From DEPARTMENT OF ONCOLOGY-PATHOLOGY
Karolinska Institutet, Stockholm, Sweden

WAR AND PEACE IN THE TUMOR MICROENVIRONMENT: TUMOR-ASSOCIATED CELLS AS FACILITATORS OR ADVERSARIES DURING TUMOR DEVELOPMENT

Margarita Bartish

Stockholm 2018

Cover illustration by Matthias Grönwall

All previously published papers were reproduced with permission from the publisher.
Published by Karolinska Institutet.
Printed by Eprint AB 2018

ISBN 978-91-7831-256-6

War and Peace in the Tumor Microenvironment - Tumor-Associated Cells as Facilitators or Adversaries during Tumor Development

THESIS FOR DOCTORAL DEGREE (Ph.D.)

Science for life laboratory (Scilifelab) gamma 2, Seminar room "Fire", Tomtebodavägen 23 Solna

Friday the 14th of December, 09:30

By

Margarita Bartish

Principal Supervisor:
Associate Professor Charlotte Rolny
Karolinska Institutet
Department of Oncology-Pathology

Co-supervisor(s):
Associate Professor Ola Larsson
Karolinska Institutet
Department of Oncology-Pathology

Opponent:
Professor Raúl Méndez
Institute for Research in Biomedicine,
IRB Barcelona
Division of Molecular Medicine

Examination Board:
Associate Professor Lisa Westerberg
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology

Professor Ola Söderberg
Uppsala Universitet
Department of Pharmaceutical Biosciences

Associate Professor Guillem Genové
Karolinska Institutet
Department of Medicine

“Here's to the future, for the dreams of youth”

To my family

“Всі вони кандидати у якісь науки. Що таке "кандидат", ми добре знаємо. Явина мати була кандидатом у депутати районної Ради. Її біографія з фотографією висіла на стіні нашої школи. Та це було недовго. Пройшли вибори, і Явина мати стала депутатом. А ці третій рік приїздять, і все іще кандидати.

— Щось довго їх не вибирають, — казав Ява. — Мабуть, не дуже вдатні до наук. Як ми з тобою.”

- Всеволод Нестайко "Тореадори з Васюківки"

ABSTRACT

The tumor microenvironment, including immune cells, fibroblasts and vasculature, profoundly affects tumor development by initially opposing, but eventually facilitating tumor growth, vascularization and spread. Though corrupted by the growing tumor, such cells remain non-transformed and thus, with proper cues, are possible to direct toward their physiological anti-tumor function. Understanding the mechanisms by which the tumor microenvironment is shaped, before and during tumor growth, has been the principal aim of this thesis.

In **paper I**, we demonstrate that the composition of tumor-associated macrophages (TAMs) can be modulated by selective proliferation of TAM subsets. We identify SEMA3A as a factor potentiating such selective proliferation of anti-tumor TAMs. In **paper II** we extend the study of SEMA3A's effect on tumor immunity by showing that it can functionally alter the phenotype of polymorphonuclear myeloid-derived suppressor cells (PMN-MDSC). As a consequence of its effects on TAMs and PMN-MDSCs, the tumor microenvironment is infiltrated by activated cytotoxic lymphocytes which act to obstruct tumor growth.

In **paper III** we show that regulation of mRNA translation shapes the phenotype of TAMs as they become increasingly pro-tumor during tumor growth. We further show that transcripts translationally activated during tumor growth in TAMs were regulated similarly upon M2-polarization of macrophages *in vitro*. Selective inhibition of the MNK2/phospho-eIF4E pathway, which impinges on mRNA translation, functionally altered *in vitro* M2-polarized macrophages toward a pro-inflammatory phenotype. This suggests that modulation of mRNA translation is a potential target in TAM-based anti-tumor therapies.

We further emphasize the importance of mRNA translation in regulating gene expression in the microenvironment in **paper IV**, where we show changes in its efficiency to drive cancer-associated gene expression alterations in the stroma of patients with chronic obstructive pulmonary disease (COPD). Depending on the lung function, two distinct gene expression programs were discovered. These were enriched for proteins previously identified in fibroblast secretomes that promoted cancer initiation in animal models, highlighting the involvement of non-transformed cells in neoplastic transformation.

In paper V, we show that class switch junctions in B cells from patients with BRCA1 mutations display decreased use of non-homologous end joining pathway in favor of the alternative end-joining pathway. This implicates a role for BRCA1 in maintaining genome stability and tumor suppression outside of its recognized role in mediating homologous recombination during cell division.

LIST OF SCIENTIFIC PAPERS

I. Wallerius M*, Wallmann T*, **BARTISH M**, Östling J, Mezheyeuski A, Tobin NP, Nygren E, Pangigadde P, Pellegrini P, Squadrito ML, Pontén F, Hartman J, Bergh J, De Milito A, De Palma M, Östman A, Andersson J, Rolny C **Guidance Molecule SEMA3A Restricts Tumor Growth by Differentially Regulating the Proliferation of Tumor-Associated Macrophages**
Cancer Research, 2016, 76(11); 3166-78

II. Wallerius M*, **BARTISH M***, Wallman T, Östling J, Joly A-L, Andersson J, Rolny C **Semaphorin3A re-educates myeloid derived suppressor cells towards a pro-inflammatory phenotype**
Manuscript

III. **BARTISH M**, Wallerius M, Wallmann T, Tong D, Liu H, Masvidal L, Joly A-L, van Hoef V, Goncalves C, Seitz C, Bergh J, Del Rincón S, Andersson J, Rolny C**, Larsson O** **Translational control of the tumor-associated macrophage phenotype**
Manuscript

IV. Sandri B J*, Masvidal L*, Murie C, **BARTISH M**, Avdulov S, Higgins LA, Markowski T, Peterson M, Bergh K, Yang P, Rolny C, Limper A H, Griffin T J, Bitterman P B, Wendt C H** and Larsson O ** **Distinct cancer-promoting stromal gene expression depending on lung function**
Under minor revision in American Journal of Respiratory and Critical Care Medicine

V. Björkman A, Qvist P, Du L, **BARTISH M**, Zaravinos A, Georgiou K, Børglum A D, Gatti R A, Törngren T, Pan-Hammarström Q **Aberrant recombination and repair during immunoglobulin class switching in BRCA1-deficient human B cells**
Proc. Natl. Acad. Sci. USA, 2015, 17;112(7):2157-62

ADDITIONAL PUBLICATIONS

Not included in this thesis

Felgentreff K, Du L, Weinacht KG, Dobbs K, **BARTISH M**, Giliani S, Schlaeger T, DeVine A, Schambach A, Woodbine LJ, Davies G, Baxi SN, van der Burg M, Bleesing J, Gennery A, Manis J, Pan-Hammarström Q, Notarangelo LD. **Differential role of nonhomologous end joining factors in the generation, DNA damage response, and myeloid differentiation of human induced pluripotent stem cells**
Proc Natl Acad Sci U S A. 2014 Jun 17;111(24):8889-94

*, ** Equal Contribution

CONTENTS

LIST OF COMMONLY USED ABBREVIATIONS

3´	Three prime
4E-BPs	4E-Binding Protein
5'	Five prime
A-EJ	Alternative-End Joining
Anota	Analysis of Translational Activity
ARE	Adenylate/uridylate-Rich Element
Arg1	Arginase 1
BMDM	Bone Marrow-Derived Macrophage
BRCA1	Breast Cancer 1
CAF	Cancer-Associated Fibroblast
CCL	Chemokine (C-C motif) Ligand
CCR	Chemokine (C-C motif) Receptor
COPD	Chronic Obstructive Pulmonary Disease
CRE	Cis-Regulatory Element
CSR	Class-Switch Recombination
CXCL	Chemokine C-X-C Ligand
DC	Dendritic Cell
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
eEF	eukaryotic Elongation Factor
eIF	eukaryotic Initiation Factor
ER	Endoplasmic Reticulum
FEVpp1	Forced Expiratory Volume in 1 sec, percent predicted
GDP	Guanosine Diphosphate
GM CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GTP	Guanosine Triphosphate
HR	Homologous Recombination
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRES	Internal Ribosome Entry Site
ISR	Integrated Stress Response
LPS	Lipopolysaccharide
M-MDSC	Monocytic-Myeloid-Derived Suppressor Cell
MAPK	Mitogen-Activated Protein Kinase
MCSF	Macrophage Colony-Stimulating Factor
MDSC	Myeloid-derived suppressor cell
MMP	Matrix Metalloproteinase
MNK	MAPK-interacting Kinase
mRNA	messenger Ribonucleic Acid
mTOR	mechanistic Target of Rapamycin
NHEJ	Non-Homologous End Joining
NP1	Neuropilin 1
PABP	Poly(A)-Binding Protein
PIC	Preinitiation Complex
PIGF	Placental Growth Factor
PMN	Polymorphonuclear
PTEN	Phosphatase and tensin homolog
RPF	Ribosome Protected Fragment
SEMA3A	Semaphorin 3A
TACD	Tumor-Associated Dendritic Cell
TAM	Tumor-Associated Macrophage
TAN	Tumor-Associated Neutrophil
TC	Ternary complex
TE	Translation Efficiency
TGF-β	Transforming growth factor-β
TLR	Toll-like receptor
TIL	Tumor-infiltrating lymphocytes
TIM	Tumor-infiltrating myeloid cells
TME	Tumor Microenvironment
TNF	Tumor Necrosis Factor
TOP	Terminal OligoPyrimidine
uORF	upstream Open Reading Frame
UTR	Untranslated region
UV	Ultraviolet
VEGF	Vascular Endothelial Growth Factor
α-SMA	α-Smooth Muscle Actin

FOREWORD

"I am not what you would call a civilized man!"
-Captain Nemo

At some point during the evolution of the eukaryotic cell, it transitioned from life as an isolated unit to a communal existence. Giving up self-sufficiency, it opted to share resources and responsibilities with its kin who, by virtue of shared genes, were united in the goal of passing them on. So began the era of multicellular organisms and with it, increasing internal and external complexity of the cells themselves and the organisms they now built up. Being a part of an organized whole enabled the components of specialized tissues and organs to become more adept at their respective functions, improving the evolutionary fitness of the organism - but it also made each individual component subordinate to the needs of the organism as a whole. A cell's life cycle thus became carefully measured, with regulatory mechanisms set in place to make sure that it expresses appropriate genes at appropriate times, divides when needed and dies if signaled to do so.

Cancer represents a mutiny against this order. Through a gradual accumulation of genomic and epigenomic alterations, cancer cells acquire several distinct abilities – hallmarks – that distinguish them from their non-transformed counterparts [1, 2]. No longer constrained by the strict etiquette governing multicellular cooperation, cancer cells produce their own growth factors, ignore suppressive signals in their microenvironment, evade hostile immune cells, proliferate indefinitely, supply themselves with nutrients and constantly evolve their genome to keep abreast of environmental changes during their - now indefinite - lifespan.

The cancer cell is thus a distinct cell type with an agenda clearly at odds to that of the organism as a whole. Indeed, upon histological examination of tissue sections, abnormal neoplastic cells are clearly distinguished in the orderly architecture of healthy tissue [3]. Its hallmark abilities notwithstanding, the growing tumor mass would be unable to sustain its internal infrastructure and weather external pressure without considerate collaboration with resident "normal" cells [4]. The scientific community only relatively recently has begun to look outside of the transformed cell to understand tumor development, but in doing so, has now discovered the involvement of tumor-associated cells behind nearly all the hallmark abilities distinguishing cancer [4]. Tumor-associated cells are found accompanying the tumor from the very early neoplasm – perhaps even contributing to its initiation [5] – and participate throughout tumor growth, eventually promoting its ultimate dissemination and colonization [6].

A transformed cell is beyond physiological control. A tumor-associated cell remains attuned to the cues of the organism. Understanding the mechanisms driving non-transformed cells to promote tumor progression, particularly on the level of gene expression, and the ways by which these mechanisms can be targeted to reverse this process, constitute the overarching aims of this thesis.

1 COMPONENTS OF THE TUMOR MICROENVIRONMENT

"…of the two natures that contended in the field of my consciousness, even if I could rightly be said to be either, it was only because I was radically both"

-Dr. Jekyll

The metazoan immune system has evolved to recognize and neutralize threats to homeostasis. A mass of renegade cells unresponsive to regulatory signals is a bona fide threat, and indeed, the immune system responds and eliminates many neoplastic cells [7]. The concept of cancer immunosurveillance was initially difficult to reconcile with the idea that the immune system's main objective is to eliminate external, not internal, danger. Nonetheless, spontaneous tumor regression sometimes seen in humans and tumor-transplantation experiments in mice suggested that there is an immunological component preventing tumor initiation [8]. The combined weight of studies in animal models and observed cancer incidence rates in immunodeficient patients over the years that followed have conclusively shown that the immune system can – and does – prevent tumor formation [9].

The inflammatory environment that initiates the removal of potentially transformed cells is stressful for the cells that inhabit it. When unresolved, it can, in a positive feedback loop, stimulate further oncogenic transformation. It has been suggested that as many as 20% of all cancers can be linked to chronic inflammation [10]. Some transformed cells are less immunogenic and thus escape immunosurveillance. What follows then is a complex tug of war between proliferating cancer cells and resident cells of the tissue. Infiltrating immune cells, vascular and lymphatic endothelial cells, and mesenchymal supporting cells (e.g., fibroblasts and pericytes) will be found in varying proportions in all established tumors [4], but their phenotype will vary depending on the stage of the tumor [11]. The current conceptual model (**Figure 1**) proposes that in the course of tumor progression, cancer cells exert increasingly stronger influence over their microenvironment. As the tumor grows, it recruits or reprograms associated cells that dampen immune response, supply growth factors,

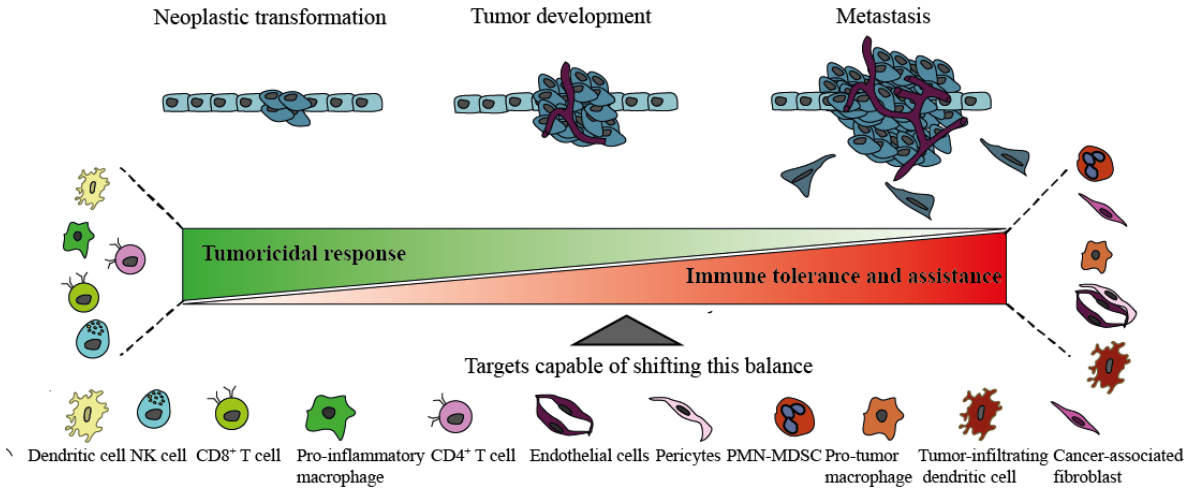

Figure 1 Conceptual model of changes in the tumor microenvironment during tumor progression. The list of cell types pictured is meant to be illustrative but not exhaustive.

nutrients and oxygen, transport waste products and remodel the extracellular matrix (ECM) to further tumor growth and spread.

The concept of duality is recurring in the study of infiltrating tumor-associated immune cells. The same – or an ontologically closely related – cells are often found to have both tumor-promoting and tumor-adverse functions (Figure 1). A macrophage, a neutrophil or a dendritic cell in the early stages of tumor development is likely to lyse tumor cells, promote inflammation, recruit and/or activate cytotoxic cells and attempt to clear the neoplasm. As the tumor develops, these cells instead will secrete immunosuppressive factors. It is worth highlighting that while terms such as "pro-" or "anti-tumor" are useful to differentiate the phenotypes these cells adopt, the cells themselves take no stand vis-à-vis tumor progression. They perform particular functions in response to environmental cues, functions that, while "pro-tumor" in the context of tumor development, are equally "pro-organism" under normal physiological conditions. Understanding these cues and how they are hijacked by the tumor would provide an inlet to manipulate the composition of the tumor microenvironment and, as a consequence, an ability to direct the overall tumor fate.

1.1 TUMOR-ASSOCIATED MACROPHAGES

1.1.1 Macrophage roles in non-pathological physiology

While the hallmark ability of the macrophages - to phagocytize - makes them integral components of innate immunity, the functional significance of these cells extends beyond the immune system [12]. Virtually all aspects of an organism's physiology – from embryogenesis to maintenance of homeostasis during adulthood – involve the participation of the macrophage. During embryonic development they participate in tissue patterning - both physically, by clearing unwanted cells while guiding tissue or vessel out-growth along defined paths [13] and chemically, by secreting factors that modulate the ECM [14]. They play important roles in angiogenesis and lymphangiogenesis, as they produce angiogenic- and lymphangiogenic factors such as Vascular Endothelial Factor (VEGF)-A [15] and VEGF-C [16]. They regulate metabolism, helping to maintain insulin sensitivity in healthy lean animals but promoting insulin resistance during pathogen invasion to fuel the actions of activated immune cells [17] [18]. Finally, they can modulate apoptosis, both inducing and protecting cells from programmed cell death [12].

1.1.1.1 Macrophage ontology

The ontological origin of macrophages is thought to comprise three separate branches, the unifying feature between (almost all of the) cells in these branches being the dependence on macrophage colony-stimulating factor 1 receptor (CSF1R) for growth, differentiation and survival [14]. In mice, the earliest macrophage progenitors appear before embryonic day 8. Some of these progenitors will migrate to the brain and establish microglia [19]. Others will instead enter the vasculature between the yolk sac and embryo and seed the second wave of

hematopoiesis in the fetal liver [20]. Due to a lack of genetic tools to uniquely distinguish fetal liver-derived macrophages, the particular contribution of this developmental stage has been debated [21] [22]. It is now believed that most tissue-specific macrophages originate as progenitors in the yolk sac, develop further in the fetal liver and migrate to the tissues in a chemokine-receptor–dependent manner during embryonic development [23] [24]. Langerhans cells, the macrophages in the skin, have been shown to have dual origin: with some progenitors seeding the tissue directly from the yolk sac, and some maturing via the liver [25]. Eventually, hematopoiesis in the liver is replaced by the bone marrow, which will give rise to circulating monocytes that differentiate to macrophages upon particular stimuli [23] (**Figure 2**).

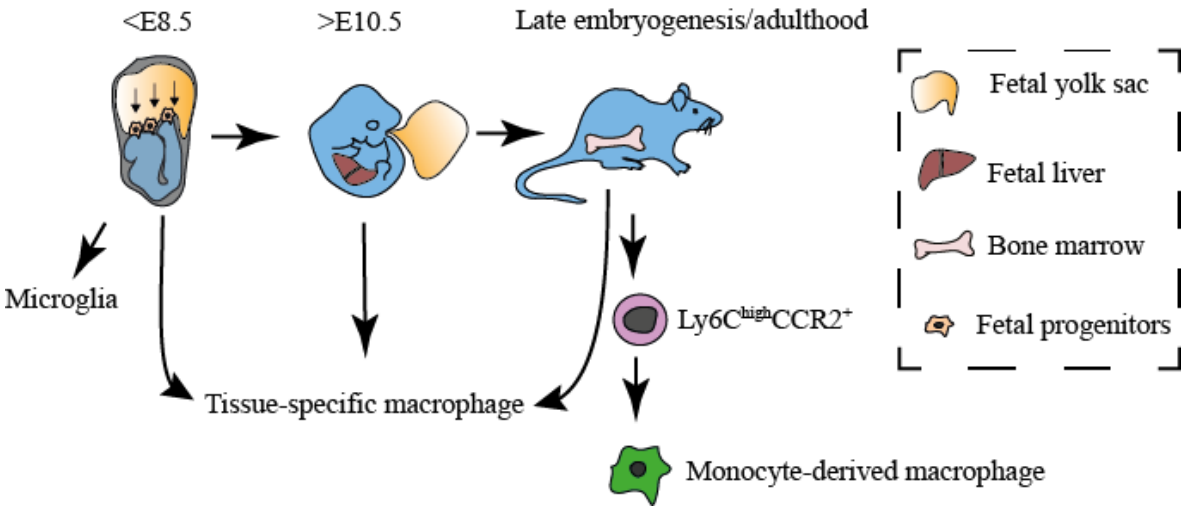

Figure 2. Schematic overview of macrophage ontology, depicting main sites of macrophage origin: fetal yolk sac, fetal liver and bone marrow, giving rise to both tissue-resident macrophages and monocytes with the capacity to differentiate into macrophages.

