* The tumour microenvironment harbours ontogenically distinct dendritic cell populations with opposing effects on tumour immunity. *Nature communications* **7**, 13720, doi:10.1038/ncomms13720 (2016).

107 Kojima, Y. *et al.* Autocrine TGF-beta and stromal cell-derived factor-1 (SDF-1) signaling drives the evolution of tumor-promoting mammary stromal myofibroblasts. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 20009-20014, doi:10.1073/pnas.1013805107 (2010).

108 Kalluri, R. The biology and function of fibroblasts in cancer. *Nature reviews. Cancer* **16**, 582-598, doi:10.1038/nrc.2016.73 (2016).

109 Guido, C. *et al.* Metabolic reprogramming of cancer-associated fibroblasts by TGF-beta drives tumor growth: connecting TGF-beta signaling with "Warburg-like" cancer

metabolism and L-lactate production. *Cell cycle* **11**, 3019-3035, doi:10.4161/cc.21384 (2012).

110 Lochter, A. *et al.* Matrix metalloproteinase stromelysin-1 triggers a cascade of molecular alterations that leads to stable epithelial-to-mesenchymal conversion and a premalignant phenotype in mammary epithelial cells. *The Journal of cell biology* **139**, 1861-1872 (1997).

111 Kalluri, R. & Weinberg, R. A. The basics of epithelial-mesenchymal transition. *The Journal of clinical investigation* **119**, 1420-1428, doi:10.1172/JCI39104 (2009).

112 Quante, M. *et al.* Bone marrow-derived myofibroblasts contribute to the mesenchymal stem cell niche and promote tumor growth. *Cancer cell* **19**, 257-272, doi:10.1016/j.ccr.2011.01.020 (2011).

113 O'Connell, J. T. *et al.* VEGF-A and Tenascin-C produced by S100A4+ stromal cells are important for metastatic colonization. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 16002-16007, doi:10.1073/pnas.1109493108 (2011).

114 Pinchuk, I. V. *et al.* PD-1 ligand expression by human colonic myofibroblasts/fibroblasts regulates CD4+ T-cell activity. *Gastroenterology* **135**, 1228-1237, 1237 e1221-1222, doi:10.1053/j.gastro.2008.07.016 (2008).

115 Hu, Y. *et al.* Fibroblast-Derived Exosomes Contribute to Chemoresistance through Priming Cancer Stem Cells in Colorectal Cancer. *PloS one* **10**, e0125625, doi:10.1371/journal.pone.0125625 (2015).

116 Sun, Y. *et al.* Treatment-induced damage to the tumor microenvironment promotes prostate cancer therapy resistance through WNT16B. *Nature medicine* **18**, 1359-1368, doi:10.1038/nm.2890 (2012).

117 Martinez-Outschoorn, U. E. *et al.* Anti-estrogen resistance in breast cancer is induced by the tumor microenvironment and can be overcome by inhibiting mitochondrial function in epithelial cancer cells. *Cancer biology & therapy* **12**, 924-938, doi:10.4161/cbt.12.10.17780 (2011).

118 Karin, M. Nuclear factor-kappaB in cancer development and progression. *Nature* **441**, 431-436, doi:10.1038/nature04870 (2006).

119 Marienfeld, R. *et al.* RelB forms transcriptionally inactive complexes with RelA/p65. *The Journal of biological chemistry* **278**, 19852-19860, doi:10.1074/jbc.M301945200 (2003).

120 Hayden, M. S. & Ghosh, S. NF-kappaB, the first quarter-century: remarkable progress and outstanding questions. *Genes & development* **26**, 203-234, doi:10.1101/gad.183434.111 (2012).

121 Barnes, P. J. & Karin, M. Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. *The New England journal of medicine* **336**, 1066-1071, doi:10.1056/NEJM199704103361506 (1997).

122 Oeckinghaus, A., Hayden, M. S. & Ghosh, S. Crosstalk in NF-kappaB signaling pathways. *Nature immunology* **12**, 695-708, doi:10.1038/ni.2065 (2011).

123 Liu, M. *et al.* The canonical NF-kappaB pathway governs mammary tumorigenesis in transgenic mice and tumor stem cell expansion. *Cancer research* **70**, 10464-10473, doi:10.1158/0008-5472.CAN-10-0732 (2010).

124 Hagemann, T. *et al.* "Re-educating" tumor-associated macrophages by targeting NF-kappaB. *The Journal of experimental medicine* **205**, 1261-1268, doi:10.1084/jem.20080108 (2008).

125 Kono, Y., Kawakami, S., Higuchi, Y., Yamashita, F. & Hashida, M. In vitro evaluation of inhibitory effect of nuclear factor-kappaB activity by small interfering RNA on pro-tumor characteristics of M2-like macrophages. *Biological & pharmaceutical bulletin* **37**, 137-144 (2014).

126 Takahashi, H., Ogata, H., Nishigaki, R., Broide, D. H. & Karin, M. Tobacco smoke promotes lung tumorigenesis by triggering IKKbeta- and JNK1-dependent inflammation. *Cancer cell* **17**, 89-97, doi:10.1016/j.ccr.2009.12.008 (2010).

127 Yang, J. *et al.* Conditional ablation of Ikkb inhibits melanoma tumor development in mice. *The Journal of clinical investigation* **120**, 2563-2574, doi:10.1172/JCI42358 (2010).

128 Karin, M. NF-kappaB as a critical link between inflammation and cancer. *Cold Spring Harbor perspectives in biology* **1**, a000141, doi:10.1101/cshperspect.a000141 (2009).

129 Chefetz, I., Holmberg, J. C., Alvero, A. B., Visintin, I. & Mor, G. Inhibition of Aurora-A kinase induces cell cycle arrest in epithelial ovarian cancer stem cells by affecting NFkB pathway. *Cell cycle* **10**, 2206-2214, doi:10.4161/cc.10.13.16348 (2011).