In mice, two distinct monocyte subsets have been identified: C-C chemokine receptor type 2 $(CCR2)^{high}Ly6C^{+}$ extravascular monocytes sometimes labelled as "inflammatory" monocytes and CX3C chemokine receptor 1 $(CX_3CR1)^{high}Ly6C^{-}$ intravascular "patrolling" monocytes [26]. The $CCR2^{high}Ly6C^{+}$ subset expresses a number of chemokine receptors, making them sensitive to signals of infection. Then, they readily differentiate to dendritic cells and macrophages. They are also short-lived, with a half-life of less than a day [27]. A recent study showed that $CX_3CR1^{high}Ly6C^{-}$ cells could be derived from $CCR2^{high}Ly6C^{+}$ monocytes [27], prompting the suggestion that these "patrolling" cells could be a population of terminally-differentiated blood macrophages [28].

Macrophages are genetically [29] and epigenetically [30] a highly heterogeneous group of cells. At the same time, they remain plastic and are attuned to environmental signals. Microglia and peritoneal macrophages were found to have lost their tissue-specific gene expression programs when removed from their local environment and cultured *in vitro* [31]. Experiments transferring macrophages from one tissue to another demonstrated the ability of the macrophage to adopt the gene expression and epigenetic signature of the host tissue [32]. A study by Suzuki et al., has confirmed this functionally, by showing that granulocyte-

macrophage colony-stimulating factor (GM-CSF) receptor-β-deficient (*Csf2rb*$^{-/-}$) alveolar macrophages could be replaced by transfer of wild-type bone marrow-derived macrophages (BMDMs) that would then adopt the genetic phenotype and functionality of the alveolar cells [33].

It was long believed that macrophages were terminally differentiated and thus would either migrate to their target tissue during embryonic development to persist there for life or be recruited from the population of circulating monocyte precursors. Isolated examples challenged this assumption. Local proliferation of a particular macrophage type, peritoneal macrophage, was described in 1982 [34]. A report published in 2009 was first to describe proliferation without the loss of functionality in mature macrophages deficient for the transcription factors *MafB/c-Maf*. These cells could furthermore contribute to *in vivo* macrophage populations when injected in mice [35]. This finding suggested that proliferation and terminal differentiation are not mutually exclusive. In 2011, using a model of Th2-driven inflammation, Jenkins et al. reported the proliferative expansion of resident tissue macrophages in an IL-4-dependent manner [36]. In the years that followed, a number of reports have concluded that mature macrophages in various contexts replenish their numbers via local proliferation without further differentiation or loss of function [37] [38]. In this respect, we have, in **study I,** shown a new mechanism to control tumor-associated macrophage (TAM) composition, by enhancing the proliferation of anti-tumor TAMs while restricting proliferation of pro-tumor TAMs [39].

1.1.1.2 Conceptual framework of M1 and M2 polarization states

Early *in vitro* experiments [40] [41] using well-defined stimuli such as Interferon (INF)γ and lipopolysaccharide (LPS) or interleukin (IL)4-treatment of BMDMs led to the adoption of the terms M1 "classically activated" or M2 "alternatively activated" macrophages respectively. These are also sometimes referred to as "pro-inflammatory" (M1) and "anti-inflammatory" (M2) on the basis of their function to either promote or stifle inflammation and their expression of cytokines and chemokines characteristic for these processes [42]. According to a conceptual model [43] [44] (**Figure 3**), when sensing tissue damage or presence of bacteria, via bacterial LPS or INFγ secreted by other immune cells, Ly6C^{+} inflammatory monocytes – or resident steady-state macrophages - migrate to the affected site, adopting a particular functional phenotype that is characterized by a production of a variety of inflammatory factors, such as chemokine C-X-C motif ligand (CXCL)9, CXCL10, CXCL11, IL-12 [45]. These "pro-inflammatory" M1 macrophages prime anti-bacterial mechanisms to clear the threat and attract and activate Th1, granulocytic and tumor infiltrating lymphocytes (TILs), i.e. NK, CD4^{+} helper T and CD8^{+} T cells to the site.

Once the tissue damage has been resolved or the bacterial threat has been cleared, guided by signals such as Transforming Growth Factor (TGF)β and IL-4, the M1-macrophage is skewed towards an M2-phenotype [40]. These cells produce anti-inflammatory cytokines such as IL-10, and factors including ornithine and the catalyst during its synthesis from L-arginine,

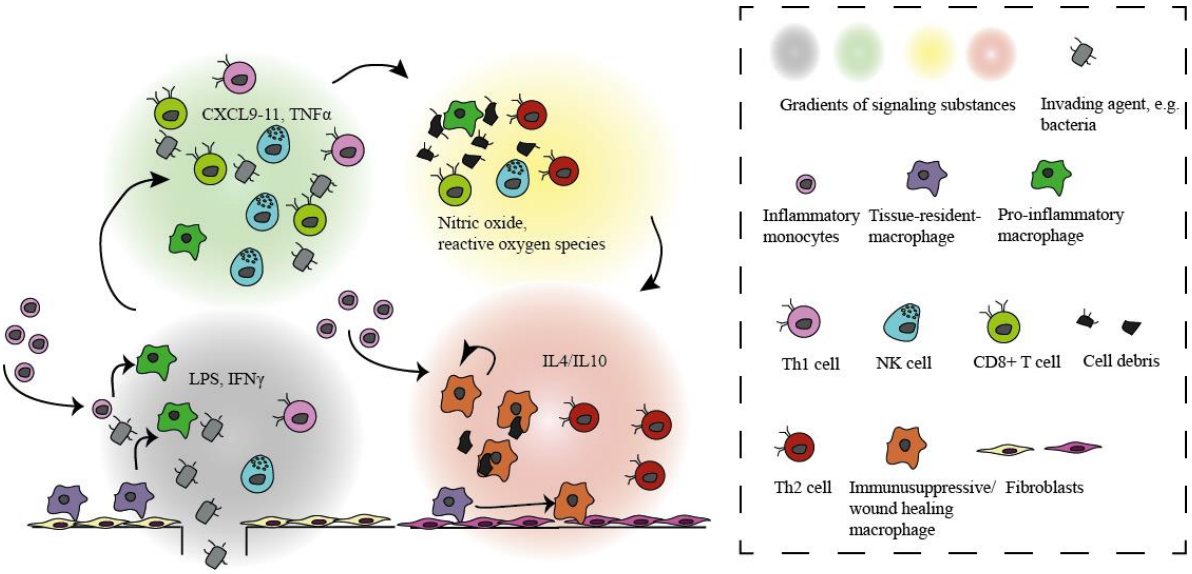

Figure 3 Schematic illustration of the activation of macrophages in an inflammatory context and in the context of resolution of inflammation.

Arginase 1 [46]. The cumulative effect of this immunosuppressive functional phenotype is deactivation of the cytotoxic T cell response and restoration of the extracellular matrix, wound healing and immune tolerance. If inflammatory signals are not controlled, surrounding tissue is compromised [10], highlighting the physiological importance of the entire range of macrophage activation.

While the IFNγ and IL-4 stimuli are clearly antagonistic *in vitro*, genetic and functional profiling of macrophages *in vivo* have called into question such a dichotomous view of macrophage biology under physiological conditions [47] [48] [49]. In tissue, whether healthy or cancerous, macrophages are exposed to numerous, often contradictory, signals and they respond accordingly, by adopting complex phenotypes whose function is not defined by the expression of isolated markers. The concept of M1/M2 macrophage activation in response to defined stimuli remains useful as an experimental model intended to simplify and simulate, as long as the stringent criteria for reporting experimental conditions as proposed by Murray and colleagues [48] are met. Ultimately, what defines a macrophage is not the activation stimulus or the genes that become up- or downregulated in response, but its function [49]. Thus, the nomenclature adopted to describe the phenotype of the cell should reflect this.

1.1.2 Tumor-Associated Macrophages

Macrophages make up a significant portion of the tumor, in some cancers up to 50% of the tumor mass [50]. The TAM population is highly heterogeneous between tumors as well as within a single tumor [51] [52] [53]. However, a general skewing towards pro-tumor macrophage phenotypes occurs as the tumor develops [54] [55].

1.1.2.1 TAM functions

Many of the functions performed by pro-tumor TAMs in the tumor, mirror the functions performed by the alternatively activated macrophage (M2) during resolution of inflammation and wound repair. For example, M2-like TAMs secrete a number of angiogenic and lymphangiogenic factors such as VEGF-A, platelet growth factor (PIGF) and VEGF-C, stimulating angiogenesis [56] and lymphangiogenesis [57] respectively. This is important to restore tissue integrity after damage, but in the tumor, this has a direct tumor- and metastasis promoting effect [58]. In the absence of macrophages, angiogenesis is severely impaired in the mouse mammary tumor virus polyoma middle-T (MMTV-PyMT) model, resulting in diminished tumor growth [56]. V*egfa* deletion specifically in the myeloid tumor-infiltrating cells altered the vasculature of the tumor in the same model, presenting less twisted network of vessels with increased pericyte coverage characteristic for healthy blood vessels [59]. As a consequence, the tumor was more susceptible to chemotherapeutic agents. Another important aspect to consider is that unregulated release of angiogenic factors in the tumor microenvironment - by TAMs, tumor cells and other components of the stroma - stimulates formation of defective "leaky" vessels that promote tumor spread [60]. Re-programming pro-tumoral M2-like TAMs toward an anti-tumoral M1-like phenotype results in vessel normalization and restriction of metastatic dissemination [61], indicating that targeting pro-tumor TAMs is a valid strategy to modulate tumor vasculature in order to enhance anti-cancer drug efficacy and limiting tumor cell dissemination to secondary organs.

Apart from producing VEGF-A directly, M2-like TAMs also secrete metalloproteases (MMPs) that, in turn, increase availability of VEGF-A, by processing it and releasing it from the ECM [62]. Metalloproteases - and other factors secreted by M2-like TAMs furthermore serve to remodel the ECM to facilitate tumor extravasation from the basement membrane [63] [64] and entry to the metastatic niche [65]. They can also stimulate tumor mobility directly. Elevated macrophage MMP production was shown to increase invasiveness of malignant tumor cell lines *in vitro*, as measured by migration through an artificial basement membrane in a Boyden chamber [66]. Benign cells were not affected. Furthermore, CSF1-recruited TAMs produce EGF, which, in a chemotactic way through a paracrine signaling loop, promotes tumor invasiveness [67]. Confirming this using multiphoton microscopy, macrophages were shown to lead tumor cells to blood vessels [68]. Once near the blood vessel, macrophage-derived VEGF-A was shown to contribute to transient vessel permeability and thus to facilitate tumor cell intravasation [69].

1.1.2.2 TAMs and the adaptive immunity

The interaction between TAMs and the adaptive immune system and the shift that occurs as TAMs adapt a more M2-like phenotype has profound consequences for the development of the tumor. As a component of the innate immune system, the macrophage is a professional antigen-presenting cell (APC), displaying antigens on the major histocompatibility complex II (MHCII) and by doing so activating $CD4^+$ T helper cells and cytotoxic $CD8^+$ T cells [70].

It also possesses a direct tumorolytic ability, by phagocytosing tumor cells and eliminating them in early neoplasms [71]. Pro-inflammatory macrophages secrete chemokines CXCL9-11 that attract cytotoxic $CD8^+$ T and NK cells, and IL12 that activates them to produce INFγ. This, in turn, promotes skewing of macrophages towards and M1-phenotype and also stimulates further accumulation of cytotoxic lymphocytes [72]. M1-like macrophages also present co-stimulatory molecules CD80 and CD86 on their cell membranes which are necessary for T cell activation [73].

Conversely, in response to signals such as IL-4 and IL-13 produced by Th2 cells in the tumor microenvironment [74], TAMs assume an immunosuppressive phenotype, downregulating IL12 and upregulating expression of arginase 1 (ARG1) and IL-10 [75]. ARG1 catabolizes the non-essential amino acid arginine into L-ornithine and urea [76], depleting arginine from the environment and thereby blocking T cell proliferation [77]. Elevated IL-10 levels stimulate increased expression of programmed death ligand 1 (PD-L1) on tumor-infiltrating monocytes, dampening the anti-tumoral T cell response [78]. Pro-tumoral TAMs also secrete CCL22 and TGF-β that recruit immune-suppressive regulatory T cells [79] [80], further attenuating the immune response. We [39] and others [61] [81] have shown that increasing the proportion of M1-like pro-inflammatory TAMs in mice models of cancer results in impeded tumor growth by stimulating activation and accumulation of cytotoxic lymphocytes. Importantly, in human breast cancer, an inverse relationship between macrophage ($CD68^+$ cells) and $CD8^+$ T cell infiltration has been documented, with the $CD68^+CD4^{high}CD8^{low}$ signature being an independent predictor of poor overall survival [82].

1.1.2.3 TAM ontology and turnover

The ontological origin of TAMs is variable [83]. In many cases, they are recruited as monocytes to the site of neoplasm by chemoattractants (C-C motif chemokine ligand (CCL) 2 [84], VEGF [85] [86], CSF1 [87] CXCL12 [88]) secreted by the tumor or tumor-associated cells. Alternatively, they can be local resident tissue macrophages corrupted by the developing tumor [89] [90]. Adding a further layer of complexity, to persist in the tumor, they might need replenishment from their respective precursors, either resident or monocyte-derived, or they might proliferate independently.

In the MMTV-PyMT spontaneous breast carcinoma model, Franklin et al. showed that $Ly6C^+CCR2^+$ monocyte progenitors are required to seed the TAM population [91]. However, a decrease in circulating $CCR2^+$ monocytes did not affect TAM numbers to the same extent as it affected the numbers of mammary tissue macrophages (MTMs), the resident macrophage population. Compared to MTMs, the TAM population had higher levels of 5-ethynyl-2'-deoxyuridine (EdU)-incorporation in the newly synthesized DNA and stronger Ki67 (a marker of G1/S/G2 phases of cell cycle [92]) staining, suggesting higher proliferation rates to account for a diminished dependency on monocyte replenishment. Interestingly, the TAMs in this study did not exhibit an alternative activation phenotype, highlighting the heterogeneity and context-dependency of TAM composition in any given tumor. Developing

this notion, Tymoszuk et al. identified two distinct TAM populations, both originating from circulating monocytes in the MMTV-Neu mouse model for HER2 dependent human breast cancer, that they distinguished by differential expression of CD11b (high or low expression) and F4/80 markers [93]. Importantly, these populations were found to occupy distinct areas of the tumor and demonstrated differential dependency on monocyte contribution. The dominant $CD11b^{low}F4/80^{high}$ population, residing in vessel-poor regions of the neoplasm, was shown to proliferate actively as measured by Ki67-expression and bromodeoxyuridine (BrdU)-DNA incorporation via M-CSFR mediated mechanisms without significant monocyte contribution. Contrastingly, the monocyte-replenished $CD11b^{high}F4/80^{low}$ population, instead, was localized near potential sites of monocyte influx and exhibited slower proliferation rates. Cumulatively, these two studies highlight monocytic origin of TAMs in mouse models of breast cancer, but also suggest that targeting TAM proliferation could represent a potential therapeutic strategy.

1.1.2.4 TAMs in human cancer

Macrophages are poorly characterized in human cancers, where the non-exclusive marker CD68 is generally used to define this cell type [94]. There is strong support that accumulation of $CD68^+$ TAM is correlated to poor prognosis in many cancers [95] [96]. However, a study of Mahmoud and colleagues examining 1322 human breast cancer tumors showed that the number of $CD68^+$ cells in a tumor is not an independent prognostic marker when included in multivariate model also containing parameters such as tumor grade and size [97]. These authors highlight the heterogeneity of the TAM population and point out that CD68 expression does not discriminate between macrophages having a pro- or anti-tumoral phenotype. Therefore, additional biomarkers that are upregulated in response to IL-4, such as scavenger receptor CD163 and mannose receptor CD206, are commonly used to define M2-like TAMs [95]. In this respect, a study that used a combination of CD68 and CD163 concluded that CD163 expression is an independent prognostic marker for shorter overall survival in basal-like breast carcinoma [98].

1.1.3 Tumor-Associated Macrophage-based therapy strategies

Given the long-established role of TAMs in aiding tumor progression, TAMs are a recognized target for anti-tumoral therapies, with strategies ranging from conceptual to having shown promise in early clinical trials [83]. In general, research is pursued in three main directions: 1) limiting TAM recruitment, proliferation and/or survival in the tumor, 2) reprogramming of TAMs towards tumor-suppressive phenotypes and 3) interfering with their effector functions. Often these approaches are pursued in synergy with other tumor-targeting therapies. For example, there is a growing understanding that the TAM composition, outside their inherent pro- or anti-tumor properties, may also expert secondary effects by either interfering with or, alternatively, superimposing the effect of certain chemotherapies on the tumor [99]. Tissue-damage signals arising as a consequence of the action of certain agents, (e.g. platinum compounds used in chemotherapy [82]) may provoke increased recruitment of

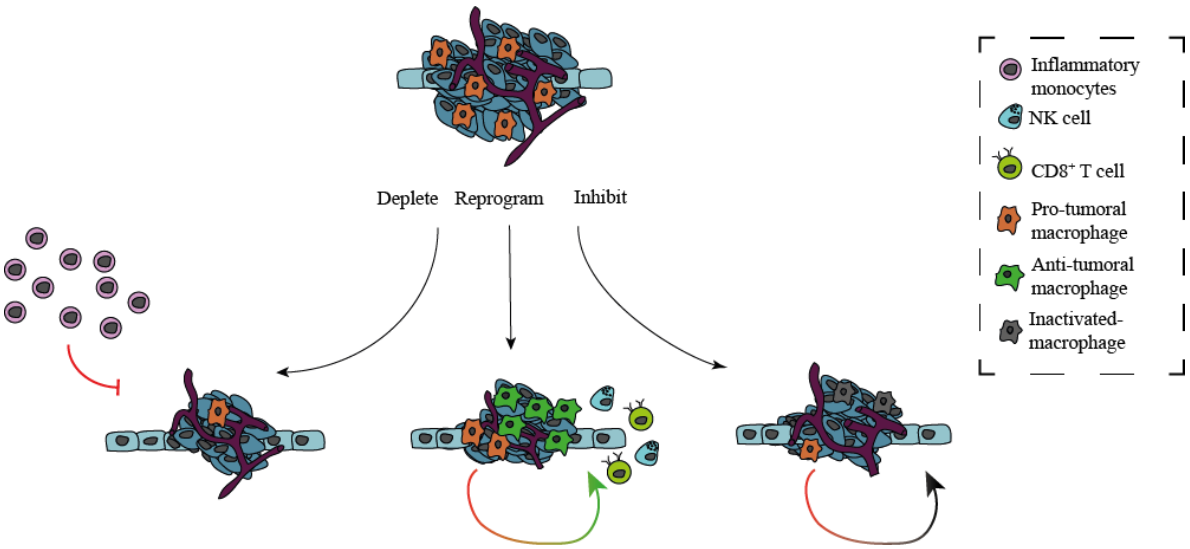

Figure 4. Overview of main TAM-based therapeutic strategies

TAMs to the tumor site and their subsequent pro-tumoral polarization. Contrastingly, other drugs, such as doxorubicin, instead may stimulate efficient recruitment and differentiation of antigen-presenting cells, resulting in efficient adaptive immune response targeting the tumor [100] [101]. In connection to the interplay between TAMs and the adaptive immunity, TAM-derived IL-10 was found to decrease the efficacy of chemotherapy (using paclitaxel and carboplatin) by suppressing IL-12 production in intra-tumoral dendritic cells, which, in turn suppressed the $CD8^+$ T cell response [102].