130 Alvero, A. B. *et al.* Molecular phenotyping of human ovarian cancer stem cells unravels the mechanisms for repair and chemoresistance. *Cell cycle* **8**, 158-166, doi:10.4161/cc.8.1.7533 (2009).

131 Huber, M. A. *et al.* NF-kappaB is essential for epithelial-mesenchymal transition and metastasis in a model of breast cancer progression. *The Journal of clinical investigation* **114**, 569-581, doi:10.1172/JCI21358 (2004).

132 Xie, T. X., Xia, Z., Zhang, N., Gong, W. & Huang, S. Constitutive NF-kappaB activity regulates the expression of VEGF and IL-8 and tumor angiogenesis of human glioblastoma. *Oncology reports* **23**, 725-732 (2010).

133 Yoshida, A., Yoshida, S., Ishibashi, T., Kuwano, M. & Inomata, H. Suppression of retinal neovascularization by the NF-kappaB inhibitor pyrrolidine dithiocarbamate in mice. *Investigative ophthalmology & visual science* **40**, 1624-1629 (1999).

134 Disis, M. L. Immune regulation of cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **28**, 4531-4538, doi:10.1200/JCO.2009.27.2146 (2010).

135 Nishida, N., Yano, H., Nishida, T., Kamura, T. & Kojiro, M. Angiogenesis in cancer. *Vascular health and risk management* **2**, 213-219 (2006).

136 Moretta, L. *et al.* Human natural killer cells: origin, receptors, function, and clinical applications. *International archives of allergy and immunology* **164**, 253-264, doi:10.1159/000365632 (2014).

137 Crowther, M., Brown, N. J., Bishop, E. T. & Lewis, C. E. Microenvironmental influence on macrophage regulation of angiogenesis in wounds and malignant tumors. *Journal of leukocyte biology* **70**, 478-490 (2001).

138 Ke, Q. & Costa, M. Hypoxia-inducible factor-1 (HIF-1). *Molecular pharmacology* **70**, 1469-1480, doi:10.1124/mol.106.027029 (2006).

139 Chung, A. S., Lee, J. & Ferrara, N. Targeting the tumour vasculature: insights from physiological angiogenesis. *Nature reviews. Cancer* **10**, 505-514, doi:10.1038/nrc2868 (2010).

140 Folkman J, K. R. *Holland-Frei Cancer Medicine*. 6th edn, (BC Decker, 2003).

141 Gilkes, D. M., Semenza, G. L. & Wirtz, D. Hypoxia and the extracellular matrix: drivers of tumour metastasis. *Nature reviews. Cancer* **14**, 430-439, doi:10.1038/nrc3726 (2014).

142 Yang, M. H. *et al.* Direct regulation of TWIST by HIF-1alpha promotes metastasis. *Nature cell biology* **10**, 295-305, doi:10.1038/ncb1691 (2008).

143 Erler, J. T. *et al.* Lysyl oxidase is essential for hypoxia-induced metastasis. *Nature* **440**, 1222-1226, doi:10.1038/nature04695 (2006).

144 Lukashev, D. *et al.* Cutting edge: hypoxia-inducible factor 1alpha and its activation-inducible short isoform I.1 negatively regulate functions of CD4+ and CD8+ T lymphocytes. *Journal of immunology* **177**, 4962-4965 (2006).

145 Philip, B., Ito, K., Moreno-Sanchez, R. & Ralph, S. J. HIF expression and the role of hypoxic microenvironments within primary tumours as protective sites driving cancer stem cell renewal and metastatic progression. *Carcinogenesis* **34**, 1699-1707, doi:10.1093/carcin/bgt209 (2013).

146 Stewart, M. D., Ritterhoff, T., Klevit, R. E. & Brzovic, P. S. E2 enzymes: more than just middle men. *Cell research* **26**, 423-440, doi:10.1038/cr.2016.35 (2016).

147 Scheffner, M., Nuber, U. & Huibregtse, J. M. Protein ubiquitination involving an E1-E2-E3 enzyme ubiquitin thioester cascade. *Nature* **373**, 81-83, doi:10.1038/373081a0 (1995).

148 Choi, S. H. & Park, J. Y. Erratum to: Regulation of the hypoxic tumor environment in hepatocellular carcinoma using RNA interference. *Cancer cell international* **17**, 69, doi:10.1186/s12935-017-0438-2 (2017).

149 Rius, J. *et al.* NF-kappaB links innate immunity to the hypoxic response through transcriptional regulation of HIF-1alpha. *Nature* **453**, 807-811, doi:10.1038/nature06905 (2008).

150 Walmsley, S. R. *et al.* Hypoxia-induced neutrophil survival is mediated by HIF-1alpha-dependent NF-kappaB activity. *The Journal of experimental medicine* **201**, 105-115, doi:10.1084/jem.20040624 (2005).

151 Joruiz, S. M. & Bourdon, J. C. p53 Isoforms: Key Regulators of the Cell Fate Decision. *Cold Spring Harbor perspectives in medicine* **6**, doi:10.1101/cshperspect.a026039 (2016).

152 Teodoro, J. G., Evans, S. K. & Green, M. R. Inhibition of tumor angiogenesis by p53: a new role for the guardian of the genome. *Journal of molecular medicine* **85**, 1175-1186, doi:10.1007/s00109-007-0221-2 (2007).

153 Ferraiuolo, M., Di Agostino, S., Blandino, G. & Strano, S. Oncogenic Intra-p53 Family Member Interactions in Human Cancers. *Frontiers in oncology* **6**, 77, doi:10.3389/fonc.2016.00077 (2016).

154 Mills, A. A. *et al.* p63 is a p53 homologue required for limb and epidermal morphogenesis. *Nature* **398**, 708-713, doi:10.1038/19531 (1999).

155 Yang, A. *et al.* p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours. *Nature* **404**, 99-103, doi:10.1038/35003607 (2000).

156 Donehower, L. A. *et al.* Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* **356**, 215-221, doi:10.1038/356215a0 (1992).