Preventing macrophage recruitment to the tumor is not straightforward. Tumors employ a diverse array of mediators to attract monocytes into the microenvironment [103], so targeting one of these will not preclude macrophage recruitment by other means. Inhibition of the CCL2 pathway, disrupting the recruitment of cells expressing the CCR2 receptor (e.g. monocytes) has, nonetheless, shown promise in several experimental models [104] [105]. Surprisingly, when initially administered but then removed, CCL2 was shown to accelerate metastasis and death of animals [106]. Despite this cautionary example, antibodies against CCL2 are in phase I and II clinical trials [107] [108].

To target macrophage survival, the CSF1/CSF1R pathway has been pursued in various ways. Following treatment with monoclonal antibody emactuzumab (RG7155), Ries and colleagues showed that $CD163^+$ M2-like TAMs were depleted in human tumor biopsies, however at efficiencies ranging from 40-90% between individual patients [109]. Further work identified IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) as factors negating the effect of emactuzumab when overexpressed in the tumor microenvironment, enabling a selection among patients most likely to benefit from this treatment [110].

When an inhibitor against CSF1R was used in a model of mouse proneural GBM model, the authors reported regression of tumor growth [111]. Interestingly, this was not due to the

depletion of TAMs but, rather, because of a shift toward the M1-like phenotype in TAMs. This suggested that reprogramming – not necessarily depletion – of TAMs can achieve the desired anti-tumoral effect. Numerous TAM reprogramming strategies have been reported to date. As previously discussed, histidine-rich glycoprotein (HRG), was found to skew M2-like TAMs towards an M1-like phenotype and to normalize intra-tumoral vasculature, enhancing the efficacy of chemotherapy [61]. A similar effect was observed using class IIa histone deacetylase (HDAC) inhibitor, TMP195, to induce systemic gene expression changes in the $CD11b^{+}$ cell that are recruited to the tumor, resulting in the accumulation of highly phagocytic M1-like macrophages that activated T cells and normalized blood vessels [112]. Paclitaxel, a common chemotherapeutic agent that acts via inducing cell cycle arrest, was also found to reprogram M2- like TAMs toward an M1-phenotype via Toll Like Receptor 4 (TLR4) in animal models of breast and melanoma tumors [113]. Targeting macrophage lysosomes by the anti-malarial drug chloroquine, Chen and colleagues demonstrated that the release of Ca^{2+} that followed chloroquine-induced increase in lysosomal pH could activate the p38 and NF-κB pathways, reprogramming TAMs [114].

Common to all these strategies is the fact that an intra-tumoral accumulation of M1-like macrophages, apart from being tumorolytic in itself, generates further downstream anti-tumor effects, as exemplified by the vessel normalization in the HRG [61] and TMP195 [112] studies and the activation of T cell-mediated anti-tumor immunity. This serves to reconstruct an actively tumoricidal microenvironment and suggests that selective reprogramming, rather than depletion of the entire population, is the more efficacious way to target TAMs.

1.2 MYELOID-DERIVED SUPPRESSOR CELLS

1.2.1 Ontological origins of MDSCs

Under normal physiological conditions, the myeloid compartment in the bone marrow contains progenitors with the potential to generate terminally differentiated cells of monocytic (e.g. macrophages or dendritic cells) and granulocytic (e.g. neutrophils, eosinophils, basophils) origins [115]. The expansion and activation of these cells usually takes place as a consequence of strong signals conveying presence of pathogens via pathogen-associated molecular patterns, (PAMPs) or non-infectious damage-associated molecular pattern molecules (DAMPs) and results in an inflammatory response [116].

Under pathological conditions, such as chronic inflammation and cancer, the differentiation of these progenitors can arrest at an intermediate stage, leading to the accumulation of cells with a strong immunosuppressive phenotype [117] [118]. Thus termed myeloid-derived suppressor cells (MDSCs) are further subdivided into polymorphonuclear PMN-MDSCs and monocytic M-MDSCs to reflect their morphological and phenotypic relation to cells from either the granulocytic or monocytic branches of myeloid differentiation [119]. In mice, they are characterized on the basis of the expression of granulocyte-differentiation antigen (GR1) and its composite epitopes Ly6C and Ly6G [120] [121]. M-MDSC will be defined by high

levels of Ly6C as $CD11b^+Ly6C^+$ cells and, conversely, PMN-MDSC are $CD11b^+Ly6G^+$ cells [115]. It should be noted that mature neutrophils will also express these markers and in mice, distinction between PMN-MDSCs and neutrophils can only be achieved by functional assays, testing the hallmark T cell deactivation ability of putative MDSCs [122]. The situation in humans is a bit different. Ficoll gradient centrifugation can be used to distinguish human high density neutrophils from low density PMD-MDSCs [123], but, on the other hand, more cell surface markers need to be included in the phenotyping panels, compared to mouse MDSCs. A current consensus denotes $CD11b^+CD14^-CD15^+$ or $CD11b^+CD14^-CD66b^+$ as PMN-MDSCs and $CD11b^+CD14^+HLA\text{-}DR^-/^{low}CD15^-$ as M-MDSCs in humans [124]. Recently, expression of lectin-type oxidized LDL receptor LOX-1 has been shown to be a marker of bona fide immunosuppressive human PMN-MDSCs [125].

According to a two-step model proposed by the laboratory of Dmitry Gabrilovich, the expansion and phenotypic activation of MDSCs relies on two distinct kinds of signals in the tumor microenvironment [126]. The first is driven by tumor-derived factors such as those activating the STAT3 pathway [127] [128] and involves the inhibition of differentiation towards fully mature monocytic or granulocytic cells. The second set of signals derive from tumor stroma, are pro-inflammatory, mediated by e.g. STAT1 [129] and NF-κB pathways [130] and result in the conversion of immature myeloid cells into bona fide MDSCs. The need for dual signals – for expansion and for phenotype acquisition – is reflected in the general lack of MDSC accumulation seen under conditions where only one of set of signals is provided [126].

The view of MDSCs as immature cells of myeloid origin is prevalent [117], but also contested [118]. It cannot, currently, be excluded that mature neutrophils or monocytes possess the capacity to assume an immunosuppressive phenotype and thus be classified as MDSCs in response to particular signals [118]. Conversion of monocytes to M-MDSCs has been demonstrated in sepsis [131] and neutrophils were redirected to a PMN-MDSC phenotype by synthetic induction of endoplasmic reticulum (ER) stress [125]. Physiological relevance of these isolated examples remains to be established.

1.2.2 The molecular basis of T cell suppression by MDSCs

The mechanisms by which MDSCs exert their immunosuppressive functions can be grouped as 1) depletion of nutrients required for T cell function 2) oxidative stress 3) impaired lymphocyte trafficking and 4) expansion of T regulatory cells with further immunosuppressive functions [132].

Both M-MDSCs and PMN-MDSCs (as well as alternatively activated macrophages) are characterized by elevated expression of arginase 1 (ARG1) [133]. ARG1 catabolizes the non-essential amino acid arginine into L-ornithine and urea [76]. Arginine is required for T cell proliferation [77]; the activity of ARG1 thus depletes it from the pool available to T cells. Arginine is also used as a substrate in the production of nitric oxide, catalysed by nitric oxide

synthase 2 (NOS2), another factor highly expressed especially by M-MDSCs [121]. The combined effect of ARG1 and NOS2 activity is a reduction in arginine levels and, consequently, impaired T cell proliferation. A similar mode of action is the sequestering of cystine by MDSCs. Cystine the precursor to the amino acid cysteine essential for T cell function [134].

PMN-MDSC are further characterized by increased production of reactive oxygen species (ROS) [115], including superoxide anion. Nitric oxide and superoxide anion react to form peroxynitrite, PNT, which in turn, induces nitration and nitrosylation of several amino acids, directly inhibiting T cell function by nitrating is receptors [135].

Apart from these direct effects on T cell viability and activation, MDSC are also known to interfere with their recruitment. In one such example, MDSC have been proposed to downregulate L-selectin (CD62L) in naïve T cells. L-selectin is a molecule that directs naïve lymphocytes to lymph nodes and to inflammatory environments, and upon its cleavage by MDSC-derived ADAM17, T cell homing is disrupted [136]. T cell migration is further affected by PNT, via nitration of T-cell specific chemokines [137].

Finally, MDCS express T cell inhibitory ligands such as programmed death-ligand (PD-L)1 and PD-L2 [121] and produce IL-10 and TGF-β [138] stimulating the expansion of regulatory T cells (Tregs), which in turn further suppress effector T cells.

1.2.3 MDSC-based therapeutic approaches

Tumor development is accompanied by the expansion of MDSCs in many mouse models and human tumors [139]. Indeed, increased MDSC accumulation is strongly associated with a shorter overall survival [140]. Apart from generating an immunosuppressive environment, MDSC also promote tumor angiogenesis [141] and metastasis [142] in animal models of cancer. Thus, the potential of targeting MDSCs in the context of anti-cancer therapy is currently being explored. Similar to the strategies employed to target TAMs, MDSC-based therapy strategies aim to impede their recruitment to the site of the tumor, interfere with their suppressive functions or selectively deplete them [143].

Much the same chemoattractants – CCL2, CXCL12 - direct the recruitment of M-MDSCs as monocyte-derived macrophages to the site of the tumor [144]. Indeed, studies that looked into the functional characteristics of $Ly6C^{+}$-monocytes in the tumor microenvironment (TME) showed them to be strongly immunosuppressive, suggesting that monocytic cells in the TME are in fact M-MDSCs [129] [121]. Further differentiation of M-MDSCs into TAMs have also been described in animal models [87] [145] [146]. This implies that therapeutic strategies targeting the CCL2-CCR2 pathway of monocyte-recruitment to the site of the tumor described in the context of tumor-associated macrophages would also be effective against M-MDSCs. To target both M-MDSCs and PMN-MDSCs, a peptibody (a peptide fused to an antibody) likely towards S100A9 was developed and shown to selectively deplete

MDSC both from tumors and spleens, with an accompanying reduction of tumor size in animal models [147]. In a pre-clinical study, increased expression of tumor necrosis factor (TNF)-related apoptosis-induced ligand receptor 2 (TRAIL-R2) was shown to mediate apoptosis in MDSCs in tumor-bearing mice, but not immature lymphoid cells from naïve mice. Targeting this receptor by an agonistic antibody proved efficient to reduce numbers of circulating MDSCs in tumor models [148]. A human monoclonal agonistic antibody targeting this receptor, DS-8273a was tested in a phase I clinical trial and also was shown to reduce the levels of circulating MDSCs [149]. Upregulation of STAT3 is a prominent feature of MDSCs development, as discussed above. Targeting STAT3 specifically in PMN-MDSCs in late-stage prostate cancer patients, by STAT3siRNA delivered via the toll-like receptor-9 (TLR9) expressed in this cell population, neutralized the suppressive effect of these cells on $CD8^+$ T cells [150].

1.3 OTHER COMPONENTS OF TUMOR STROMA

MDSCs share many features with pro-tumoral TAMs. Indeed, the consequences of the presence of these respective cell types in a tumor converge on their interaction with infiltrating tumor lymphocytes and their involvement in tumor vascularization and metastasis. But in a broader context, both MDSCs and TAMs share these features with a wider category of cells – tumor-infiltrating myeloid cells (TIMs) [151]. This group includes, in addition to MDSCs and TAMs, also tumor-associated neutrophils (TANs) and tumor-associated dendritic cells (TADCs). Just as TAMs, TANs and TADCs are plastic cells that, in their non-tumor-associated state (as dendritic cells or neutrophils), are vital in eliminating early neoplasms by engaging both the innate and adaptive arms of the immune system [9]. Initial inflammation, associated with neoplasm-driven remodeling of the tissue, guide macrophages and also neutrophils to the site. Cytokines and chemokines released by these cells, drive the accumulation of TILs, that react to processed tumor-specific antigens presented by professional antigen-presenting macrophages and dendritic cells. When the tumor adapts to the inflammatory conditions and is no longer eradicated by immune cells, equilibrium and eventually tumor escape follow, adapting with it the phenotype of the TIMs and TILs to the malign TME, as discussed above.

There is an interconnection between the cells that comprise the TIM population, manifested by factors they secrete and respond to. TGF-β or VEGF produced by TAMs or MDSCs or TANs recruit more TAMs, MDSCs and TANs to the site of the tumor [151]. This may explain why depleting a cell type, as has been done with TAMs, shows limited promise as a monotherapy [152] – compensatory mechanisms may prevail. Recently, one such mechanism has been proposed by Kumar and colleagues [153]. They could show that CSF1R blockade (targeting TAMs) induced PMN-MDSC recruitment to the tumor site, negating the effect of this treatment. Interestingly, cancer-associated fibroblasts were implicated in mediating the PMN-MDSC recruitment, illustrating complex crosstalk between cells in the TME.

The way the TIM phenotype is established also shares many similarities. For example, the STAT3 pathway mediates the immunosuppressive properties of MDSCs, as discussed, but also TAMs, TADCs and TANs [154] [155]. Consistently, the expression of the downstream target of STAT3, *Arg1*, with the accompanying suppression of $CD8^+$ T cell response, is typical for M- and PMN-MDSCs and TAMs, as discussed, and also TANs [156] and TADCs [157]. It is possible to envision how this interconnectedness can be exploited therapeutically, by identifying common targets to direct the phenotype of these cells towards their original tumorolytic role as a group and, as a consequence, amplifying the anti-tumoral response.

The crosstalk between cells in the TME, the way they influence and amplify each other, is not limited to infiltrating immune cells. The stroma around a tumor consists of basement membrane, fibroblasts, pericytes, ECM, and the vasculature [158]. Immune cells are a composite part of this active and adaptive environment. In analogy to the phenotype shift in TIMs, the stroma will be altered in the course of tumor progression and eventually promote growth, invasion, and metastasis [159].

One of the key actors in tumor stroma is the fibroblast, which synthesizes, deposits and remodels much of the ECM surrounding the tumor [160]. Much like immune cells, fibroblasts exhibit pathological cancer-associated activation states - as cancer-associated fibroblasts, CAFs - that reflect their function in wound healing and repair under cancer-free conditions [161]. Expression of alpha-smooth muscle actin (α-SMA) is characteristic for fibroblasts in both these contexts, but, contrary to activated fibroblasts, CAFs do not apoptose [158].

CAFs and immune cells interact with each other. Depleting CAFs in the 4T1 model of breast carcinoma polarized the tumor microenvironment toward a pro-inflammatory phenotype [162]. The paracrine growth factors secreted by CAFs – for example VEGF, TGFβ - fuel cancer growth [158] but also attract immune cells, as previously discussed. CAF-derived IL6 and GM-CSF together were shown to promote monocyte differentiation toward pro-tumoral TAMs in mice models of colon cancer [163]. In the clinic, co-localization of CAFs and TAMs is observed and is associated with aggressive features and high-risk classification in neuroblastoma [164].

Apart from adding to the repertoire of signaling factors of an already established tumor microenvironment, fibroblasts can also initiate expression programs of secreted factors that favor initial cell transformation. Studies in animal models have shown that senescent fibroblasts stimulate pre-malignant and malignant epithelial cell proliferation, in part due to factors secreted by the fibroblasts [165]. Loss of tumor-suppressor phosphatase and tensin homolog (*Pten)* in stromal fibroblasts but not in the tumor-initiating cells, was reported to promote the malignant transformation of mammary epithelial tumors through remodeling of the ECM and increased immune infiltration [166]. In **study IV** we used chronic obstructive pulmonary disease (COPD) as a model of cancer-initiating inflammation to identify molecular mechanisms operating in stromal cells promoting lung cancer development.

2 FROM GENOTYPE TO PHENOTYPE WITH A FOCUS ON mRNA TRANSLATION

The genetic material of a eukaryotic cell - deoxyribonucleic acid, DNA [167] - is ensconced in the cell's nucleus [168]. This aims to safeguard its integrity but, at the same time, it also introduces a particular regulatory aspect to the way this information is accessed and utilized. While the instructions encoded in the DNA have to remain intact, some of these instructions – but not all, and not always - also need to be copied, processed and executed to assemble three-dimensional polymers of amino acids – proteins - elsewhere in the cell [169]. The many ways by which regulatory aspect is addressed, bestows the different tissue types in an organism their particular and distinct functionalities despite identical genomes.

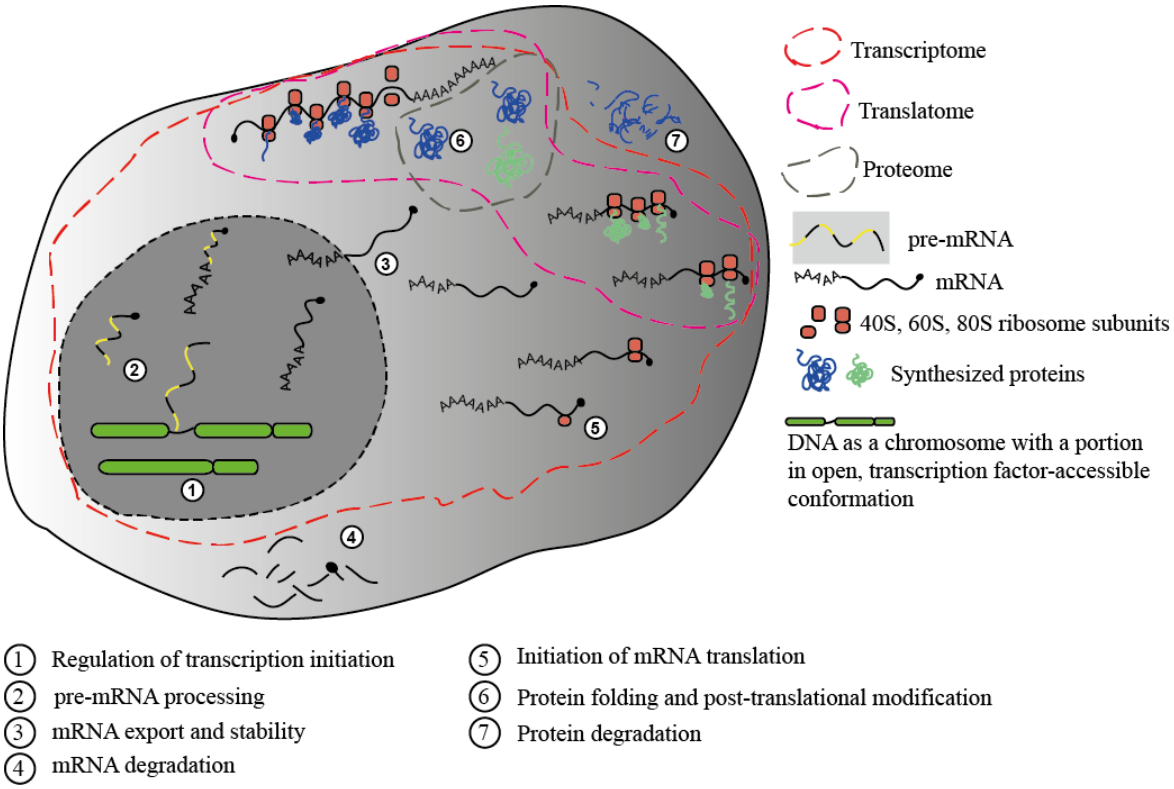

Figure 5. Schematic overview of the main steps in the regulation of gene expression

To become a functional protein, a gene's position on the DNA polymer - its locus - has to be located by the RNA polymerase [170]. For this to occur, the chromatin containing the locus has to be in an open conformation [171], the DNA itself unaffected by covalent modifications [172] and not bound by repressors [173]. The general transcription factors [174], Mediator [175], and activators [176] have to be present in the nucleus. The resulting RNA molecule - the messenger RNA, mRNA - has to be protected from degradation by the addition of 5' cap [177] and 3' polyA-tail [178], spliced to excise introns [179] and exported to the cytoplasm. There it has to avoid RNA degradation [180] and associate with ribosomes, initiating translation of the codon message into amino acids [181]. The resulting amino acid sequence has to be properly folded [182] and modified to yield a mature, functional protein [183]. Finally, it has to be delivered to its cellular or extracellular niche and eventually degraded at

an appropriate time point [184]. All of these events constitute tightly regulated events, indispensable for the cell to carry out its proper physiological program . Comprehensively, this is known as regulation of gene expression (Figure 5).

2.1 DISCREPANCIES BETWEEN mRNA AND PROTEIN ABUNDANCE

Messenger RNA is a capricious molecule to work with [185]. Chemically unstable and – unprotected - the target of many ubiquitously present cleaving enzymes [186], it degrades fast *in vitro* unless stringent preventive measures are taken [187]. Yet, RNA has a particular advantage over protein to the researcher studying gene activity – it is straightforward and relatively inexpensive to amplify and study even trace amounts of RNA. So while the proteome [188] – the expression of all proteins in a cell – arguably is the biologically more relevant parameter to understand cellular behaviour, gene expression programs normally are studied by assessing the cell's transcriptome (see figure 5 for definitions). Consequently, when investigating the effects of perturbations on the cell, conclusions are drawn on the basis of resulting changes in mRNA levels, with the underlying assumption that this is proxy for changes in the proteome [189] [190].

Several pioneering studies, published before the advent of next generation -omics technologies, addressed the veracity of this assumption. One of the earliest looked at mRNA and protein levels of a selection of genes in *S. cerevisiae* and detected that for some of the mRNA transcripts, or, conversely, proteins, found in similar concentrations in the cell, the corresponding protein or mRNA levels could differ manifold [191]. In humans, a study of 76 lung adenocarcenomas as well as 9 non-transformed tissue samples reported that among the approximately hundred genes quantified on mRNA and protein levels, only a fifth showed statistically significant correlation between these parameters [192]. Another report, in contrast, found a high correlation between 39 out of 40 proteins (and their corresponding mRNA) in a set of bladder cancer samples [193]. Arguably, being technically limited to the detection of only a fraction of cellular proteins, these and similar studies [194] [195] of the time could not provide a comprehensive overview of protein diversity in a cell and its relationship to corresponding mRNA levels. They did, however, introduce the notion that post-transcriptional may play important regulatory roles in the expression of some – or many - proteins.

Recent technological advances in the field of mass spectrometry have enabled genome-wide quantification of protein levels and corresponding mRNA levels to be made side-by-side [189]. Surprisingly, the discrepancy between these numbers persists. Schwanhäusser et al. published a landmark study in 2011, where they reported the first genome-wide quantification of mRNA and proteins levels in parallel to measured rates of protein turnover [196]. They concluded that only 40% of genome-wide protein level variability in the mouse cultured fibroblasts they studied was explained by mRNA levels.

The interpretation of these and similar data is a source of contention in the scientific community [197]. While the scientists behind the Schwanhäusser study postulate that factors downstream of DNA transcription dominate in shaping the proteome of the (mammalian) cell, others maintain that correcting for "experimental noise", transcription levels remain the best predictor of protein abundance [198] [199]. Yet others attempt to allay the debate by emphasizing the great organism-, tissue- timing- and context dependancy of this question [200].

Aiming to quantify the contribution of translation control to cellular protein levels, a recent publication revised the mathematical relationship between mRNA and protein abundances [201]. Schwanhäusser et al. originally suggested that translation rates of mRNA, i.e. how many proteins are synthesized from a given mRNA per unit time is the driving factor shaping the cell's proteome [196]. Accounting for differential translation rates enables a more congruous approximation of protein levels based on mRNA levels. Li et al. [201] argue that splitting translation rate into mRNA abundance-dependent and independent components, allows for more precise quantification of the contribution of translation, which, they maintain, remains limited compared with the contribution of transcription in their model organism, *C. Serevisae*. They further argue that certain features of the mRNA sequence, such as length or codon usage, dictate the relative contribution of the abundance-dependent and independent components, suggesting why mRNA:protein correlation for certain mRNAs would be stronger than others.

The nuances in the relationship between mRNA and protein abundance – as well as the nuances in the techniques by which it is studied - are too many and too varied for this relationship to be distilled to a universal number. Cells undergoing pertubation will behave differently to those under steady state conditions, pathological cells will differ from healthy, single cell organisms have other regulatory mechanisms than cells in multicellular organisms. Amidst this wealth of contradictory data, accumulated research converges on that whatever its precise numerical contribution in a given system in a given context, regulation of mRNA translation is indispensable for proper cellular function. Dysregulation of translation is indeed a prominent feature of many malignancies, including cancer [202].

2.2 METHODS TO STUDY CHANGES IN TRANSLATIONAL EFFICIENCY

The process of mRNA translation comprises four distinct phases: initiation, elongation, termination and ribosome recycling [181]. Of these, the initiation phase - i.e. step-wise recruitment of the ribosomal subunits while scanning the mRNA for the initiation codon until the fully assembled 80S ribosome is ready to synthesize peptide bonds - is considered rate-limiting. Recent studies have approximated initiation to take place 1-3 times per minute, while between 200-600 amino acids can be synthesized during this time [203-206]. It thus follows that investment of resources in the form of ribosome complexes recruited to the mRNA will reflect the number of protein molecules to be obtained from it, assuming that the

elongation rate along the mRNA molecule itself is equal across a pool of mRNAs. Empirical kinetic measurements by Ignolia et al., 2011 support this assumption [207].

Regulation of mRNA translation is, in some cases, a binary on-off switch: upon receiving particular stimuli, a pool of mRNA shifts from having no ribosomes to multiple ribosomes associated with it. mRNAs with the so called "5' Terminal OligoPyrimidine" (5' TOP) motif are one such example [208]. In other cases, changes in mRNA translation efficiency entails a subtle addition of ribosomes to already translating mRNAs [209]. If the ribosome occupancy per mRNA were to be plotted for a given mRNA transcript, the resulting distribution is approximately normal (providing, of course, this mRNA is translated at all) [210]. Importantly, the modes – most frequent numbers – of ribosome occupancy that reflect biologically relevant changes in translational efficiency of that population appear to lie on either side of 3 ribosomes (Figure 6). Therefore, efficiently translated mRNAs are commonly defined as mRNAs that are associated with more than 3 ribosomes in the cytoplasm. This – in general – applies for both on/off and gradual types of translation regulation [210].

Building on this discussion, studies of the translatome – efficiently translated mRNAs – are usually performed using either one of two major methods, both correlating translation efficiency to ribosome density on an mRNA molecule.

Polysome profiling was the first method to be established and is therefore referred to as either the "gold standard of the field" [211] or "an earlier version" [200] of the more recently developed ribosome profiling. "Polysomes" – multiple ribosome complexes on an mRNA – are "profiled" by stratifiyng total RNA, and any ribosomes bound to it, in a cell along a linear sucrose gradient. Following ultracentrifugation, components of the sample are separated on the basis of their sedimentation rate, the heavier the component the faster the rate. Monitoring the RNA content of the gradient by its UV-absorbance profile, enables collection of fractions containing mRNA bound to the 40S ribosome subunit, 60S, the 80S monosome and the mRNA associated with polysomes (Figure 6).

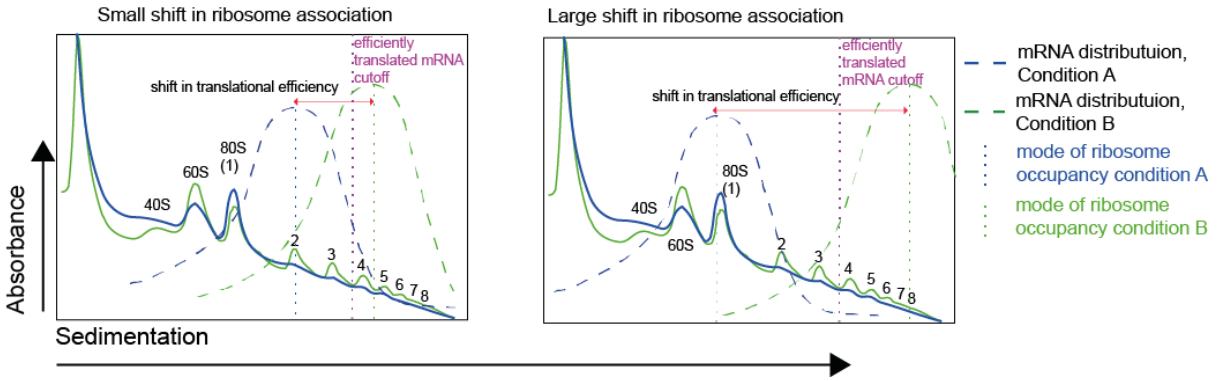

Figure 6. A sucrose sedimentation profile of the mRNA content, also displaying shifts in the distribution of ribosomes on the mRNA between two hypothetical conditions, A and B and between mRNA displaying small and large shifts. Numbers refer to number of ribosomes bound to the mRNA.

Addressing the concern that the spread of RNA over multiple fractions dilutes the RNA, increases processing times as well as introduces potential sample handling errors, our lab has recently optimized the classical protocol [212]. By replacing the linear 5-50% gradient with a modified two-step version, efficiently translated mRNA could be contained in a single fraction without compromising RNA yield and quality.

Polysome profiling physically separates mRNAs in a sample into defined pools, but it preserves the integrity of the molecules in each pool. Herein lies an important distinction from ribosome-profiling, which degrades the mRNA leaving only ribosome-protected parts for analysis (Figure 7). In practical terms, mRNA is treated with RNAseI [213]. When the resulting mixture of fragments is purified and sequenced, only the parts – around 30 nucleotides long – sterically protected by the presence of a ribosome can generate sequencing reads. Following the ribosome "footprint" along an mRNA, translation can be monitored on a codon-by-codon basis, giving unmatched resolution and providing insights into such aspects as translational dynamics, use of alternative start codons or translation from upstream ORFs [214]. On the other hand, translational efficiency is an indirect metric in ribosome profiling, inferred rather than measured based on the number of ribosome-protected fragments, RPFs (Figure 7). There are inherent analytical challenges piecing together 30 nucleotide-long snippets to recreate an mRNA. Adding to the challenge, these fragments may originate from *n* ribosomes in positions 1, 2, … on the same mRNA molecule or one ribosome each in position 1, 2… but on *n* different mRNAs. As these scenarios reflect

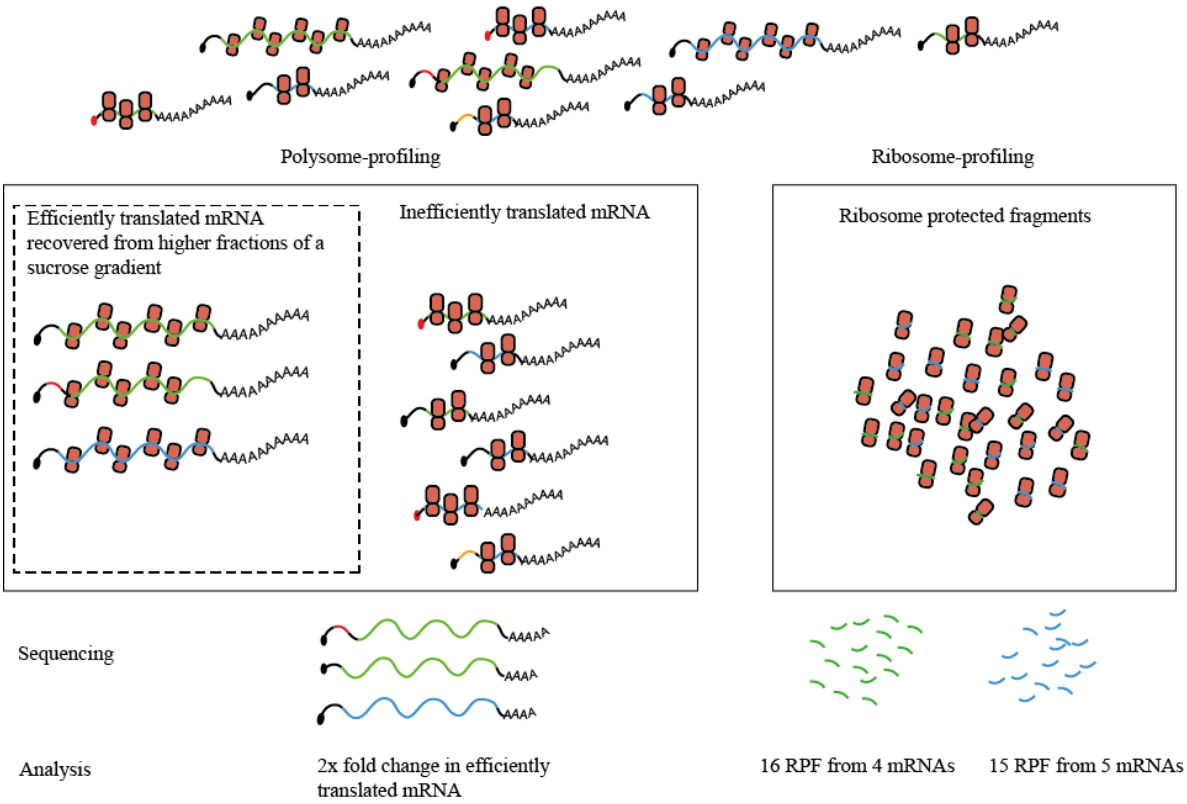

Figure 7: Schematic illustration of the principal methodological difference between poly- and ribosome-profiling and how it influences the ability of each method to capture differential translation. Note the presence of two kinds of mRNA (or same mRNA at two conditions) – blue and green. Also note presence of particular defining characteristics in the 5' UTR region (red or orange areas) that are lost during ribosome-profiling

different translational efficiencies they need to be differentiated. Furthermore, technical limitations such as sub-optimal sequencing depths may cloud the proportion of mRNAs with fewer RPFs among the RPFs from highly expressed and efficiently translated mRNAs, even if a translational efficiency shift has occurred in the former population [215]. As a consequence of these methodological nuances, when analyzing differential translation between several conditions using classical statistical significance thresholds, extremes – multiple ribosomes on an mRNA molecule versus very few ribosomes - tend to dominate in ribosomal profiling, overshadowing less pronounced but biologically relevant shifts in mean ribosome occupation [210] [215]. In addition, because all characteristics of the mRNA not protected by the ribosome at the moment of sample preparation are lost, so are important regulatory dimensions. If translation took place from an mRNA transcript isoform, this would not be apparent unless a ribosome happened to span some identifying characteristic of this isoform [216].

While polysome- and ribosome profiling remain the pillars of translatome research, other methods exist, addressing specific needs [211]. Recognizing sucrose density centrifugation underlying polysome profiling as essentially the separation of proteins on the basis of size, Yoshikawaka et al. replaced this time-consuming step with the otherwise widely used in the context of protein separation size-exclusion chromatography coupled with ultra high pressure liquid chromatography (SEC-uHPLC) [217]. By experimenting with pore sizes, serial column arrangements and flow rates, they overcame the initial concern that polysomes are too large to be efficiently separated into n-mers, monosomes and the 40S and 60S subunits by this method. The authors conclude that the output generated by the thus established Ribo Mega-Sec is comparable to polysome-profiling but has further advantages such as a shorter processing times.

To study translation in a defined cell type from a mixed population, engineered affinity-tagged proteins under the control of tissue-specific promoters can be used to “fish out” associated ribosomal complexes [218]. Known as translating ribosome affinity purification, TRAP, this technique does not discriminate between polysomes and monosomes, so it does not provide a fully quantitative view of translational regulation, to the same extent as poly- or ribosome-profiling. Its advantage lies in the insights into translation it provides in contexts such as rare cell types in the nervous system, insights that would be unattainable by other methods. Unfortunately, as it relies on prior genetic engineering it cannot be used to study, for example, patient samples.

2.3 BIOINFORMATICAL METHODS IN TRANSLATOME RESEARCH

In stringent terminology of the (polysome-profiling) translatome field, an increase - or decrease - in translational activity of an mRNA is defined as a shift of the ribosome occupancy distribution on that mRNA towards heavier – or lighter - polysomal fractions between two conditions (Figure 8). As regulatory mechanisms upstream of translation initiation – e.g. mRNA stability - affect the availability of mRNA for translation, a change in

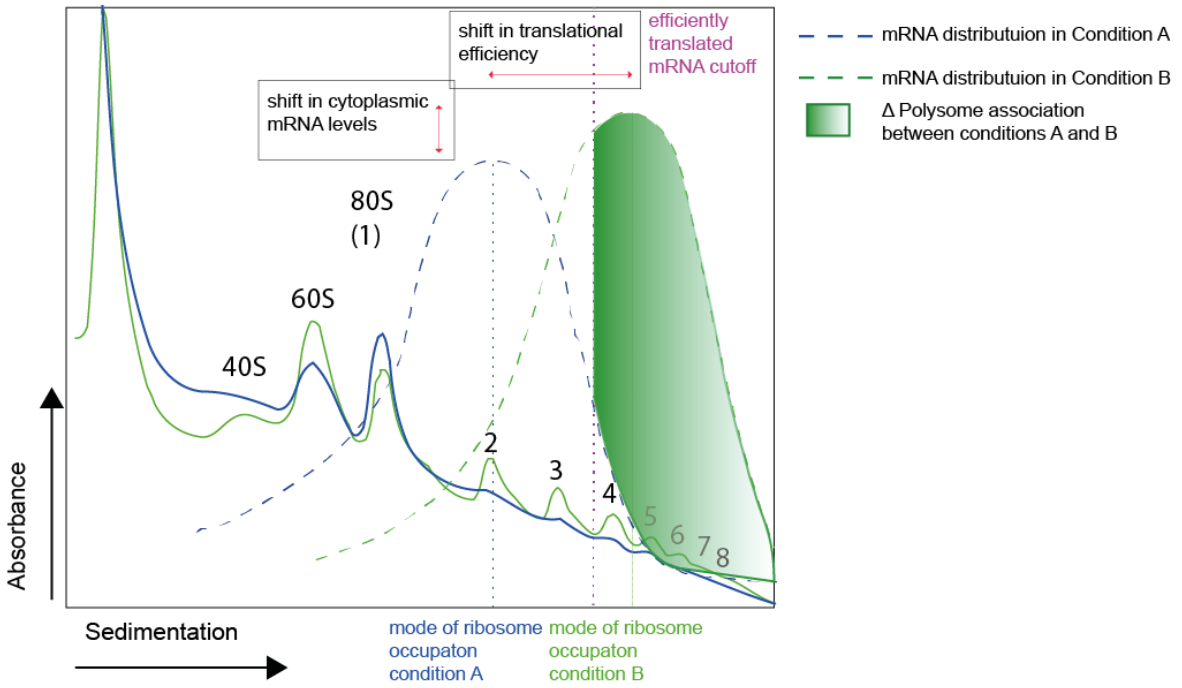

Figure 8. A change in polysome association is the change in RNA content between two conditions beyond a defined threshold of efficient translation. Illustrated here on a ribosome sedimentation plot but the principle of ribosome distribution applies for ribosome profiling as well

cytoplasmic mRNA levels would contribute to an increase of mRNAs bound to more than 3 ribosomes. Translated mRNA in translatome analyses thus needs to be collected and analysed in parallel with cytoplasmic mRNA from the same cells and this applies regardless of the method chosen to study the translatome.

An intuitive way to analyze changes in a quantity at different conditions is to calculate a ratio of this quantity at these conditions. Since logarithms are easier to manipulate mathematically, such a ratio is usually log-transformed. In translatome research, the level of an mRNA found in the polysomal fraction is compared with the level of this mRNA found in the cytoplasmic fraction. The log-ratio between these two quantities is defined as a score of translational efficiency (TE) and by looking at changes in TE many studies conclude change in translational activity in their setting. However, because of an underlying mathematical property of such a ratio known as spurious correlation – as well as empirical observation in a range of translational activity datasets – a log ratio of polysomal and cytoplasmic RNA levels often correlates with cytoplasmic RNA levels, *i.e.* the more of a transcript there is in the cytoplasm, the higher or lower (the correlation often is negative) the TE without there being any biology behind this [219].

Approaching translational activity as a change between two conditions enables the differences in polysomal mRNA levels related to differences in cytoplasmic mRNA levels to be modelled using a linear regression model [220]. In doing so, the potential for spurious correlation is removed.

$$\Delta Polysomal\ mRNA \sim \Delta cytoplasmic\ mRNA + \Delta translational\ efficiency$$

Polysomal mRNA levels (defined as mRNA levels in a pool of mRNA having a mean ribosome occupancy beyond a set threshold, see discussion above) are in this model a sum of cytoplasmic mRNA levels plus a factor that describes changes in polysomal mRNA levels of a gene that cannot be described by changes in cytoplasmic mRNA levels. This factor is the change in translational efficiency and is the key to understand regulation of gene expression at the level of mRNA translation. When there is no change in translational efficiency between two conditions, changes in polysomal mRNA levels will be completely described by changes in cytoplasmic mRNA levels.

This model constitutes the basis for analysis of translational activity (anota) algorithm developed and used in our lab to study changes in transcriptome-wide translational activities independent of cytosolic mRNA levels. In practice, anota calculations of differential translation can be visualized by plotting the cytosolic and polysomal levels of a given mRNA in the conditions studied. A least square linear regression model is then fitted to the data points assuming that the slope of the regression line is the same in all conditions. Changes in translational efficiency between two conditions are the differences in the y-intercept of the regression lines of these two conditions (Figure 9).