157 Kaghad, M. *et al.* Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. *Cell* **90**, 809-819 (1997).

158 Pietsch, E. C., Sykes, S. M., McMahon, S. B. & Murphy, M. E. The p53 family and programmed cell death. *Oncogene* **27**, 6507-6521, doi:10.1038/onc.2008.315 (2008).

159 Talos, F., Nemajerova, A., Flores, E. R., Petrenko, O. & Moll, U. M. p73 suppresses polyploidy and aneuploidy in the absence of functional p53. *Molecular cell* **27**, 647-659, doi:10.1016/j.molcel.2007.06.036 (2007).

160 Dotsch, V., Bernassola, F., Coutandin, D., Candi, E. & Melino, G. p63 and p73, the ancestors of p53. *Cold Spring Harbor perspectives in biology* **2**, a004887, doi:10.1101/cshperspect.a004887 (2010).

161 Niklison-Chirou, M. V. *et al.* How Does p73 Cause Neuronal Defects? *Molecular neurobiology* **53**, 4509-4520, doi:10.1007/s12035-015-9381-1 (2016).

162 Du, W. *et al.* TAp73 enhances the pentose phosphate pathway and supports cell proliferation. *Nature cell biology* **15**, 991-1000, doi:10.1038/ncb2789 (2013).

163 Murray-Zmijewski, F., Lane, D. P. & Bourdon, J. C. p53/p63/p73 isoforms: an orchestra of isoforms to harmonise cell differentiation and response to stress. *Cell death and differentiation* **13**, 962-972, doi:10.1038/sj.cdd.4401914 (2006).

164 Rufini, A. *et al.* p73 in Cancer. *Genes & cancer* **2**, 491-502, doi:10.1177/1947601911408890 (2011).

165 Petrenko, O., Zaika, A. & Moll, U. M. deltaNp73 facilitates cell immortalization and cooperates with oncogenic Ras in cellular transformation in vivo. *Molecular and cellular biology* **23**, 5540-5555 (2003).

166 Tomasini, R. *et al.* TAp73 knockout shows genomic instability with infertility and tumor suppressor functions. *Genes & development* **22**, 2677-2691, doi:10.1101/gad.1695308 (2008).

167 Lucena-Araujo, A. R. *et al.* High DeltaNp73/TAp73 ratio is associated with poor prognosis in acute promyelocytic leukemia. *Blood* **126**, 2302-2306, doi:10.1182/blood-2015-01-623330 (2015).

168 Nurse, P. A long twentieth century of the cell cycle and beyond. *Cell* **100**, 71-78 (2000).

169 Gaiddon, C. *et al.* Cyclin-dependent kinases phosphorylate p73 at threonine 86 in a cell cycle-dependent manner and negatively regulate p73. *The Journal of biological chemistry* **278**, 27421-27431, doi:10.1074/jbc.M300251200 (2003).

170 Lee, C. W. & La Thangue, N. B. Promoter specificity and stability control of the p53-related protein p73. *Oncogene* **18**, 4171-4181, doi:10.1038/sj.onc.1202793 (1999).

171 Strano, S. *et al.* The transcriptional coactivator Yes-associated protein drives p73 gene-target specificity in response to DNA Damage. *Molecular cell* **18**, 447-459, doi:10.1016/j.molcel.2005.04.008 (2005).

172 Toh, W. H., Nam, S. Y. & Sabapathy, K. An essential role for p73 in regulating mitotic cell death. *Cell death and differentiation* **17**, 787-800, doi:10.1038/cdd.2009.181 (2010).

173 Urist, M., Tanaka, T., Poyurovsky, M. V. & Prives, C. p73 induction after DNA damage is regulated by checkpoint kinases Chk1 and Chk2. *Genes & development* **18**, 3041-3054, doi:10.1101/gad.1221004 (2004).

174 Tomasini, R. *et al.* TAp73 regulates the spindle assembly checkpoint by modulating BubR1 activity. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 797-802, doi:10.1073/pnas.0812096106 (2009).

175 Beitzinger, M. *et al.* p73 poses a barrier to malignant transformation by limiting anchorage-independent growth. *The EMBO journal* **27**, 792-803, doi:10.1038/emboj.2008.13 (2008).

176 Stiewe, T., Zimmermann, S., Frilling, A., Esche, H. & Putzer, B. M. Transactivation-deficient DeltaTA-p73 acts as an oncogene. *Cancer research* **62**, 3598-3602 (2002).

177 Stiewe, T. *et al.* Inactivation of retinoblastoma (RB) tumor suppressor by oncogenic isoforms of the p53 family member p73. *The Journal of biological chemistry* **278**, 14230-14236, doi:10.1074/jbc.M300357200 (2003).

178 Chang, H. Y. & Yang, X. Proteases for cell suicide: functions and regulation of caspases. *Microbiology and molecular biology reviews : MMBR* **64**, 821-846 (2000).

179 Harms, K., Nozell, S. & Chen, X. The common and distinct target genes of the p53 family transcription factors. *Cellular and molecular life sciences : CMLS* **61**, 822-842, doi:10.1007/s00018-003-3304-4 (2004).

180 Di, C. *et al.* Mechanisms, function and clinical applications of DNp73. *Cell cycle* **12**, 1861-1867, doi:10.4161/cc.24967 (2013).

181 Schilling, T. *et al.* Active transcription of the human FAS/CD95/TNFRSF6 gene involves the p53 family. *Biochemical and biophysical research communications* **387**, 399-404, doi:10.1016/j.bbrc.2009.07.063 (2009).

182 Terrasson, J. *et al.* p73-dependent apoptosis through death receptor: impairment by human cytomegalovirus infection. *Cancer research* **65**, 2787-2794, doi:10.1158/0008-5472.CAN-04-2019 (2005).

183 Muller, M. *et al.* TAp73/Delta Np73 influences apoptotic response, chemosensitivity and prognosis in hepatocellular carcinoma. *Cell death and differentiation* **12**, 1564-1577, doi:10.1038/sj.cdd.4401774 (2005).