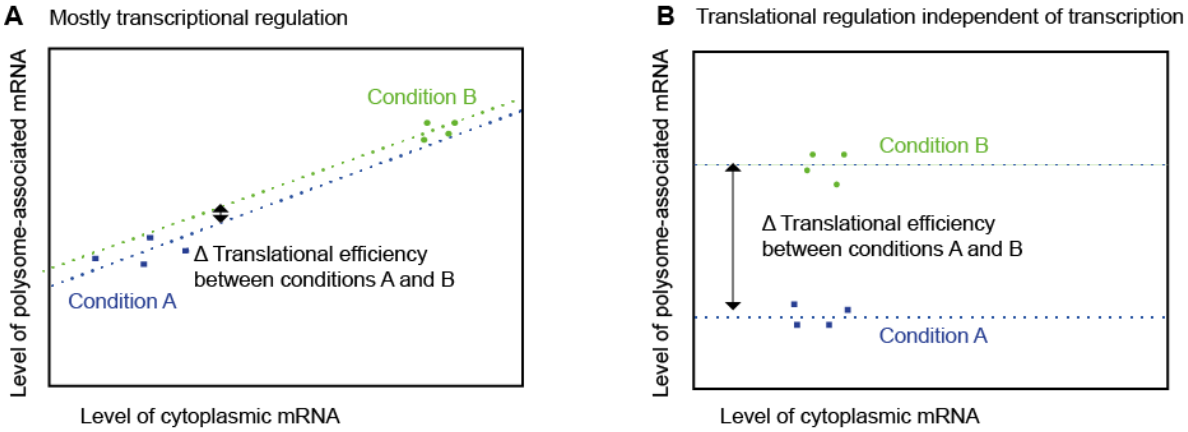

Figure 9. Illustration of the principle behind the anota algorithm in two hypothetical scenarios: one where mRNA levels change mostly on the level of total, cytoplasmic RNA, with minimal change in translational efficiency (A) and one where the change occurs mostly on the level of ribosome-association, while cytoplasmic levels remain constant (B). Dashed lines represent linear regression models fitted to have the same slope in conditions A and B. The distance between the lines is the change in translational efficiency

This algorithm can also be generalized to quantify differential translation between more than two conditions or along continuous variables, as has been done in **study III**. When the number of replicates allows for estimation of parameters for more than one phenotypic measurement, interaction terms can also be evaluated, as in **study IV**.

The anota algorithm was designed to analyze normalized continuous data such as those derived from a DNA-microarray. Seeking to extend the scope of the algorithm to cover analysis of increasingly popular RNAseq experiments as well as to be able to identify more

complex modes of translational regulation, members of our lab have recently developed an updated algorithm, anota2seq (Oertlin et al. 2018 bioRxiv doi: 10.1101/106922). It is found to consistently outperform other available methods to analyze translational activity in RNAseq experiments.

2.4 CAP-DEPENDENT mRNA TRANSLATION INITIATION

Translational control in a cell may be global – affecting the translation of most mRNAs in a cell – or selective, targeting a subset of mRNAs or even single mRNAs [221]. Both the global and selective modes of translational regulation often hinge on the availability and biochemical modulation of various translation initiation factors, in line with translation initiation being the rate limiting step in protein synthesis.

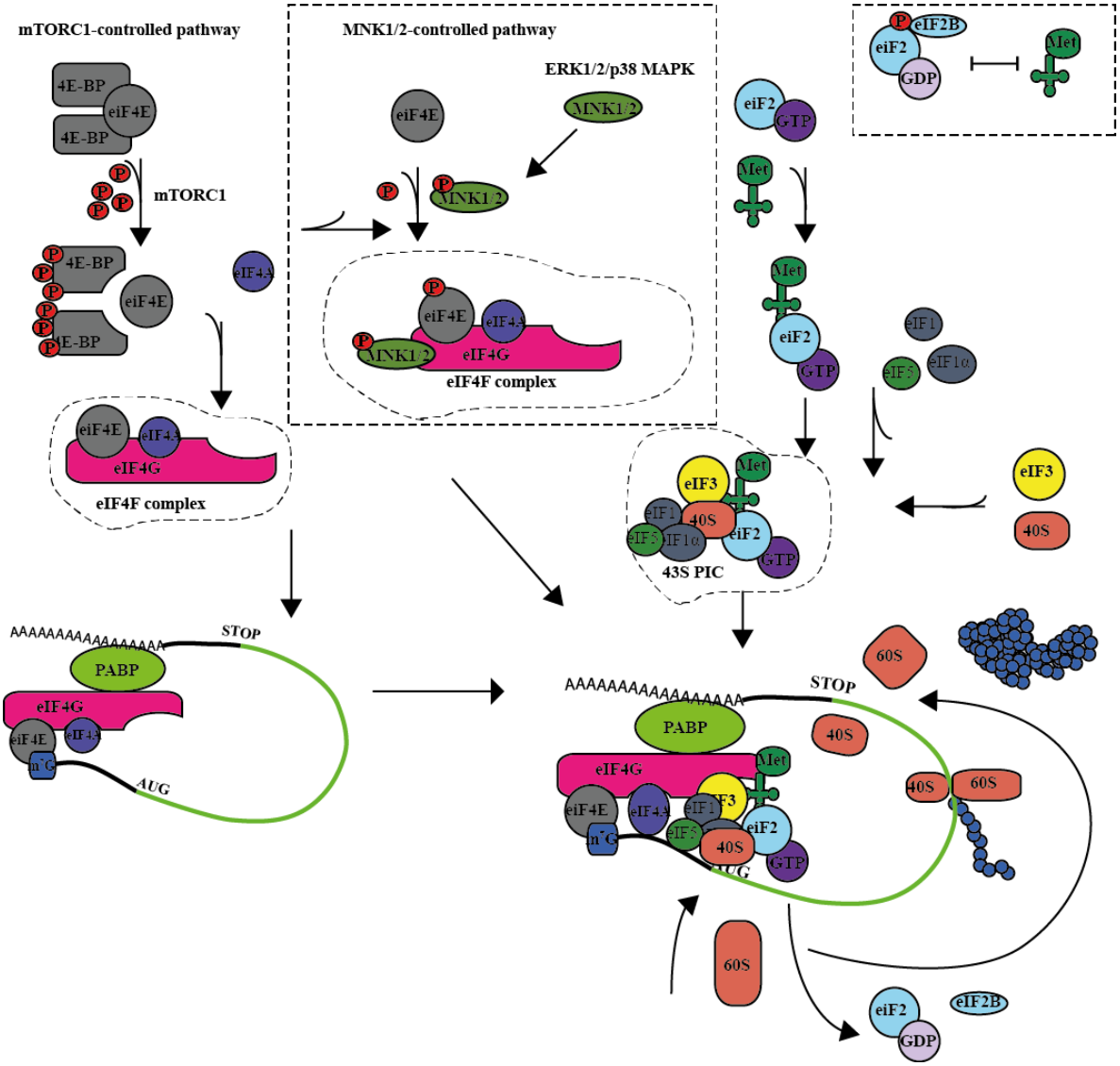

Figure 10. Schematic overview of the steps leading to cap-dependent translation initiation. For clarity, not all participating factors are shown. See text for a full description.

The description of translation initiation – as well as the description of its regulation – is commonly centered around eukaryotic initiation factor (eIF) 4 (Figure 10). As the limiting component in the translation initiation machinery, found at much lower levels compared to the other components it forms a complex with [222] (Figure 10), its stoichiometry alone can influence cellular behavior. Indeed, many cancers are characterized by increased expression of eIF4E (reviewed in [202]), it is a negative prognostic marker in some cancers [223] [224, 225] and induced overexpression of eIF4E causes cellular transformation *in vitro* [226] and *in vivo* [227]. Interestingly, heterozygous *Eif4e*$^{+/-}$ mice (homozygocity for *Eif4e* is lethal), while otherwise viable, developmentally normal and fertile, are resistant to oncogenic transformation [228].

While eIF4E is also active in the nucleus, facilitating nuclear export of certain mRNAs (some of which are involved in the process of translation) [229] [230], its central function is in the cytoplasm, where it facilitates the recruitment and assembly of factors guiding the 40S ribosomal subunit to the start codon of an mRNA [181]. Together with the scaffold protein eIF4G and the DEAD-box RNA helicase eIF4A, it forms the eIF4F complex, which, in turn, recruits mRNA via the strong affinity of eF4E to the 7-methylguanosine (m^7G) cap [231] found on almost all eukaryotic mRNAs [232].

The eIF4F – via its interaction with polyA-binding protein PABP [233] - coordinates the positioning of the mRNA in a circular loop formation (see Figure 10) favorable for translation [181, 234]. Prior to this, the small ribosomal 40S unit separately assembles with eIF3, eIF1, eiF1A, eIF5 and the so called ternary complex (TC), consisting of eIF2-GTP bound to the Met-tRNA cognate to the initiation codon, AUG. Once assembled, this is known as the 43S pre-initiation complex, PIC. eIF4G then acts as an adaptor protein via its binding to eIF3 [235] [236] to facilitate the positioning of the 43S PIC along the mRNA. The PIC initiates the process of mRNA scanning, utilizing the helicase eIF4A to resolve any secondary structures in the UTR region of the mRNA. Once the initiation codon is reached, complementarity between Met-$tRNA_i^{Met}$ causes hydrolysis of the eIF2-bound GTP and the release of the resulting eIF2-GDP. The large ribosomal 60S unit is recruited to the complex, the remaining initiation factors are released and the 80S fully assembled ribosome proceeds to the elongation phase of translation. [237] [238]

Because of the key role played by the eIF4E-m7G cap interaction to recruit ribosomal subunits to the 5' UTR of the mRNA, the mechanism outlined above is referred to as cap-dependent translation initiation and describes the vast majority of translation initiation in eukaryotic cells. The use of eIF4E, though prevalent, is not a prerequisite for cap-dependent translation initiation. Recently, a cap-dependent, but eIF4E-independent, mechanism of translation initiation proceeding via DAP5 has been described [239]. DAP5 is a homolog to a member of the scaffold protein eIF4G family, eIF4GII [240] [241]. It lacks the N terminal domain found in eIF4GII needed for PAPB and eIF4E binding, but it was found to bind an alternative cap-binding protein eIF3d [242] and initiate translation of a number of mRNAs involved in for example cell death, survival, proliferation and mobility.

Translation can proceed in the absence of eIF4F, *i.e*. in cap-independent way [243]. Certain viruses are known to rely on internal ribosome entry sites (IRESs) when translating their uncapped mRNAs in the cytoplasm [244]. Under particular conditions such as cellular stress, certain mammalian mRNAs may bypass cap-dependent translation initiation too [245] [246], but whether this occurs via an IRES-like mechanism is debated [247] [248]. Recently, the concept of cap-independent translational enhancers (CITEs) located in the sequence of certain mRNAs, binding components of the translation machinery necessary to initiate translation, has been proposed [249].

2.5 REGULATION OF CAP-DEPENDANT TRANSLATION INITIATION

As the limiting component in the rate-limiting initiation step of translation, eIF4E is found at the node of two of the major and most-well characterized pathways regulating translation initiation. In one of these pathways, the capacity of eIF4E to bind to eIF4G is modulated by the mTORC1 complex. In the second one, eIF4E is the target of direct phosphorylation by MNK1/2 kinases (Figure 10).

2.5.1 Regulation of mRNA translation initiation via mTORC1

Mechanistic target of rapamycin, (mTOR), is a threonine/serine kinase [250] that coordinates upstream signals concerning, for example, nutrient availability, growth factor signaling and oxygen levels with appropriate downstream responses [251]. It thus serves as a nexus point in critical cellular pathways that commonly are deregulated in cancer. It exists in two complexes - mTORC1 [252] and mTORC2 [253] – associating with different effector proteins and influencing mostly non-overlapping processes in the cell. The involvement of mTORC2 in protein synthesis is limited to facilitating proper protein folding of a specific target, AKT, by direct association with the ribosome [254]. Indirectly, it can regulate mTORC1 – the other mTOR complex regulating cell metabolism, proliferation and growth - via phosphorylation of AKT in a signaling chain that culminates in RHEB-GTP activation of mTORC1 [255]. Among the many downstream targets of mTORC1, in turn, two central ones are eIF4E-Binding Proteins (eIF4E-BPs) [256] and S6 kinases (S6Ks) 1 and 2 [257], both of which have prominent roles in regulating translation.

2.5.1.1 mTORC1-S6Ks

The S6Ks phosphorylate components of the translational machinery, such as ribosomal protein S6 [258] and eukaryotic elongation factor (eEF) 2 kinase [259]. eEF2 facilitates ribosome translocation to the next codon during elongation. When phosphorylated by eEF2 kinase, its interaction with the ribosome is impaired [260], providing a well-characterized example of translation being regulated at the level of elongation, rather than PIC assembly and initiation. Similarly to translation initiation, the regulation of eEF2 kinase has been shown to be targeted by oncogenic signaling to facilitate efficient protein synthesis required for cancer cell function [261]. *In vivo*, targeting the mTORC1–S6K–eEF2K–eEF2 pathway

in a model of colorectal cancer by rapamycin treatment (which inhibits S6K phosphorylation by mTORC1, and, consequently, eEF2K activity), suppressed tumour development [262].

As evidenced by gene deletion studies in mice [263] and *in vitro* [264], the combined effect of S6K activity appears to converge on modulating cell (and organism) size. Via their interaction with eIF4B [265] and PDCD4 [266] (which, when unphosphorylated, sequesters eIF4A), S6Ks are also indirectly involved in regulating the pre-initation complex.

2.5.1.2 mTORC1-4E-BPs

The assembly of the eIF4F is a critical point in initiation of translation (Figure 10). In resting cells, it is restricted by the binding of repressive eIF4E-BPs to eIF4E, preventing its association to the scaffold of the complex, eIF4G. Upon sequential phosphorylation by MTORC1, eIF4E-BP lose affinity for eIF4E, freeing it for eIF4G binding and, consequently, eIF4F assembly [267]. Studies in 4E-BP DKO mice (lacking all three known 4E-BP proteins) have separated the effect of MTORC1-4E-BP signaling from the MTORC1-S6K pathway, by showing that MTORC1-4E-BP regulates cellular proliferation but not growth [268]. In concordance, 4E-BP have been shown to act as tumor suppressors, by increasing the capacity of the cell to resist oncogenic transformation when overexpressed [269] [270] and by being inactivated in aggressive breast carcinoma cells [271].

2.5.1.3 mTOR-sensitive mRNAs

Based on the features of their 5´-UTR, certain mRNAs are particularly sensitive to perturbations in mTOR signaling. A subset of these mRNAs, in particular key proteins involved in proliferation (for example MYC [272] and cyclin D1 [273]), are sensitive to the levels of available eIF4E, mediated by mTORC1-4E-BP signaling. Many of these "eIF4E-sensitive" mRNAs have long and complex 5'-UTR. eIF4A, the helicase in eIF4F-complex, is considerably more active when a part of the complex compared to when it is free [232]. It is thus proposed that the efficient translation of these eIF4E-sensitive mRNAs is dependent on eIF4E to assemble the eIF4F complex, including eIF4A, which would then facilitate 5'UTR scanning by unwinding secondary structures [274]. On the other hand, it has recently been shown that mRNAs with extremely short 5'-UTR also are eIF4E-, but not eIF4A-sensitive [171].

Another class of mTOR-sensitive mRNAs are the TOP mRNAs, carrying a so called "TOP"-motif (a cysteine immediately after the 5' cap and a string of 4-15 purimidines). Most of these mRNAs encode components of the protein synthesis machinery, e.g. initiation factors and PABP [224]. In recent ribosome profiling studies, they were identified as almost exclusive targets of mTOR-signaling [275] [276], contradicting the well-known eIF4E dependency of non-TOP mRNAs discussed above. It has been argued that the limitations of the ribosome profiling method, discussed in **section 2.2**, led to the loss of non-TOP mRNAs displaying less pronounced efficiency shifts in the conditions of these studies [215]. The conclusion that the translation of TOP mRNAs proceeds via 4E-BP, is furthermore

contradicted by examples showing it not being dependent on 4E-BP, or RAPTOR, the 4E-BP recruiting unit of mTORC1 [277] [278]. Currently, it is proposed that La-related protein 1 (LARP1) [279] regulates the translation of TOP mRNAs, possibly via binding to the 5' cap and thus inhibiting eIF4E association [280]. LARP1 itself associates with RAPTOR and is phosphorylated by mTORC1 which is thought to modulate its mRNA-binding activity [281].

Collectively, these examples of mTOR-sensitive translation illuminate the principle of selective – in this case, on the basis of specific 5'-UTR characteristics - regulation of mRNA translation. Importantly, eIF4A [282] and eIF4G [283] also have a set of associated "sensitive" mRNAs.

2.5.2 Regulation of mRNA translation initiation by MNK1/2-eIF4E phosphorylation

Apart from being regulated on the basis of expression level and capacity to bind to eIF4G, eIF4E can also be biochemically modulated by direct phosphorylation at a sole amino acid – serine 209 [284]. The only known kinases capable of phosphorylating eIF4E are MNK1 and MNK2 [285]. In humans, these exist in two isoforms each: MNK1a and 1b [286], MNK2a and b [287]. In mice there is only the "a" isoform [288]. Both MNK1 and MNK2 need to be phosphorylated to become active [289]. MNK2 has been shown to possess high basal activity [290], via its stable binding to phosphorylated ERK, protecting it against dephosphorylation and inactivation [291]. MNK1 activity, on the other hand, is induced as a consequence of Erk1/2 and p38 MAPK signaling [292]. When phosphorylated, they bind to eIF4G [293] and, in turn, phosphorylate eIF4E.

The role of eIF4E phosphorylation in translation initiation is poorly understood, although circumstantial evidence showing its importance are abundant. On one hand, *Mnk1* and *Mnk2* double knockout mice (*Mnk1/2* DKO) are fully viable and developmentally normal [285] and so are mice in which serine at position 209 in eIF4E has been replaced by alanine, which cannot be phosphorylated (eIF4E$^{s209a/s209a}$) [284]. This indicates that phosphorylation of eIF4E is not a prerequisite for normal physiological development. On the other hand, it is clear that phosphorylated eIF4E has a role in oncogenic transformation and metastasis. The ability of mutated non-phosphorylatable eIF4E to transform cells *in vitro* is substantially reduced [294]. eIF4E$^{s209a/s209a}$ mice and MNK DKO are resistant to tumorigenesis in animal models of prostate cancer [284] and *Pten*-deficient lymphoma [295] respectively. Metastasis is impaired in MMTV-PyMT eIF4E$^{s209a/s209a}$ model of mammary cancer [296]. In humans, increased phosphorylation of eIF4E is seen in many cancers [297] and is associated with poor prognosis in non-small cell lung cancer [298] and melanoma [299]. The effect of eIF4E phosphorylation on global mRNA translation is context-specific, correlating with increased translation in a model of HSV-1 viral infection [300] but, conversely, constitutively active MNK1 and MNK2 were found to decrease cap-dependent translation in HEK293T cells [301]. Importantly, there are differences in the subsets of eIF4E and phospho-eIF4E sensitive mRNAs, with chemokines being an important class of genes whose translation is affected by the absence of eIF4E phosphorylation [284].

2.5.3 Regulation of Ternary Complex formation and the Integrated Stress Response

While the mTOR and/or eIF4E-centric pathways of translational regulation are demonstratedly important, they are by no means the only means to regulate translation. TC formation is a key step in 43S PIC assembly (Figure 11). The binding of Met-tRNA$_i^{Met}$ to the P site of the ribosome is accompanied by hydrolysis of GTP bound to eIF2 [302]. eIF2, now bound to GDP dissociates from the complex. To participate in the TC association in the next round of translation initiation, it requires the guanine nucleotide exchange factor eIF2B to facilitate the exchange of GDP to GTP [303] (Figure 11). The levels of available eIF2B in a cell are limited, and phosphorylation of eIF2 at the alpha subunit greatly increases the affinity of eIF2B towards eIF2 [304], thus sequestering it. As a consequence, Met-tRNA$_i^{Met}$, which has a much higher affinity towards eIF2-GTP [305], is not bound as efficiently and the TC is not formed, leading to a global reduction of translation. This mechanism is employed by the cell as a part of so called integrated stress response (ISR) to various stresses, such as starvation [306], haem-deficiency [307] virus [308] or accumulation of unfolded proteins in the endoplasmic reticulum (ER) [309]. For example, the protein unfolding response (UPR) proceeds by inducing the activity of the eIF2α kinase PERK [310], impeding global translation and promoting preferential translation of particular mRNAs such as mRNAs encoding transcriptional stress-response regulators (e.g., ATF4) which are inefficiently translated under non-stress conditions. This is followed by recovery of translation, via induction of GADD34 [311], a stress-induced gene that binds to PP1 phosphatase and dephosphorylates eIF2α [312] thus reinstating TIC formation.