184 Chau, B. N., Chen, T. T., Wan, Y. Y., DeGregori, J. & Wang, J. Y. Tumor necrosis factor alpha-induced apoptosis requires p73 and c-ABL activation downstream of RB degradation. *Molecular and cellular biology* **24**, 4438-4447 (2004).

185 Schuster, A. *et al.* DeltaNp73beta is oncogenic in hepatocellular carcinoma by blocking apoptosis signaling via death receptors and mitochondria. *Cell cycle* **9**, 2629-2639, doi:10.4161/cc.9.13.12110 (2010).

186 Liu, T., Roh, S. E., Woo, J. A., Ryu, H. & Kang, D. E. Cooperative role of RanBP9 and P73 in mitochondria-mediated apoptosis. *Cell death & disease* **4**, e476, doi:10.1038/cddis.2012.203 (2013).

187 White, E. The role for autophagy in cancer. *The Journal of clinical investigation* **125**, 42-46, doi:10.1172/JCI73941 (2015).

188 Crighton, D., O'Prey, J., Bell, H. S. & Ryan, K. M. p73 regulates DRAM-independent autophagy that does not contribute to programmed cell death. *Cell death and differentiation* **14**, 1071-1079, doi:10.1038/sj.cdd.4402108 (2007).

189 He, Z. *et al.* p73 regulates autophagy and hepatocellular lipid metabolism through a transcriptional activation of the ATG5 gene. *Cell death and differentiation* **20**, 1415-1424, doi:10.1038/cdd.2013.104 (2013).

190 Rosenbluth, J. M., Mays, D. J., Pino, M. F., Tang, L. J. & Pietenpol, J. A. A gene signature-based approach identifies mTOR as a regulator of p73. *Molecular and cellular biology* **28**, 5951-5964, doi:10.1128/MCB.00305-08 (2008).

191 Yan, W., Zhang, J., Zhang, Y., Jung, Y. S. & Chen, X. p73 expression is regulated by RNPC1, a target of the p53 family, via mRNA stability. *Molecular and cellular biology* **32**, 2336-2348, doi:10.1128/MCB.00215-12 (2012).

192 Rufini, A. *et al.* TAp73 depletion accelerates aging through metabolic dysregulation. *Genes & development* **26**, 2009-2014, doi:10.1101/gad.197640.112 (2012).

193 Agostini, M. *et al.* p73 Regulates Primary Cortical Neuron Metabolism: a Global Metabolic Profile. *Molecular neurobiology*, doi:10.1007/s12035-017-0517-3 (2017).

194 Amelio, I. *et al.* TAp73 opposes tumor angiogenesis by promoting hypoxia-inducible factor 1alpha degradation. *Proceedings of the National Academy of Sciences of the United States of America* **112**, 226-231, doi:10.1073/pnas.1410609111 (2015).

195 Dulloo, I., Hooi, P. B. & Sabapathy, K. Hypoxia-induced DNp73 stabilization regulates Vegf-A expression and tumor angiogenesis similar to TAp73. *Cell Cycle* **14**, 3533-3539, doi:10.1080/15384101.2015.1078038 (2015).

196 Fernandez-Alonso, R. *et al.* p73 is required for endothelial cell differentiation, migration and the formation of vascular networks regulating VEGF and TGFbeta signaling. *Cell death and differentiation* **22**, 1287-1299, doi:10.1038/cdd.2014.214 (2015).

197 Galtsidis, S. *et al.* Unravelling a p73-regulated network: The role of a novel p73-dependent target, MIR3158, in cancer cell migration and invasiveness. *Cancer letters* **388**, 96-106, doi:10.1016/j.canlet.2016.11.036 (2017).

198 Rodhe, J., Kavanagh, E. & Joseph, B. TAp73beta-mediated suppression of cell migration requires p57Kip2 control of actin cytoskeleton dynamics. *Oncotarget* **4**, 289-297, doi:10.18632/oncotarget.833 (2013).

199 Tamura, M. *et al.* Forkhead transcription factor FOXF1 is a novel target gene of the p53 family and regulates cancer cell migration and invasiveness. *Oncogene* **33**, 4837-4846, doi:10.1038/onc.2013.427 (2014).

200 Thakur, A. K. *et al.* TAp73 loss favors Smad-independent TGF-beta signaling that drives EMT in pancreatic ductal adenocarcinoma. *Cell death and differentiation* **23**, 1358-1370, doi:10.1038/cdd.2016.18 (2016).

201 Zhang, Y., Yan, W., Jung, Y. S. & Chen, X. Mammary epithelial cell polarity is regulated differentially by p73 isoforms via epithelial-to-mesenchymal transition. *The Journal of biological chemistry* **287**, 17746-17753, doi:10.1074/jbc.M112.358143 (2012).

202 Zhang, Y., Young, A., Zhang, J. & Chen, X. P73 tumor suppressor and its targets, p21 and PUMA, are required for madin-darby canine kidney cell morphogenesis by maintaining an appropriate level of epithelial to mesenchymal transition. *Oncotarget* **6**, 13994-14004, doi:10.18632/oncotarget.4374 (2015).

203 Steder, M. *et al.* DNp73 exerts function in metastasis initiation by disconnecting the inhibitory role of EPLIN on IGF1R-AKT/STAT3 signaling. *Cancer cell* **24**, 512-527, doi:10.1016/j.ccr.2013.08.023 (2013).

204 Dominguez, G. *et al.* DeltaTAp73 upregulation correlates with poor prognosis in human tumors: putative in vivo network involving p73 isoforms, p53, and E2F-1. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **24**, 805-815, doi:10.1200/JCO.2005.02.2350 (2006).

205 Meier, M. *et al.* Differential expression of p73 isoforms in relation to drug resistance in childhood T-lineage acute lymphoblastic leukaemia. *Leukemia* **20**, 1377-1384, doi:10.1038/sj.leu.2404288 (2006).

206 Alla, V. *et al.* E2F1 confers anticancer drug resistance by targeting ABC transporter family members and Bcl-2 via the p73/DNp73-miR-205 circuitry. *Cell cycle* **11**, 3067-3078, doi:10.4161/cc.21476 (2012).

207 Rees, D. C., Johnson, E. & Lewinson, O. ABC transporters: the power to change. *Nature reviews. Molecular cell biology* **10**, 218-227, doi:10.1038/nrm2646 (2009).

208 Trock, B. J., Leonessa, F. & Clarke, R. Multidrug resistance in breast cancer: a meta-analysis of MDR1/gp170 expression and its possible functional significance. *Journal of the National Cancer Institute* **89**, 917-931 (1997).

209 Chartrain, M. *et al.* Melanoma chemotherapy leads to the selection of ABCB5-expressing cells. *PloS one* **7**, e36762, doi:10.1371/journal.pone.0036762 (2012).

210 Johnson, R. A., Shepard, E. M. & Scotto, K. W. Differential regulation of MDR1 transcription by the p53 family members. Role of the DNA binding domain. *The Journal of biological chemistry* **280**, 13213-13219, doi:10.1074/jbc.M414646200 (2005).

211 Vilgelm, A. *et al.* DeltaNp73alpha regulates MDR1 expression by inhibiting p53 function. *Oncogene* **27**, 2170-2176, doi:10.1038/sj.onc.1210862 (2008).

212 Talos, F. *et al.* p73 is an essential regulator of neural stem cell maintenance in embryonal and adult CNS neurogenesis. *Cell death and differentiation* **17**, 1816-1829, doi:10.1038/cdd.2010.131 (2010).

213 Wetzel, M. K. *et al.* p73 regulates neurodegeneration and phospho-tau accumulation during aging and Alzheimer's disease. *Neuron* **59**, 708-721, doi:10.1016/j.neuron.2008.07.021 (2008).

214 Fontemaggi, G. *et al.* The transcriptional repressor ZEB regulates p73 expression at the crossroad between proliferation and differentiation. *Molecular and cellular biology* **21**, 8461-8470, doi:10.1128/MCB.21.24.8461-8470.2001 (2001).

215 Kommagani, R., Whitlatch, A., Leonard, M. K. & Kadakia, M. P. p73 is essential for vitamin D-mediated osteoblastic differentiation. *Cell death and differentiation* **17**, 398-407, doi:10.1038/cdd.2009.135 (2010).

216 Gonzalez-Cano, L. *et al.* p73 deficiency results in impaired self renewal and premature neuronal differentiation of mouse neural progenitors independently of p53. *Cell death & disease* **1**, e109, doi:10.1038/cddis.2010.87 (2010).

217 He, Z., Agostini, M., Liu, H., Melino, G. & Simon, H. U. p73 regulates basal and starvation-induced liver metabolism in vivo. *Oncotarget* **6**, 33178-33190, doi:10.18632/oncotarget.5090 (2015).

218 Ebelt, H. *et al.* Directed expression of dominant-negative p73 enables proliferation of cardiomyocytes in mice. *Journal of molecular and cellular cardiology* **45**, 411-419, doi:10.1016/j.yjmcc.2008.06.006 (2008).

219 Carrasco, G. *et al.* Overexpression of p73 as a tissue marker for high-risk gastritis. *Clinical cancer research : an official journal of the American Association for Cancer Research* **16**, 3253-3259, doi:10.1158/1078-0432.CCR-09-2491 (2010).

220 Wilhelm, M. T. *et al.* Isoform-specific p73 knockout mice reveal a novel role for delta Np73 in the DNA damage response pathway. *Genes & development* **24**, 549-560, doi:10.1101/gad.1873910 (2010).

221 Inoue, S. *et al.* TAp73 is required for spermatogenesis and the maintenance of male fertility. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 1843-1848, doi:10.1073/pnas.1323416111 (2014).

222 Nemajerova, A. *et al.* TAp73 is a central transcriptional regulator of airway multiciliogenesis. *Genes & development* **30**, 1300-1312, doi:10.1101/gad.279836.116 (2016).

223 Lai, J. *et al.* Transcriptional regulation of the p73 gene by Nrf-2 and promoter CpG methylation in human breast cancer. *Oncotarget* **5**, 6909-6922, doi:10.18632/oncotarget.2230 (2014).

224 Liu, P., Garbutt, C., Hornicek, F. J., Liu, F. & Duan, Z. Aberration of p73 Promoter Methylation in Chondrosarcoma. *Anticancer research* **37**, 2939-2945, doi:10.21873/anticanres.11647 (2017).

225 Corn, P. G. *et al.* Transcriptional silencing of the p73 gene in acute lymphoblastic leukemia and Burkitt's lymphoma is associated with 5' CpG island methylation. *Cancer research* **59**, 3352-3356 (1999).

226 Kawano, S. *et al.* Loss of p73 gene expression in leukemias/lymphomas due to hypermethylation. *Blood* **94**, 1113-1120 (1999).

227 Alexandrova, E. M. & Moll, U. M. Role of p53 family members p73 and p63 in human hematological malignancies. *Leukemia & lymphoma* **53**, 2116-2129, doi:10.3109/10428194.2012.684348 (2012).

228 Ibrahim, N. *et al.* BRCA1-associated epigenetic regulation of p73 mediates an effector pathway for chemosensitivity in ovarian carcinoma. *Cancer research* **70**, 7155-7165, doi:10.1158/0008-5472.CAN-10-0668 (2010).

229 Daskalos, A. *et al.* Global DNA hypomethylation-induced DeltaNp73 transcriptional activation in non-small cell lung cancer. *Cancer letters* **300**, 79-86, doi:10.1016/j.canlet.2010.09.009 (2011).

230 Gomez, L. C. *et al.* TP73 DNA methylation and upregulation of DeltaNp73 are associated with an adverse prognosis in breast cancer. *Journal of clinical pathology*, doi:10.1136/jclinpath-2017-204499 (2017).