Recently, a different ISR mechanism has been described, operating under conditions of chronic ER stress [313]. While also employing PERK, it entails a consistent, not transient, translation of uORF mRNAs concurrent with a partial translational recovery. eIF2B remains

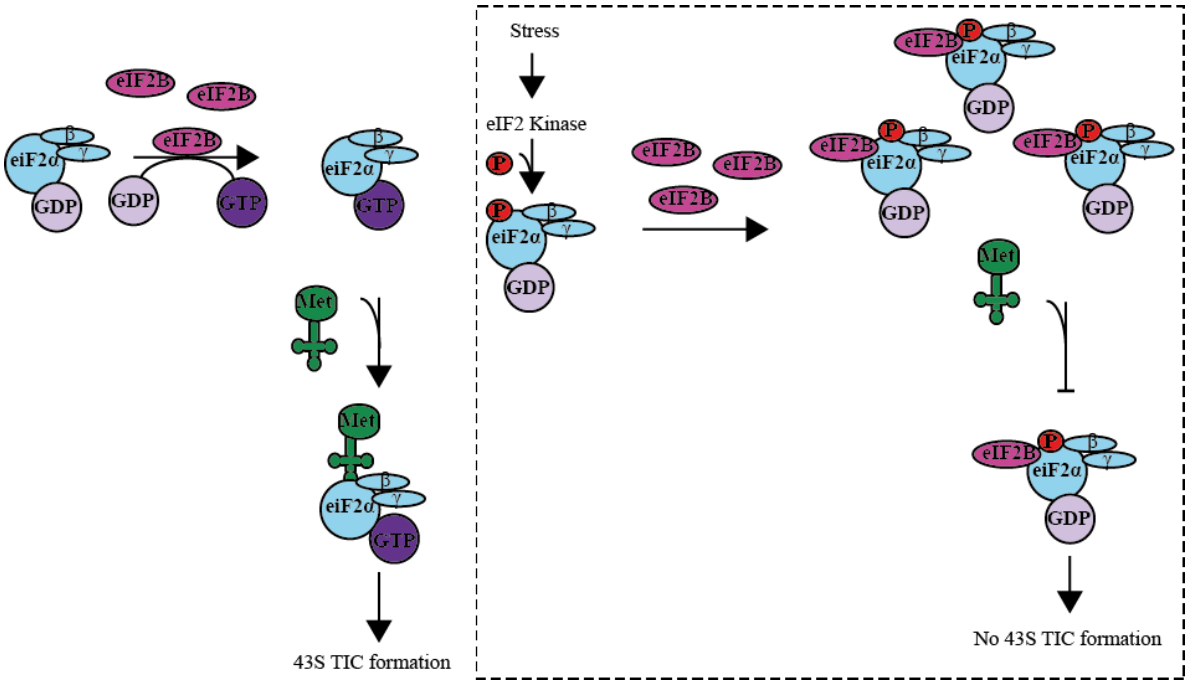

Figure 11 Illustration of regulation of translational initiation at the level of ternary complex assembly

suppressed however, suggesting there exists an alternative way to transport Met-$tRNA_i^{Met}$ to translation start site. Translation during chronic ER stress was furthermore found to be mediated by eIF3d, the alternative cap-binding protein discussed previously.

2.5.4 Regulation of mRNA translation by RNA-Binding Proteins

While the discussion until now has focused on the pathways involving translation initiation factors, a very significant part of regulation of mRNA translation is mediated by other means. Many mRNAs are regulated specifically by the action of particular RNA-binding proteins (RPBs) that recognize motifs (so called cis-elements) often in the 3' UTR of the mRNA and direct translation by binding [314]. Relevant to the discussion of translational regulation in immune cell function, many key components of signaling pathways in immune cells are regulated by RPBs. A classic example of such regulation is the GAIT translational repressor complex. In humans, it consists of the aminoacyl-tRNA synthetase EPRS, the RBP NSAP1, the ribosomal protein L13a and glycolysis enzyme GAPDH [315]. Its binding to a GAIT element (a stem-loop secondary structure with an asymmetric internal bulge) in the 3′ UTR of ceruloplasmin mRNA, disrupts its translation, thus abrogating ceruplasmid protein levels, while the mRNA levels remain unaffected [316]. Ceruloplasmin is an acute-phase plasma protein with bactericidal activity [317] produced by hepatocytes and activated monocytes and macrophages in response to IFNγ [318]. It's timely synthesis but, likewise, inhibition of translation, is an example of the importance of swift adaptation to external stimuli in immune cells. Since the discovery of the GAIT element on the 3' UTR of ceruloplasmin, it was also identified on VEGF-A [319] and several chemokine ligands and receptors discussed in this text, such as CCL22 and CXCR3 [320].

Transforming growth factor-β-activated kinase 1 (TAK1) has recently been identified as the target of translational regulation by two distinct RBP-mediated pathways: via binding of heterogeneous nuclear ribonucleoprotein K (hnRNP K) [321] and cytoplasmic-element-binding (CPEB) protein [322]. TAK1, a mitogen-activated protein kinase kinase kinase (MAP3K) participates in a signaling cascade cultimating in the activation of the NF-κB pathway and the subsequent expression of several pro- and anti-inflammatory cytokines [323]. Through a genome-wide RNA Immunoprecipitation chip (RIP-Chip) analysis of RAW 264.7 macrophage cell line and BMDMs with HnRNP K as bait, with or without LPS stimulation, several TLR4 signalling candidates were found to co-precipitate with HnRNP K in response to the LPS treatment [321]. TAK1 was selected for further analysis, and hnRNP K was found to bind a regulatory sequence, containing AU-rich element (ARE) in the TAK1 mRNA 3′-UTR, suppressing its translation. Upon siRNA-mediated hnRNP K knockdown, TAK1 mRNA translation is enhanced (while mRNA levels remained stable), resulting in elevated TNF-α, IL-1β, and IL-10 mRNA expression. The authors speculate that upon LPS stimulation, hnRNP K may become phosphorylated abrogating its RNA binding capacity and thus releasing TAK1 mRNA. Similarly, CPEB, binding a putative cytoplasmic polyadenylation element (CPE) in the 3'-UTR of TAK1, was shown to regulate its translation, thus controlling the NF-kB-mediated inflammatory response. In its absence, LPS-

challenged CPEB double knockout mice display hypersensitivity to endotoxic shock but this is lost upon TAK1 inhibition [322]. Cumulatively, the presence of regulatory GAIT elements, AREs and CPEs in the 3'-UTR of genes related to inflammation, highlight the importance of translation regulation in controlling the inflammatory immune response.

2.5.5 Regulation of mRNA translation by secondary structures in the 3'-UTR

The cis-elements may not need RBPs to regulate translation – secondary structures in the mRNA that affect initiation factor binding are one such example [324]. Recently, this was illustrated in the translational regulation of MCPIP1 by proinflammatory cytokines IL-1 and IL-17 [325]. MCPIP1, also named ZC3H12A or regnase-1, is induced by LPS treatment in macrophages and suppresses inflammatory processes by inhibiting inflammatory gene expression and NF-κB activation in response to LPS [326]. Its translation is inhibited by presence of 5'-uORF but also by the presence of putative stem-loop-forming sequence in its 3'-UTR, previously associated with rapid degradation of the mRNA [327]. Binding of IL1 or IL17 to the 3'-UTR stabilized the mRNA and increased its translation.

2.5.6 Regulation of mRNA translation by microRNA

Micro-RNAs (miRNAs) can also affect protein synthesis [328]. While many miRNA induce mRNA degradation of target mRNAs by recruiting deadenylases or decapping enzymes, promoting the degradation of these protective structures and the subsequent mRNA degradation through the mRNA decay pathways, some repress its translation without affecting mRNA levels [329]. Three proposed mechanisms by which they do so are promoting PAPB displacement [330], disrupting mRNa circularization, and/or recruitment of translational repressors [331] and/or dissociation of eIF4A from eIF4E [332]. Many inflammatory processes in innate immune cells are regulated by miRNA repression [333], one of the first such miRNAs to be identified was miR-146, whose expression is induced by NF-kB signaling and thought to regulate toll-like receptor and cytokine signaling [334].

3 THESIS FINDINGS IN CONTEXT

"Let us reflect. Let us reason. Let us – *enfin*! – employ our little grey cells!"
-Hercule Poirot

The work presented in this thesis has touched upon the themes of cancer initiation – highlighting the importance of DNA repair mechanisms to safeguard against neoplastic transformation **(Study V)** and the contribution of the stromal environment in fueling it **(Study IV)**. It has applied a previously implicated biological target – SEMA3A – to shift the balance of tumor-associated macrophages in an established tumor **(Study I)** and to phenotypically alter tumor-promoting MDSCs, **(Study II)**, in sum priming immune cells in the tumor microenvironment to resist tumor development. Finally, it has extended the search for novel such anti-tumor targets to a global scale by looking for clues in the regulation of gene expression on the level of mRNA translation in tumor-associated macrophages **(Study III)**. Though each individual study in itself is but a snapshot of a bustling research area, together they form a continuum highlighting important themes in contemporary cancer research.

3.1 STUDY I

Guidance molecule SEMA3A restricts tumor growth by differentially regulating the proliferation of tumor-associated macrophages

Semaphorin3A (SEMA3A) is a signaling protein expressed in most human and mouse tissues. It was first identified due to its involvement in directing axon growth but is now believed to regulate many physiological and pathological processes, including angiogenesis, immune response and tumor development [335] [336]. SEMA3A binds a receptor complex consisting of the ligand-binding subunit neuropilin 1 (NP1) and plexin A family (1-4) that acts as signal transduction molecules [337]. Gene expression studies in human cancers suggest inverse correlation of SEMA3A expression with tumor malignancy in most tumors [336]. Indeed, in this study, we show that SEMA3A is downregulated in higher grades of ductal breast carcinoma, and, in addition, its expression is positively correlated with the expression of M1-like TAMs markers i.e. CD80 and CD86, as well as CD8 T cell and NK cell-associated markers, suggesting its elevated expression in tumor microenvironments characterized by more activated associated cytotoxic cells [39].

Existing data concerning the role of SEMA3A in tumor immunity is contradictory. On one hand, its overexpression in cancer models has been shown to inhibit tumor growth by several mechanisms, including impeded tumor angiogenesis [338]. It has also been reported to recruit a subset of NP1-expressing monocytes with anti-tumor properties from the bone marrow [339]. Other studies suggest, however, that it can also facilitate accumulation of pro-tumor macrophages in hypoxic areas of the tumor, thus contributing to tumor progression [340].

We therefore sought to clarify the functional role of SEMA3A in the tumor microenvironment, particularly its involvement in shaping the composition of the TAM population. Using lentiviral gene transfer, 4T1 tumor cell line was engineered to overexpress SEMA3A and injected in the mammary fat-pad of naïve mice, growing orthotopically (i.e. in the anatomically correct site) and thus exposed to breast-specific microenvironment during growth. Consistent with previous reports [338], SEMA3A efficiently hampered tumor growth. The SEMA3A-overexpressing tumors displayed an accumulation of M1-like TAMs, as characterized by elevated levels of antigen-presenting molecules MHCI and MHCII, T cell activating molecules CD80 and CD86, as well as M1-marker CD11c and a decrease of M2-marker MRC1 (CD206). On RNA level, we could detect upregulation of T cell recruiting and activating chemo- and cytokines. Importantly, SEMA3A-overexpression increased the accumulation of $CD8^+$ and NK cells to the tumor site and these cells in SEMA3A-tumors expressed higher levels of IFNγ and CD69, suggesting an activated phenotype.

In order to elucidate the role of TAMs in SEMA3A-mediated inhibition of tumor growth, we performed a proof-of concept experiment where we depleted TAMs by targeting CSF1 with a neutralizing antibody (clone 5A1). Depletion of TAMs revoked the SEMA3A-mediated tumor growth reduction and, importantly, TIL accumulation. We then depleted $CD8^+$ T cells and NK cells and we observed that in the absence of these cells, SEMA3A-overexpression, likewise, failed to reduce tumor growth. We thus conclude that SEMA3A-mediated reduction of tumor growth is dependent on the accumulation of anti-tumor TAMs, which, in turn, recruit activated cytotoxic T and NK cells to the tumor. Surprisingly, TAM depletion in itself did not impede tumor growth. This is in concordance with many similar findings [153] [61] [82]. It appears that targeting CSF1 is most effective when used in synergy with other treatments, such as chemotherapy [82] but that the TAM population is too heterogeneous and reliance on CSF1 is too stage-specific for this to have an universal detrimental effect on tumor development [152].

SEMA3A has previously been proposed to act as an attractant for NP1-expressing mononuclear phagocytes, including macrophages [341]. Casazza et al. [340] illustrate an example of such chemotactic mechanism, whereby hypoxia-upregulated tumor-derived SEMA3A (as well as VEGF-A, also a binding partner of NP1) act as macrophage attractants, guiding TAM localization, and subsequent entrapment, in hypoxic areas inside the tumor. Hypoxia fine-tunes the pro-tumor phenotype of TAMs [342], and, cumulatively, this suppresses tumor immunity and promotes angiogenesis and metastasis. Disrupting this chemotaxis via NP1-deletion in macrophages, limits the localization of TAMs to normoxic areas, consequently maintaining a more immune-active TAM phenotype and obstructing tumor development.

In contrast, when we investigated the effect of SEMA3A overexpression on the circulation of inflammatory and patrolling monocytes, we could not detect any statistically significant differences in the monocyte-recruitment to SEMA3A-tumors, compared to controls. Neither did we detect SEMA3A-induced chemokine expression differences in the tumor cells that

would explain increased monocyte recruitment. Finally, SEMA3A had no effect on the migration capacity of BMDMs polarized to either M1- or M2 phenotypes. Cumulatively, this led us to conclude that the effect of SEMA3A on intratumoral immune cell composition in our tumor model does not stem from increased or differential recruitment of monocytes or anti-tumor TAMs.

Surprisingly, SEMA3A had no direct effect on BMDM polarization, suggesting that the altered composition of the TAM population in SEMA3A tumors is not due to direct polarizing or reprogramming effects of SEMA3A. Given recent studies implicating proliferation as a mechanism to expand TAM population [91, 93], we investigated if proliferation of M1-like and M2-like macrophages was affected by SEMA3A. Indeed, both M1-like TAMs and M1-polarized BMDM showed increased incorporation of BrdU, while the effect in M2-like TAMs and M2-polarized BMDMs was reversed in SEMA3A-tumors.

While the capacity of macrophages to proliferate has been established prior to our study, the concept of differential proliferation between macrophages in different polarization states is novel. The expression of NP1 was previously shown to be induced upon M2-polarization, and correspondingly, reduced upon M1-polarization in human monocyte-derived macrophages [343]. In this report, SEMA3A was furthermore shown to induce apoptosis in macrophages resistant to Fas-mediated cell death and the authors speculate that downregulation of NP1 could be a mechanism by which M1-macrophages escape SEMA3A-mediated apoptosis during inflammatory conditions. Consistently, we show significantly higher expression of NP1 in M2-polarized BMDMs compared to M1-polarized BMDMs. Furthermore, we show that the differential effect of SEMA3A on M1- or M2 macrophage proliferation is NP1-dependant; the differential effect of SEMA3A treatment on AKT and MAPK phosphorylation in M1- and M2-macrophages in BMDMs lacking NP1 was lost.

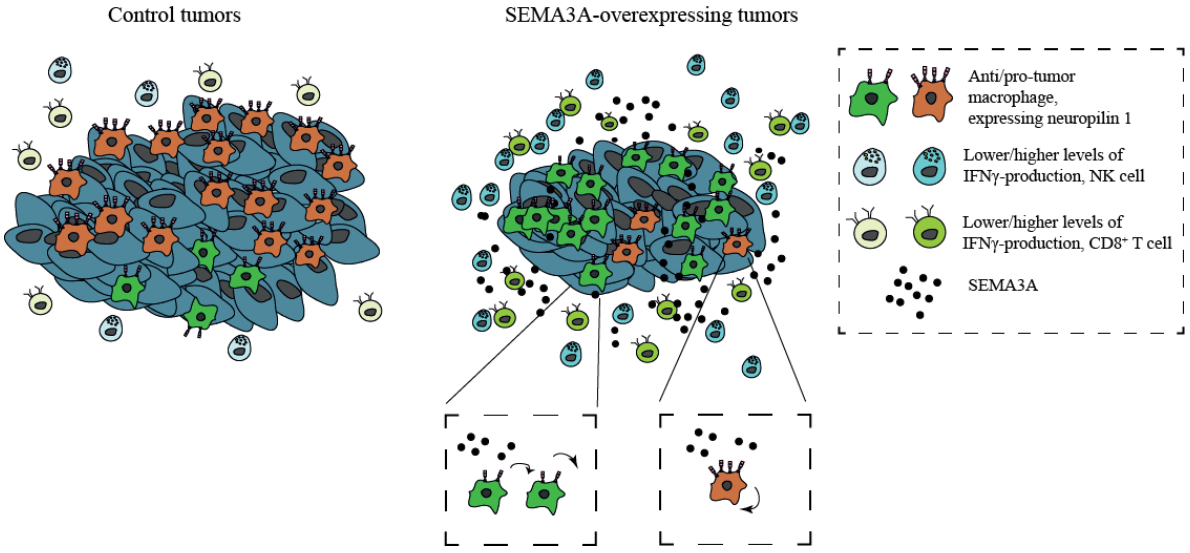

Figure 12. Schematic summary of key findings of study I. SEMA3A overexpression by tumor cells leads to selective proliferation of macrophages with M1-like pro-inflammatory characteristics. These, in turn, recruit cytotoxic cells to the site of the tumor, contributing to diminished tumor growth

It is thus plausible that, like during its induction of apoptosis in human monocyte-derived M2-macrophages, SEMA3A exerts some sort of regulatory "stop signal"-effect downstream of its signaling, an effect that M1-macrophages escape due to downregulated NP1-levels. Given that NP1, in itself, lacks a signal-transducing cytoplasmic domain, and can thus only transmit signals when in complex with members of the plexin A family, it is plausible that the differential SEMA3A signaling in M1 and M2-like macrophages may furthermore depend on the identity of plexins in the assembled NP1 receptor complex. This would require further investigation. In conclusion, our study is the first study to show that the TAM composition in the TME of a mammary tumor model can be modulated by differential proliferation of particular macrophage subsets. By selectively increasing proliferation of M1-like macrophages while impeding proliferation of M2-like macrophages we achieved a tumor-suppressing microenvironment populated by recruited and activated T and NK cells.

3.2 STUDY II

Semaphorin3A re-educates myeloid-derived suppressor cells towards a pro-inflammatory phenotype

Apart from an increase in, and altered composition of, the TAM population, SEMA3A overexpression in our 4T1 tumor model also resulted in a decrease in the intratumoral $CD11b^{+}Ly6G^{+}$ population. This was also seen in the spleens of tumor-bearing mice. The spleen is an important site of extra-medullary (i.e. occurring outside of the bone marrow) myelopoiesis prominent during pathological conditions such as cancer and is a potential site of the origin of MDSCs [118]. Importantly, the $CD11b^{+}Ly6C^{+}$ population was not affected. Furthermore, we established that both $CD11b^{+}Ly6C^{+}$ and $CD11b^{+}Ly6G^{+}$ cells express the NP1 receptor to a similar extent and that SEMA3A treatment in itself did not affect this expression. We thus hypothesized that SEMA3A may exert an effect on the intratumoral $CD11b^{+}Ly6G^{+}$ population that is separate from its effect in stimulating the proliferation of M1-like TAMs, and, cumulatively, this would result in the observed diminished tumor growth in the SEMA3A overexpressing tumors.

The mouse model we chose to use in **study I** and **study II** – the 4T1 model – is particularly suitable to assess the role of $Ly6G^{+}$ cells on the tumor development. In itself, it is a fast-growing and aggressive tumor model that resembles triple negative breast cancer (TNBC) and in later stages recapitulates characteristics of stage IV TNBC [344]. Relevant for this study, we observe higher granulocytic infiltration in these tumors compared to other breast cancer models we employ.