231 Ding, Y. *et al.* Molecular cloning and functional characterization of the upstream promoter region of the human p73 gene. *DNA research : an international journal for rapid publication of reports on genes and genomes* **6**, 347-351 (1999).

232 Seelan, R. S. *et al.* The human p73 promoter: characterization and identification of functional E2F binding sites. *Neoplasia* **4**, 195-203, doi:10.1038/sj/neo/7900237 (2002).

233 Yu, J., Baron, V., Mercola, D., Mustelin, T. & Adamson, E. D. A network of p73, p53 and Egr1 is required for efficient apoptosis in tumor cells. *Cell death and differentiation* **14**, 436-446, doi:10.1038/sj.cdd.4402029 (2007).

234 Flinterman, M. *et al.* E1A activates transcription of p73 and Noxa to induce apoptosis. *The Journal of biological chemistry* **280**, 5945-5959, doi:10.1074/jbc.M406661200 (2005).

235 Logotheti, S. *et al.* Sp1 binds to the external promoter of the p73 gene and induces the expression of TAp73gamma in lung cancer. *The FEBS journal* **277**, 3014-3027, doi:10.1111/j.1742-4658.2010.07710.x (2010).

236 Vilgelm, A. E. *et al.* Characterization of DeltaNp73 expression and regulation in gastric and esophageal tumors. *Oncogene* **29**, 5861-5868, doi:10.1038/onc.2010.319 (2010).

237 Watanabe, K. *et al.* Physical interaction of p73 with c-Myc and MM1, a c-Myc-binding protein, and modulation of the p73 function. *The Journal of biological chemistry* **277**, 15113-15123, doi:10.1074/jbc.M111281200 (2002).

238 Grob, T. J. *et al.* Human delta Np73 regulates a dominant negative feedback loop for TAp73 and p53. *Cell death and differentiation* **8**, 1213-1223, doi:10.1038/sj.cdd.4400962 (2001).

239 Nakagawa, T. *et al.* Autoinhibitory regulation of p73 by Delta Np73 to modulate cell survival and death through a p73-specific target element within the Delta Np73 promoter. *Molecular and cellular biology* **22**, 2575-2585 (2002).

240 Kartasheva, N. N., Contente, A., Lenz-Stoppler, C., Roth, J. & Dobbelstein, M. p53 induces the expression of its antagonist p73 Delta N, establishing an autoregulatory feedback loop. *Oncogene* **21**, 4715-4727, doi:10.1038/sj.onc.1205584 (2002).

241 Vossio, S. *et al.* DN-p73 is activated after DNA damage in a p53-dependent manner to regulate p53-induced cell cycle arrest. *Oncogene* **21**, 3796-3803, doi:10.1038/sj.onc.1205465 (2002).

242 Mantovani, F. *et al.* Pin1 links the activities of c-Abl and p300 in regulating p73 function. *Molecular cell* **14**, 625-636, doi:10.1016/j.molcel.2004.05.007 (2004).

243 Costanzo, A. *et al.* DNA damage-dependent acetylation of p73 dictates the selective activation of apoptotic target genes. *Molecular cell* **9**, 175-186 (2002).

244 Gonzalez, S., Prives, C. & Cordon-Cardo, C. p73alpha regulation by Chk1 in response to DNA damage. *Molecular and cellular biology* **23**, 8161-8171 (2003).

245 Jones, E. V., Dickman, M. J. & Whitmarsh, A. J. Regulation of p73-mediated apoptosis by c-Jun N-terminal kinase. *The Biochemical journal* **405**, 617-623, doi:10.1042/BJ20061778 (2007).

246 Tsai, K. K. & Yuan, Z. M. c-Abl stabilizes p73 by a phosphorylation-augmented interaction. *Cancer research* **63**, 3418-3424 (2003).

247 Munarriz, E. *et al.* PIAS-1 is a checkpoint regulator which affects exit from G1 and G2 by sumoylation of p73. *Molecular and cellular biology* **24**, 10593-10610, doi:10.1128/MCB.24.24.10593-10610.2004 (2004).

248 Wang, X. Q., Ongkeko, W. M., Lau, A. W., Leung, K. M. & Poon, R. Y. A possible role of p73 on the modulation of p53 level through MDM2. *Cancer research* **61**, 1598-1603 (2001).

249 Zhu, J., Jiang, J., Zhou, W. & Chen, X. The potential tumor suppressor p73 differentially regulates cellular p53 target genes. *Cancer research* **58**, 5061-5065 (1998).

250 Lau, L. M., Nugent, J. K., Zhao, X. & Irwin, M. S. HDM2 antagonist Nutlin-3 disrupts p73-HDM2 binding and enhances p73 function. *Oncogene* **27**, 997-1003, doi:10.1038/sj.onc.1210707 (2008).

251 Peirce, S. K. & Findley, H. W. The MDM2 antagonist nutlin-3 sensitizes p53-null neuroblastoma cells to doxorubicin via E2F1 and TAp73. *International journal of oncology* **34**, 1395-1402 (2009).

252 Paliwal, P., Radha, V. & Swarup, G. Regulation of p73 by Hck through kinase-dependent and independent mechanisms. *BMC molecular biology* **8**, 45, doi:10.1186/1471-2199-8-45 (2007).

253 Munarriz, E. *et al.* Calpain cleavage regulates the protein stability of p73. *Biochemical and biophysical research communications* **333**, 954-960, doi:10.1016/j.bbrc.2005.05.188 (2005).

254 Sayan, B. S. *et al.* Differential control of TAp73 and DeltaNp73 protein stability by the ring finger ubiquitin ligase PIR2. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 12877-12882, doi:10.1073/pnas.0911828107 (2010).

255 Bernassola, F. *et al.* Ubiquitin-dependent degradation of p73 is inhibited by PML. *The Journal of experimental medicine* **199**, 1545-1557, doi:10.1084/jem.20031943 (2004).

256 Peschiaroli, A., Scialpi, F., Bernassola, F., Pagano, M. & Melino, G. The F-box protein FBXO45 promotes the proteasome-dependent degradation of p73. *Oncogene* **28**, 3157-3166, doi:10.1038/onc.2009.177 (2009).

257 Minty, A., Dumont, X., Kaghad, M. & Caput, D. Covalent modification of p73alpha by SUMO-1. Two-hybrid screening with p73 identifies novel SUMO-1-interacting proteins and a SUMO-1 interaction motif. *The Journal of biological chemistry* **275**, 36316-36323, doi:10.1074/jbc.M004293200 (2000).

258 Sang, M. *et al.* Plk3 inhibits pro-apoptotic activity of p73 through physical interaction and phosphorylation. *Genes to cells : devoted to molecular & cellular mechanisms* **14**, 775-788, doi:10.1111/j.1365-2443.2009.01309.x (2009).

259 Zaika, A. I. *et al.* DeltaNp73, a dominant-negative inhibitor of wild-type p53 and TAp73, is up-regulated in human tumors. *The Journal of experimental medicine* **196**, 765-780 (2002).

260 Ushiku, T. *et al.* p73 gene promoter methylation in Epstein-Barr virus-associated gastric carcinoma. *International journal of cancer* **120**, 60-66, doi:10.1002/ijc.22275 (2007).

261 Meier, C., Hardtstock, P., Joost, S., Alla, V. & Putzer, B. M. p73 and IGF1R Regulate Emergence of Aggressive Cancer Stem-like Features via miR-885-5p Control. *Cancer research* **76**, 197-205, doi:10.1158/0008-5472.CAN-15-1228 (2016).

262 Casciano, I. *et al.* Expression of DeltaNp73 is a molecular marker for adverse outcome in neuroblastoma patients. *Cell death and differentiation* **9**, 246-251, doi:10.1038/sj.cdd.4400993 (2002).

263 Uramoto, H. *et al.* Expression of deltaNp73 predicts poor prognosis in lung cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* **10**, 6905-6911, doi:10.1158/1078-0432.CCR-04-0290 (2004).

264 Zhu, W. *et al.* Expression and prognostic significance of TAp73 and DeltaNp73 in FIGO stage I-II cervical squamous cell carcinoma. *Oncology letters* **9**, 2090-2094, doi:10.3892/ol.2015.3052 (2015).

265 Concin, N. *et al.* Clinical relevance of dominant-negative p73 isoforms for responsiveness to chemotherapy and survival in ovarian cancer: evidence for a crucial p53-p73 cross-talk in vivo. *Clinical cancer research : an official journal of the American Association for Cancer Research* **11**, 8372-8383, doi:10.1158/1078-0432.CCR-05-0899 (2005).

266 Kilic, E. *et al.* Increased expression of p73Deltaex2 transcript in uveal melanoma with loss of chromosome 1p. *Melanoma research* **18**, 208-213, doi:10.1097/CMR.0b013e3283036aa1 (2008).

267 Tuve, S., Wagner, S. N., Schittek, B. & Putzer, B. M. Alterations of DeltaTA-p 73 splice transcripts during melanoma development and progression. *International journal of cancer* **108**, 162-166, doi:10.1002/ijc.11552 (2004).

268 Soldevilla, B. *et al.* Prognostic impact of DeltaTAp73 isoform levels and their target genes in colon cancer patients. *Clinical cancer research : an official journal of the American Association for Cancer Research* **17**, 6029-6039, doi:10.1158/1078-0432.CCR-10-2388 (2011).

269 Emmrich, S., Wang, W., John, K., Li, W. & Putzer, B. M. Antisense gapmers selectively suppress individual oncogenic p73 splice isoforms and inhibit tumor growth in vivo. *Molecular cancer* **8**, 61, doi:10.1186/1476-4598-8-61 (2009).

270 Engelmann, D., Meier, C., Alla, V. & Putzer, B. M. A balancing act: orchestrating amino-truncated and full-length p73 variants as decisive factors in cancer progression. *Oncogene* **34**, 4287-4299, doi:10.1038/onc.2014.365 (2015).

271 Le Guelte, A. & Gavard, J. Role of endothelial cell-cell junctions in endothelial permeability. *Methods in molecular biology* **763**, 265-279, doi:10.1007/978-1-61779-191-8_18 (2011).

272 Le Guelte, A., Dwyer, J. & Gavard, J. Jumping the barrier: VE-cadherin, VEGF and other angiogenic modifiers in cancer. *Biology of the cell* **103**, 593-605, doi:10.1042/BC20110069 (2011).

273 Weis, S., Cui, J., Barnes, L. & Cheresh, D. Endothelial barrier disruption by VEGF-mediated Src activity potentiates tumor cell extravasation and metastasis. *The Journal of cell biology* **167**, 223-229, doi:10.1083/jcb.200408130 (2004).

274 Bates, D. O. Vascular endothelial growth factors and vascular permeability. *Cardiovascular research* **87**, 262-271, doi:10.1093/cvr/cvq105 (2010).

275 Cao, Y. *et al.* Vascular endothelial growth factor C induces angiogenesis in vivo. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 14389-14394 (1998).

276 Kaur, B., Brat, D. J., Devi, N. S. & Van Meir, E. G. Vasculostatin, a proteolytic fragment of brain angiogenesis inhibitor 1, is an antiangiogenic and antitumorigenic factor. *Oncogene* **24**, 3632-3642, doi:10.1038/sj.onc.1208317 (2005).

277 Ravi, R. *et al.* Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1alpha. *Genes & development* **14**, 34-44 (2000).

278 Dulloo, I. *et al.* Hypoxia-inducible TAp73 supports tumorigenesis by regulating the angiogenic transcriptome. *Nature cell biology* **17**, 511-523, doi:10.1038/ncb3130 (2015).

279 Borsig, L., Wolf, M. J., Roblek, M., Lorentzen, A. & Heikenwalder, M. Inflammatory chemokines and metastasis--tracing the accessory. *Oncogene* **33**, 3217-3224, doi:10.1038/onc.2013.272 (2014).