We choose to refer to the intratumoral $CD11b^{+}Ly6G^{+}$ population as polymorphonuclear myeloid-derived suppressor cells, MDSCs. The choice of terminology is contentious. Under normal physiological conditions, $CD11b^{+}Ly6G^{+}$ cells would be identified as neutrophils and their function would be to lyse and phagocytose cells and activate the immune system. Due

to the current lack of verified MDSC-specific expression markers in mice, only functional tests can distinguish neutrophils and MDSCs [345]. In the tumor, the situation becomes even more complex as TANs, much like TAMs, have been proposed to exist along an anti-tumorigenic (N1) and pro-tumorigenic (N2) functional continuum. Whether N2-like TANs and MDSCs are indeed different cells and, if so, what are the defining features of these respective cell types is not yet firmly established in the scientific community [346].

By performing a Ly6G-depletion (1A8 clone) experiment, we observe that the control tumors without $CD11b^{+}Ly6G^{+}$ cells display reduced growth at later stages of the growth curve. This leads us to conclude that the cells we deplete had a pro-tumor phenotype. Depleting $Ly6G^{+}$ cells in itself did not increase the activation of $CD8^{+}$ T cells, and only marginally increased the activation of $CD4^{+}$ T and NK cells. This is counterintuitive given the hallmark immunosuppressive identity of PMN-MDSC. On the other hand, it has been established that M-MDSCs ($CD11b^{+}Ly6C^{+}$) are more potent immunosuppressors on a per cell basis compared with PMN-MDSCs [347] and our depletion strategy would not affect this cell population. Additionally, in the TAM-depletion experiments performed in **study I**, we could not detect increased T and NK cell activation as a direct consequence of the anti-CSF1 treatment, despite the TAMs we depleted in the control group verifiably being skewed toward $MHCII^{low}$ pro-tumor immune-suppressive phenotype.

Importantly, in the SEMA3A tumors, the $CD11b^{+}Ly6G^{+}$ population displays an increase in the Th1-associated CXCL9-11 and the CCL2 and CCL5, that are associated with a pro-inflammatory phenotype. This suggests that in the SEMA3A-overexpressing tumors, $CD11b^{+}Ly6G^{+}$ are less immunosuppressive. Indeed, as in **Study I**, $CD8^{+}$ T cells and NK cells from SEMA3A-tumors express higher levels of INFγ, suggesting higher activation, and this effect is less pronounced in the absence of $CD11b^{+}Ly6G^{+}$ cells. We thus propose that the effects of SEMA3A on tumor-infiltrating lymphocytes are mediated both by TAMs but also by intratumoral $CD11b^{+}Ly6G^{+}$ cells. We also observe a downregulation of *S100a9* in the $CD11b^{+}Ly6G^{+}$ cells in SEMA3A tumors. This gene has been implicated in MDSC expansion [348] and migration [349] to the site of the tumor. This might explain the decreased $CD11b^{+}Ly6G^{+}$ infiltration in the SEMA3A tumors we observe.

Neutrophils, in under physiological conditions, are strongly cytolytic and phagocytic [350]. It is possible that, in addition to an indirect anti-tumor effect of SEMA3A, mediated via a less immunosuppressive phenotype of the $CD11b^{+}Ly6G^{+}$ population, the intratumoral $CD11b^{+}Ly6G^{+}$ cells themselves have acquired functional features of neutrophils. This should be explored further with cytotoxicity assays. Furthermore, PMD-MDSCs suppress T and NK-cell mediated immunity via a variety of different mechanisms, as discussed previously. It would be interesting to study if SEMA3A affected, for example, the levels of ROSs in the $CD11b^{+}Ly6G^{+}$ cells.

Much like the differential effect of SEMA3A on the proliferation of TAMs, the differential effect of SEMA3A on $CD11b^+Ly6G^+$ cell recruitment, compared with the recruitment of $CD11b^+Ly6C^+$ cells, despite near-identical extent of NP1-expression needs further mechanistic exploration. It is possible that in "re-programming" $CD11b^+Ly6G^+$ cells, SEMA3A does not act on the cells directly but, rather, disrupts the "two-factor signaling" mediated by the tumor and tumor stroma proposed by Gabrilovich as necessary to establish the PMN-MDSC phenotype. The observed effect on $CD11b^+Ly6G^+$ cells in SEMA3A-overexpressing tumors would thus not be a direct consequence of SEMA3A-NP1 downstream signaling in the $CD11b^+Ly6G^+$ cells but, rather, reflect altered repertoire of signals in the tumor microenvironment. The effect of direct SEMA3A addition to cultures of PMN-MDSCs as well as *Np1* knockdown studies are required to clarify this point.

In summary, while the results presented in **study II** (Figure 13) are preliminary and require further substantiation, on the basis of **study I** (Figure 12) and **study II** (Figure 13), we propose that SEMA3A has a cumulative anti-tumorigenic effect, mediated via "reshaping" of the tumor microenvironment. These findings motivate further exploration of the therapeutic potential of SEMA3A. **Study I** and **II** further highlight the plasticity of tumor-associated immune cells and add to the growing repertoire of targets capable of altering their phenotype in an anti-tumor direction. Aiming to extend our knowledge of such targets, we initiated **study III.**

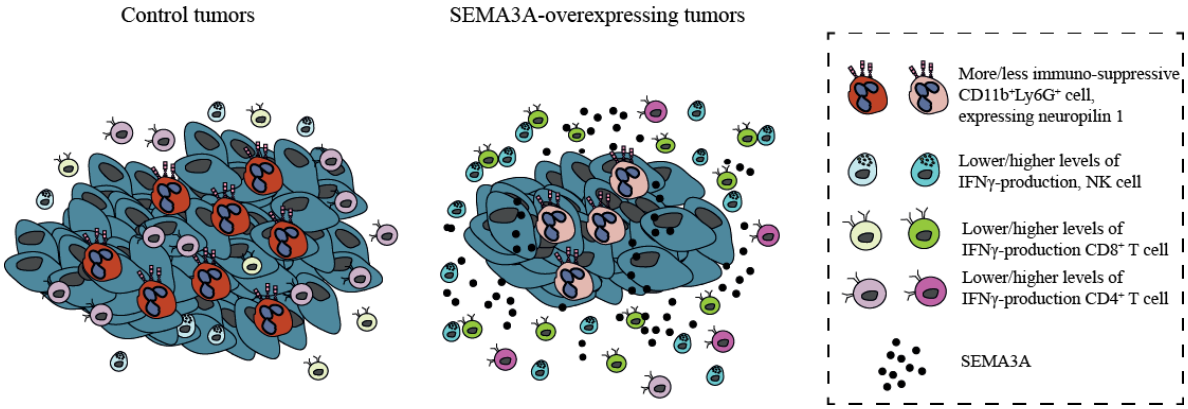

Figure 13. Schematic summary of key findings of study II. SEMA3A overexpression by tumor cells leads to a less immunosuppressive phenotype in the intratumoral $CD11b^+Ly6G^+$ population. This, in turn, increases the IFNγ production of intra-tumoral lymphocytes.

3.3 STUDY III

Translational control of tumor-associated macrophage phenotype

The discovery of macrophage targets with a potential to bring about an anti-tumor response can be serendipitous, but, in general, it is the result of systematic research, based on prior knowledge of molecular pathways important for macrophage function. Macrophages are plastic cells and their plasticity implies mechanisms set in place to sense environmental signals and modulate gene expression accordingly. Regulation of mRNA translation results

in rapid changes in protein levels as *de novo* transcription, mRNA processing and nuclear export are bypassed. Given existing data implicating translational control as a mechanism modulating many aspects of immune cell function [351], we hypothesized that genes important for the anti-tumor-to-pro-tumor progression in TAMs are translationally regulated.

3.3.1 Translational regulation during TAM progression to a pro-tumor phenotype *in vivo*

To address our hypothesis, we used the MMTV-PyMT model, where the tumor-inducing oncogene polyoma middle T (PyMT) is under the control of mammary tumor virus promoter (MMTV), restricting the development of the neoplasm exclusively to the mammary epithelium. In transgenic mice, this model provides an aggressive, autochthonous (i.e. occurring spontaneously) model of luminal B-type mammary carcinoma characterized by progression through the four stages of tumor development: hyperplasia, adenoma/mammary intra-epithelial neoplasia and early and late carcinoma [352]. Importantly, late-stage carcinogenesis and metastasis in this model are regulated by CSF1 and macrophages [353] and many seminal TAM studies cited throughout this text applied this model [112] [56] [91]. However, multiple tumors arise in the same animal in MMTV-PyMT mice. As the macrophage infiltration to any given tumor, would be affected by the tumors in the vicinity, we believe this would not reflect TAM development within an isolated tumor as needed to address our hypothesis. Instead, we used a cell line derived from the late stages of spontaneous MMTV-PyMT tumor and we induced tumors by orthotopic injection of this cell line into the mammary fat pad of syngeneic FVB mice. We do not expect the tumors that arise to progress through the defined stages of carcinoma development characterizing the spontaneous MMTV-PyMT model, as these are driven by the stepwise accumulation of genomic alterations which have already taken place before the cell line was derived. We believe, however, that this provides a more homogenous model to study TAM development during tumor growth.

To verify that the TAM composition shifts during tumor development, we characterized their phenotype, using tumor size as a proxy for developmental stage. Using two gating strategies, $Ly6C^{+/-}MHCII^{low/high}$ as well as $CD11^{+}/MRC1^{+}$ we confirmed that during tumor growth, the TAMs shift towards the $CD11b^{+}Ly6G^{-}Ly6C^{low}MHCII^{low}$ and $CD11b^{+}F4/80^{+}MRC1^{+}$ pro-tumor phenotype.

We thus collected and sequenced polysome-associated and total RNA from TAMs from 13 tumors of different sizes. Using a modified version of the anota algoritm described in **section 2.3**, we quantified differential translation, using tumor weight as a continuous variable.

Surprisingly, we found that the number of genes regulated by translation greatly outnumber the genes regulated via changes in total mRNA levels, at a ratio of approximately 10:1. We next performed Generally Applicable Gene-set Enrichment for Pathway Analysis (GAGE) [354] to identify enrichment of cellular functions (represented by GO terms) among genes

identified as translationally regulated. Interestingly, we identified proliferation as one function enriched in translationally regulated genes. In **study I**, we investigated differential translation of macrophage subsets as a mechanism affecting the intratumor TAM composition. By performing flow cytometry analysis in the context of **study III**, we could confirm that M2-like TAMs proliferate to a higher extent than M1-like TAMs (as assessed by Ki67 staining), this difference diminishing – but persisting – as the tumor grows larger (**study III**, Supplementary figure S2) This indicates that differential proliferation in TAM subsets is a possible mechanism by which the TAM composition is established.

Macrophage activation drives differential metabolism, as the activating signals often intersect with metabolic pathways [355]. The role of IL-4-induced ARG1, the classical M2 marker, in arginine metabolism has been discussed throughout this text. One of the major differences between pro-inflammatory and anti-inflammatory polarization states of macrophages *in vitro* is energy metabolism [356]. While M1-polarized macrophages preferentially produce ATP from glycolysis, M2-polarized macrophages do so through oxidative phosphorylation of fatty acids and glutamine. Interestingly, INFγ signaling via TRL2 has been shown to result in a translatome reprogramming, targeting, in particular, metabolic pathways [357].

Consistent with the notion of a metabolic shift accompanying differential macrophage activation states, we found genes involved in various metabolic processes to be translationally upregulated in TAMs during tumor growth. For example, human carbonyl reductase 1 (CBR1) has been shown to catabolize S-Nitrosoglutathione, as a means to degrade NO [358], the elevated levels of which is a typical feature of M1 pro-inflammatory macrophage. *Cbr1*, but also other members of this family such as *Cbr2, 3* and *4* were found translationally upregulated in TAMs during tumor growth. Similarly, we found genes involved in glutathione metabolism (*Gclm, Gpx3, Gstm1, Gsto1, Gstp1, Gstp2, Gstt3, Hagh, Sod2*). Glutathione is an antioxidant that acts by reducing ROS [359], the production of which also is a characteristic of M1-phenotype.

We selected three key genes from the categories we believe to be key in macrophage biology: proliferation (*Cdk4*), metabolism (*Cbr2*) and cytokine production (*Grap*) [360] and confirmed their elevated protein levels in pro-tumor macrophages by both flow cytometry and immunofluorescence staining of tumor sections, but also in *in vitro* macrophages polarized to either M1- or M2-phenotypic states. To further consolidate that the TAM phenotype shift along the tumor weight axis reflects a progression from an anti-tumor M1-like phenotype to a pro-tumor M2-like phenotype, we subjected *in vitro* M1- and M2-polarized macrophages to polysome profiling. Translational regulation was overall less abundant in the *in vitro* model, perhaps reflecting the stability of these cells compared to TAMs exposed to a complex and varied microenvironment. Nonetheless, when we compared *in vivo* translationally regulated genes to the *in vitro* data set, we found *in vivo* upregulated genes to be enriched among those efficiently translated in the M2-polarized macrophage.

Vice versa, *in vivo* downregulated genes were enriched among those efficiently translated in M1-polarized macrophages.

In summary, we conclude that changes in translational efficiencies occurring in the TAM population during tumor growth reflect, at least partially, a shift from an overall M1-like to an overall M2-like phenotype.

3.3.2 MNK2-mediated effect on M2-phenotype *in vitro*

We started looking for translation factors whose activity could explain observed changes in translation during M1-to-M2-progression. We found that phosphorylation of eIEF4 is strongly induced in *in vitro* M2-polarized macrophages (IL-4) compared to M1-macrophages (IFNγ). We hypothesized that the M2-phenotype *in vitro* is established by selective mRNA translation mediated by phosphorylation of eIF4E. Surprisingly, upon inhibition of MNK activity by the MNK-inhibitor cercosporamide, the phenotype of the M2-polarized macrophages underwent a drastic change. We found elevated transcription of Th1-associated chemokines *Cxcl9-11*, as well as T cell activating *Il-12* and *Il-1* in treated cells. These cells, furthermore, increased cell surface expression of co-stimulatory CD86 and antigen-presenting MHCII, and decreased protein levels of the M2-associated marker MRC1. Most importantly, cercosporamide-treated M2-polarized macrophages induced IFNγ production in T cells at levels comparable to M1-polarized macrophages (Figure 14). Upon shRNA-mediated MNK2 knockdown, this effect was lost. Importantly, the MNK inhibition decreased protein levels of CDK4 and GRAP (described earlier) but not their mRNA levels.

Cercosporamide inhibits MNK function, with a stronger affinity toward MNK2 [361]. However, cercosporamide also inhibits JAK3 [361]. JAK3 lies immediately downstream of the IL-4 receptor (IL-4R) and phosphorylates STAT6 [362], the well-recognized mediator of the IL-4-polarized M2 gene expression signature. Phosphorylated STAT6 translocates to the nucleus and initiates the transcription of many typical M2-signature genes such as *Arg1*, chitinase 3–like protein 3 (*Ym1*), resistin-like alpha (*Fizz-1*) [363] [364]. It has also been shown to inhibit the NF-Kβ pathway important for M1 phenotypic activation via activation of and cooperation with Krüppel-like factor 4 (KLF4) [365]. As we use IL-4 to induce an M2-phenotype *in vitro*, we compared the consequences of JAK3 inhibition by a selective JAK inhibitor CP690550 [366] in M2-polarized macrophages to that of cercosporamide treatment. Importantly, neither the elevated Th1 cytokine expression by cercosporamide nor the ability of cercosporamide-treated M2 macrophages to stimulate IFNγ production of $CD8^+$

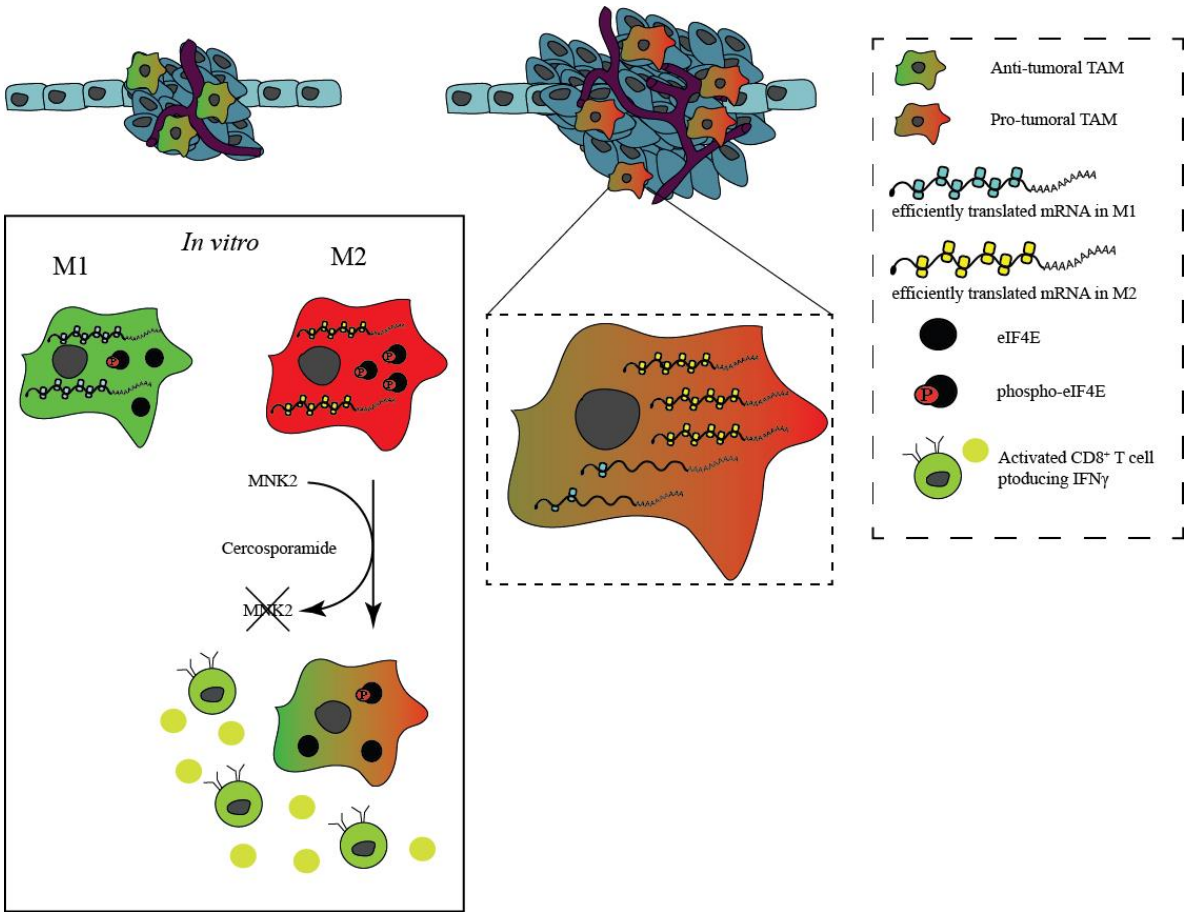

Figure 14 Summary of key findings of study III. TAM phenotype during tumor growth is regulated by differential mRNA translation. Importantly, the mRNAs that are upregulated in TAMs at later stages of tumor growth also are upregulated in M2-polarized *in vitro* BMDMs. Upon *in vitro* inhibition of MNK2 by cercosporamide, M2-BMDMs increase their capacity to activate $CD8^+$ T cells, indicating a shift towards a pro-inflammatory phenotype.

T cells was mirrored by CP690550. To verify that the MNK-driven pro-tumor effect we observe is general and not IL-4 specific, it would be interesting to study its inhibition in IL-10-polarized M2 macrophages. IL-10 signals via the STAT3 pathway, mediating the transcription of a different set of genes, aimed at attenuating the inflammatory response [367] [368]. STAT3 and STAT6 synergize in amplifying the expression of certain genes (such as *Arg1*) [369] and in increasing cathepsin secretion by TAMs needed for tumor progression [370]. Importantly, in the complex *in vivo* microenvironment, TAMs are likely to be exposed to both IL-4, IL-10 as well as IL-13 (signaling via STAT6 and IL4-R) and IL-6 (signaling via STAT3), suggesting the need to confirm the MNK effect we observe under all these contexts [44] [362].