280 Deshmane, S. L., Kremlev, S., Amini, S. & Sawaya, B. E. Monocyte chemoattractant protein-1 (MCP-1): an overview. *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research* **29**, 313-326, doi:10.1089/jir.2008.0027 (2009).

281 Lim, S. Y., Yuzhalin, A. E., Gordon-Weeks, A. N. & Muschel, R. J. Targeting the CCL2-CCR2 signaling axis in cancer metastasis. *Oncotarget* **7**, 28697-28710, doi:10.18632/oncotarget.7376 (2016).

282 Liu, G. & Chen, X. The C-terminal sterile alpha motif and the extreme C terminus regulate the transcriptional activity of the alpha isoform of p73. *The Journal of biological chemistry* **280**, 20111-20119, doi:10.1074/jbc.M413889200 (2005).

283 Koeppel, M. *et al.* Crosstalk between c-Jun and TAp73alpha/beta contributes to the apoptosis-survival balance. *Nucleic acids research* **39**, 6069-6085, doi:10.1093/nar/gkr028 (2011).

284 Ueda, Y., Hijikata, M., Takagi, S., Chiba, T. & Shimotohno, K. New p73 variants with altered C-terminal structures have varied transcriptional activities. *Oncogene* **18**, 4993-4998, doi:10.1038/sj.onc.1202817 (1999).

285 Kitamura, T. *et al.* CCL2-induced chemokine cascade promotes breast cancer metastasis by enhancing retention of metastasis-associated macrophages. *The Journal of experimental medicine* **212**, 1043-1059, doi:10.1084/jem.20141836 (2015).

286 Teng, K. Y. *et al.* Blocking the CCL2-CCR2 Axis Using CCL2-Neutralizing Antibody Is an Effective Therapy for Hepatocellular Cancer in a Mouse Model. *Molecular cancer therapeutics* **16**, 312-322, doi:10.1158/1535-7163.MCT-16-0124 (2017).

287 Zhang, J., Lu, Y. & Pienta, K. J. Multiple roles of chemokine (C-C motif) ligand 2 in promoting prostate cancer growth. *Journal of the National Cancer Institute* **102**, 522-528, doi:10.1093/jnci/djq044 (2010).

288 Zhang, J. *et al.* CCL2 expression correlates with Snail expression and affects the prognosis of patients with gastric cancer. *Pathology, research and practice* **213**, 217-221, doi:10.1016/j.prp.2016.12.013 (2017).

289 Eggert, T. *et al.* Distinct Functions of Senescence-Associated Immune Responses in Liver Tumor Surveillance and Tumor Progression. *Cancer cell* **30**, 533-547, doi:10.1016/j.ccell.2016.09.003 (2016).

290 Mora, E., Guglielmotti, A., Biondi, G. & Sassone-Corsi, P. Bindarit: an anti-inflammatory small molecule that modulates the NFkappaB pathway. *Cell cycle* **11**, 159-169, doi:10.4161/cc.11.1.18559 (2012).

291 Ueda, A. *et al.* NF-kappa B and Sp1 regulate transcription of the human monocyte chemoattractant protein-1 gene. *Journal of immunology* **153**, 2052-2063 (1994).

292 Lerebours, F. *et al.* NF-kappa B genes have a major role in inflammatory breast cancer. *BMC cancer* **8**, 41, doi:10.1186/1471-2407-8-41 (2008).

293 Kishore, N. *et al.* A selective IKK-2 inhibitor blocks NF-kappa B-dependent gene expression in interleukin-1 beta-stimulated synovial fibroblasts. *The Journal of biological chemistry* **278**, 32861-32871, doi:10.1074/jbc.M211439200 (2003).

294 Oeckinghaus, A. & Ghosh, S. The NF-kappaB family of transcription factors and its regulation. *Cold Spring Harbor perspectives in biology* **1**, a000034, doi:10.1101/cshperspect.a000034 (2009).

295 Yang, L. *et al.* Loss of negative feedback control of nuclear factor-kappaB2 activity in lymphocytes leads to fatal lung inflammation. *The American journal of pathology* **176**, 2646-2657, doi:10.2353/ajpath.2010.090751 (2010).

296 Roca, H. *et al.* CCL2 and interleukin-6 promote survival of human CD11b+ peripheral blood mononuclear cells and induce M2-type macrophage polarization. *The Journal of biological chemistry* **284**, 34342-34354, doi:10.1074/jbc.M109.042671 (2009).

297 Masoud, G. N. & Li, W. HIF-1alpha pathway: role, regulation and intervention for cancer therapy. *Acta pharmaceutica Sinica. B* **5**, 378-389, doi:10.1016/j.apsb.2015.05.007 (2015).

298 Ohh, M. Ubiquitin pathway in VHL cancer syndrome. *Neoplasia* **8**, 623-629, doi:10.1593/neo.06442 (2006).

299 Semenza, G. L. Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics. *Oncogene* **29**, 625-634, doi:10.1038/onc.2009.441 (2010).

300 Zheng, S. S., Chen, X. H., Yin, X. & Zhang, B. H. Prognostic significance of HIF-1alpha expression in hepatocellular carcinoma: a meta-analysis. *PloS one* **8**, e65753, doi:10.1371/journal.pone.0065753 (2013).

301 Bao, L. *et al.* Increased expression of P-glycoprotein and doxorubicin chemoresistance of metastatic breast cancer is regulated by miR-298. *The American journal of pathology* **180**, 2490-2503, doi:10.1016/j.ajpath.2012.02.024 (2012).

302 Yang, J. Y., Ha, S. A., Yang, Y. S. & Kim, J. W. p-Glycoprotein ABCB5 and YB-1 expression plays a role in increased heterogeneity of breast cancer cells: correlations with cell fusion and doxorubicin resistance. *BMC cancer* **10**, 388, doi:10.1186/1471-2407-10-388 (2010).