MNK1 and MNK2 are the only kinases known to phosphorylate eIF4E [285], but the MNK1/2 proteins themselves have other biological targets. For example, MNK have been found to regulate the translation of TNFα, the proinflammatory cytokine produced by classically activated macrophages. It does so by phosphorylating heterogeneous nuclear ribonucleoprotein (hnRNP) A1 [371]. This leads to hnRNP A1's de-association from ARE in the 3' UTR of TNFα mRNA and the subsequent translation of TNFα. Later, a different protein, polypyrimidine tract-binding protein (PTB)3-associated splicing factor (PSF)

together with $p54^{nrb}$, was identified as an MNK-target [372]. Its binding to ARE in the 3' UTR of TNFα mRNA was enhanced by MNK-mediated phosphorylation. Since PSF is nuclear protein [373], the authors speculate that its binding to TNFα mRNA may be involved in its nuclear export [372]. Sprouty2, a negative regulator of the MAP kinase ERK1/2 pathways [374], has been shown to be a direct target of both MNK1 [375] and MNK2 [376]. Finally, phosphorylation of eIF4E by MNKs also affects its function in mediating nuclear export of certain mRNAs [294]. Collectively, this suggests that the functional consequences of MNK activity could potentially be mediated by mechanisms other than phosphorylated eIF4E regulating the translation initiation of specific mRNAs. Our shMNK2-results make an indirect connection between eIF4E phosphorylation status and functional phenotype of the M2-polarized macrophages, by showing that comparable phospho-eIF4E levels mirror similar macrophage phenotypes in the shMNK2 experiment. This is, however, only correlation at this moment.

Interestingly, MNK1 and MNK2 have been suggested to possess different substrate specificities. For example, PSF discussed earlier is preferentially phosphorylated by MNK2 at one of its residues [372]. Furthermore, in a muscle cell line, only MNK2 and not MNK1 selectively inhibited proteins involved in the translational machinery i.e. eIF4G and mTOR [377]. The phosphorylation of eIF4G at Ser^{1108} is associated with protein synthesis [378]. In the absence of inhibitory MNK2, this phosphorylation was increased. In the same study, MNK2 was also shown to inhibit mTOR-target p70S6K through a mechanism that was unrelated to its function as a kinase, by interacting with mTORC1. In sum, these examples highlight that MNK can act independently of eIF4E phosphorylation.

We chose to pursue MNK2 in our shRNA knockdown experiments, because of the higher affinity of cercosporamide towards MNK2 compared to MNK1 [361], and we could show that the effect of cercosporamide is indeed mediated by MNK2. It would be valuable to investigate the effect of MNK1 knockdown and the cumulative effect of MNK1 and MNK2 deficiency. It is possible that a stoichiometrical relationship exists between levels of MNK1 and MNK2, influencing the downstream effect of their activity in the cell. It is also possible that, as indicated by the differences in their activity, with MNK1 being induced upon p38/ERK signaling and MNK2 possessing high basal activity, they are active at different times during macrophage polarization.

To expand our understanding of the mechanisms by which MNK reprograms M2-macrophages, we need to understand the identity and function of the genes that are affected. For instance, a recent study has suggested that MNKs regulate the translation of mRNAs with both a 5' cap and a 5' hairpin structure [379]. This study was performed using a different MNK-inhibitor, CGP57380, using a cell-free translation system and is thus somewhat artificial. We therefore plan to perform translatome profiling on shMNK2-macrophages, which, we believe, would provide a more comprehensive identification of mRNAs whose translation is regulated by MNK2 activity in M2-polarized macrophages.

However, one should keep in mind that our experiments showing functional reprogramming of M2-macrophages were performed on *in vitro* macrophages with an established M2-phenotype, as we added the inhibitor 24h after we added the polarization stimulus (IL-4). These experiments do not allow us to draw conclusions of the role of MNK activity *during* the process of M2-polarization and its role in establishing macrophage phenotype in a complex *in vivo* milieu. In a tumor, there will be a continuous flux of recruited monocytes. There would also be macrophages that are not yet skewed toward a pro-tumor phenotype and macrophages performing functions not necessarily related to immune suppression. Bone marrow transplantation of MNK-deficient macrophage progenitors in animal models is one possible way to study the effect of MNK activity on TAM-polarization, but it is not the most clinically relevant. It would be interesting to study the effect of inducible macrophage-specific MNK-knock down, to determine the temporal dependency of MNK activity/eIF4E phosphorylation on the establishment of the TAM phenotype.

The finding that MNK2, possibly via phosphorylating eIF4E, drives the M2-phenotype has potential therapeutic implications. MNK1/2 activity, as well as eIF4E phosphorylation, are dispensable for normal physiological development [284, 285]. This implies that targeting eIF4E phosphorylation would not impair physiological function of non-transformed cells. Furthermore, increased eIF4E phosphorylation is implicated in promoting malignancy of tumor cells, selectively stimulating translation of mRNAs involved in invasion and metastasis [295] [296]. In a recent study, disruption of eIF4E phosphorylation in cells of the TME – not tumor - was found to decrease metastasis, which was linked to reduced survival of pro-metastatic neutrophils [380]. Cumulatively, the association of eIF4E phosphorylation to malignancy, both in transformed cells and in tumor-associated cells, highlight the clinical potential of our finding and motivates further investigation of MNK activity during TAM polarization.

3.4 STUDY IV

Distinct cancer-promoting stromal gene expression depending on lung function

Chronic obstructive pulmonary disease (COPD) is a general term encompassing several progressing pathological states such as chronic bronchitis, emphysema (destruction of the alveoli) and refractory (non-responsive to medication) asthma [381]. Smoking is a common cause of COPD, and consequently, many COPD patients later develop lung cancer [382]. Importantly, COPD diagnosis in itself, independently of smoking, is a risk factor of lung cancer [383].

Presence of emphysema entails a microenvironment characterized by chronic inflammation [384]. The link between inflammation and cancer development has been discussed throughout this thesis and indeed, in lung cancer, the tumor tends to arise near areas of severe emphysema [385]. This suggests that damaged lung stroma plays a role in lung cancer initiation, but the molecular mechanisms by which it does so are not established. We thus

sought to investigate the relationship between COPD and cancer initiation, by characterizing the lung stroma on the mRNA-, translatome- and proteome levels across a spectrum of COPD severity in patients with and without cancer.

To quantify COPD severity, we used forced expiratory volume at one second (FEV1), which is a diagnostic measure of lung function and a correlate for COPD state. We used samples from patients graded on the GOLD scale [386], which scores lung function based on percentage of predicted FEV1 (FEV1pp), e.g. a GOLD0/1 score is assigned when FEV1 is ≥80% of a healthy individual, the score 2 reflects FEV1pp 50-80%, and 3/4 - FEV1pp <50%, this being the most severe COPD status.

After performing iTRAQ-based mass spectroscopy [387] on material from COPD patients and controls with or without lung cancer, changes in the proteome were analyzed using Analysis of Covariance (ANCOVA) looking for protein expression patterns common to cancer/non cancer patients and patients based on FEV1pp score concurrently in our model:

$$Ex = \gamma_{age} + \gamma_{cancer} + \gamma_{fev1pp} + \varepsilon$$

where Ex is the expression (protein, and in later analyses also total mRNA or quantified western blots), γ_{age} is the linear relationship to age, γ_{cancer} is the difference between cancer and non-cancer samples, γ_{fev1pp} is the linear relationship between expression and FEV1pp, and ε is the error.

We could not detect any cancer-specific protein expression. However, when we added an interaction term for interaction between cancer status and FEV1pp (cancer status-FEV1pp interaction, CFI), i.e.

$$Ex = \gamma_{age} + \gamma_{cancer} + \gamma_{fev1pp} + \varepsilon + \gamma_{cancer} * \gamma_{fev1pp}$$

where $\gamma_{cancer} * \gamma_{fev1pp}$ the interaction between cancer status and FEV1pp, we detected differential protein expression between cancer and non-cancer patients depending on their FEV1pp (i.e. lung function). This implies that lung stromal protein expression depends on an interaction between cancer status and lung function.

Polysome-prolifing revealed this relationship to be mediated by changes in translational efficiency of the implicated mRNA targets and not changes in their steady mRNA levels, suggesting differential translational efficiency mediating changes in protein levels during COPD progression.

When relating differentially expressed genes accounting for CFI to the FEV1pp status of the patients, we found that certain genes increased their expression in cancer patients with mild COPD and certain genes, on the other hand, decreased. Furthermore, there was a converse relationship in the non-cancer group, with an intersection between the expression relationship

in the cancer and non-cancer group at an intermediate Fev1pp. This suggests that two different gene expression programs are operating in cancer patients based on COPD severity.

Looking at the genes within these distinct expression patterns, we found mRNAs whose translation was previously shown to be sensitive to mTOR-activity [388] as translationally activated in the stroma of cancer patients with high FEV1pp. Correspondingly, mRNAs previously shown to exhibit more efficient translation in fibroblasts in response to fibrotic ECM [389] were more efficiently translated in the stroma of cancer patients with low FEV1pp. Thus, depending on COPD severity, mTOR- or ECM-derived signals appear to differentially coordinate cancer-associated translatomes. We hypothesized that the stroma of COPD patients may promote tumor formation by secreting tumor-initiating factors. We further hypothesized that the two discrete gene expression programs we identified on the basis of COPD severity would contain such cancer-initiating factors. To address our hypothesis, we applied data from published secretomes derived from fibroblasts in the absence of cancer (PTEN null [390] and senescent fibroblasts [391]) and from CAFs [392] [393] onto the gene expression profiles we identified. We did not detect selective regulation in the CAF secretomes, but PTEN null and senescent fibroblasts displayed opposing regulation dependent on COPD status, with PTEN null secretome being more efficiently translated in advanced COPD and the senescence secretome, conversely, tending to more efficient translation in patients with low COPD.

To confirm the cellular origin of the genetic targets implicated by these secretomes, we performed immunofluorescence stainings of key candidate genes in patient-derived lung

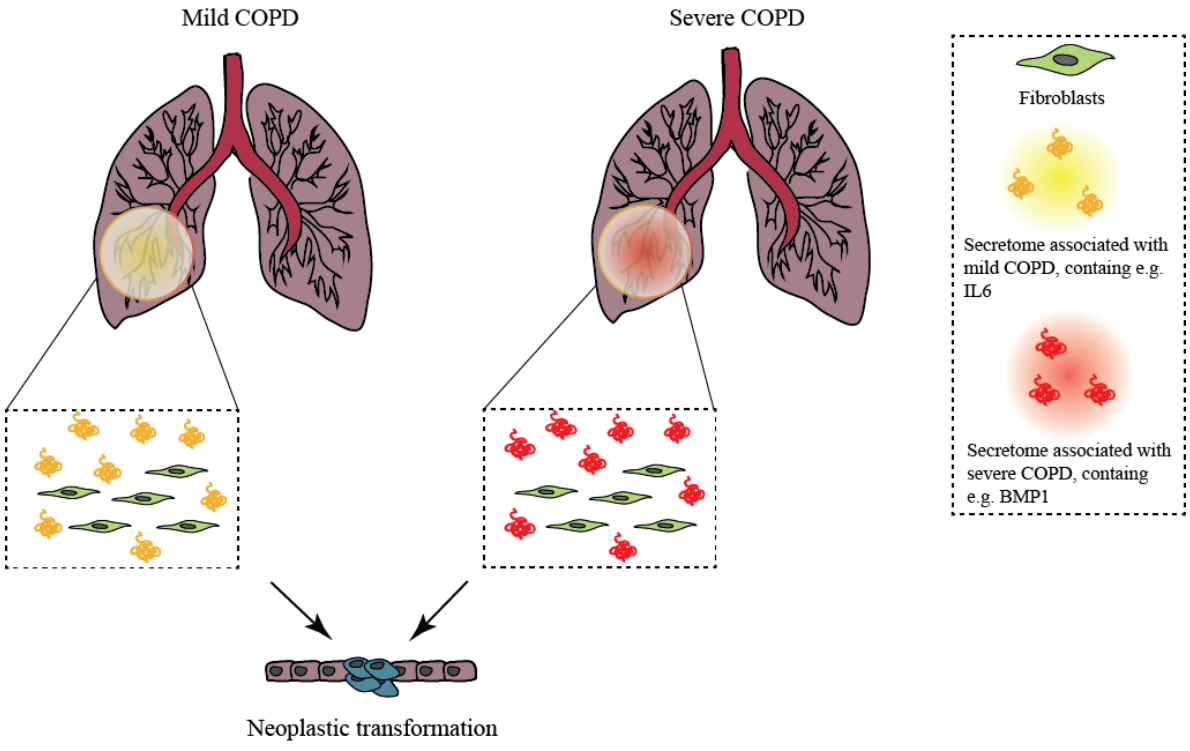

Figure 15 Summary of key findings of study IV. Depending on COPD status, tumor stroma initiates different gene expression programs, resulting in different secreted factors, both favoring tumor initiation.

stroma. We could verify that activated myofibroblasts (α-SMA positive cells) expressed these proteins in human lung tissue.

On the basis of these data, we propose that the FEV1 status (indicating COPD severity) of a patient drives the lung microenvironment to carry out two distinct secretory programs beneficial for tumor initiation (Figure 15). These secretory programs, in turn, are regulated mostly – with the exception of secretome of the senescent fibroblasts, where the largest regulation occurred on total mRNA level – on the level of translational efficiency.

3.5 STUDY V

Aberrant recombination and repair during immunoglobulin class switching in BRCA1-deficient human B cells

At its core, cancer is a genetic disease developing as a consequence of genomic alterations. The immune system plays a role in eliminating occurring neoplasms, as discussed, but the toxic inflammatory environment with accompanying increased levels of ROS and free radicals can contribute to further damage to the DNA. Ionizing radiation, exposure to chemicals or mistakes during cell division by the efficient, but not fool-proof, DNA replication machinery add to the repertoire of factors that compromise the integrity of the genetic material.

DNA damage can also be intentional, with a defined role in normal physiology. The response of the immune system – to neoplasms, bacteria or parasites – relies on the versatility of the cells of the adaptive immunity. This versatility is generated by the "shuffling" of the genes coding for B and T cell receptors during the maturation of these cell types. The shuffling process involves the infliction – and repair – of double strand breaks in the DNA molecule. Indeed, the concept of immunosurveillance of cancer was substantiated by studies in immunocompromised mice, lacking recombination activating gene 1 or 2 (RAG1/2) necessary for the infliction of double strand breaks in the immunoglobulin heavy chain (IGH) locus and co-ordination of their repair. As a consequence, these mice lacked the B and T cells required for mounting an efficient immune response and developed cancer at higher frequencies compared to control mice [394] [395] [396].

The cell has evolved sophisticated machinery set in place to sense and repair intended and accidental DNA damage. The main pathways regulating the repair of double strand breaks are homologous recombination (HR) [397] and non-homologous end joining (NHEJ) [398]. These differ in the requirement of a DNA template to repair the damage and thus in the fidelity to the original DNA sequence [399]. HR makes use of the complementary chromatid available during G2/S phase of the cell cycle to repair the damaged strand and thus is the most accurate of the pathways. NHEJ joins the two DNA strands without using a template and is thus the prevalent pathway throughout the cell cycle. When components required for NHEJ are deficient, alternative end joining (A-EJ) is activated [274]. A-EJ employs so called

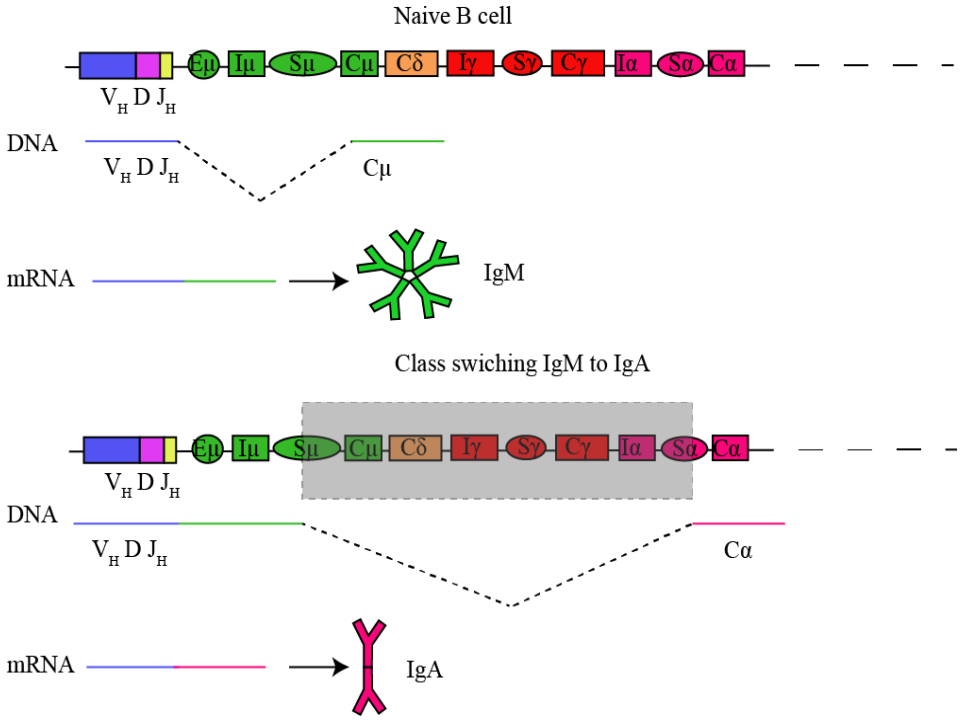

Figure 16 Illustration of the Immunoglobulin Heavy Locus (IGH) on chromosome 14 in humans and its rearrangement during class switching. For clarity, not all genes, processes and aspects of the antibody molecule are shown. Upon class switching from IgM to IgA, switch junctions Sμ-Sα are brought into proximity by a circularization-deletion process, the grey shaded part is excised and (after further modification) the VDJ region and the constant α region are translated and assembled to a secreted IgA protein.

microhomology in the sequence on each side of the break but it has also been proposed to add insertions. The variability that is introduced by NHEJ during two key processes in B cell maturation, namely VDJ recombination, which generates the variable region of the B (and also T cell) cell receptor, and immunoglobulin class-switching (CSR), contributes to a diverse and versatile repertoire of B cells.

A B cell that has not yet encountered an antigen will contain an immunoglobulin heavy chain of the so-called IgM and IgD class (also called isotype) as a part of the membrane-bound B cell receptor [70]. As a step in the B cell maturation process, the heavy chain portion of the secreted part of the B cell receptor, the antibody, undergoes a switch to IgG, IgE or IgA class. This increases the versatility of the antibody, as different antibody classes perform different immune functions in different physiological locations. The switching happens on a genetic level, via NHEJ of the IgM to IgG, IgE or IgA coding parts on the IGH locus in the DNA. The resulting junctions are called switch junctions and are designated Sμ-Sε, -Sα or -Sγ. For a simplified representation, see Figure 16.

Many cancers, including lymphomas, present mutations in the genes coding for the components of the DNA repair machinery [400]. BRCA1 is instrumental during homologous recombination [401] and the consequences of its impaired function are well-established in breast and ovarian cancer [402] [403]. We hypothesized that it is also involved in NHEJ, a connection that was not well-explored. To address out hypothesis, we used patient material from patients with mutations in different functional domains of BRCA1, as well as mutations

its partners BRCA2, BRIP1, CtIP and RNF168, and we analyzed class switch junctions in the B cells of these patients. Compared to previous *in vitro* studies of the role of BRCA1 during NHEJ [404] [405], we believe class-switched B cells serve as a physiologically relevant *in vivo* representation of the functions of BRCA1 during the NHEJ pathway.

In practical terms, CSR fragments from switched B-cells were amplified by a nested-polymerase chain reaction (PCR) described previously [406] [407] and sequenced. The CSR junctions were subsequently analysed by alignment to reference Sμ-Sα and -Sγ sequences. If the two switch regions were joined directly, the repair pattern was called direct end-joining. Other repair patterns included added nucleotide(s) at the junction, not matching either S region, which we call insertions, and stretches of nucleotides at the recombined junctions, perfectly matching both S regions. These were characterized as MHs. Furthermore, intra-switch recombination and sequential switching events, as well as inversions can be found by sequencing the CSR-junctions.

In total, 227 Sμ-Sα junctions from B cells of BRCA1-deficient patients were sequenced. We could observe change in the repair pattern that occurred in this group, compared to age-matched controls. Instead of displaying direct end-joining and short MHs (1-3bp) as seen in controls, we observed longer MHs (>6bp). This indicated a shift from NHEJ to A-EJ. We then grouped the mutations, based on their effect on the functional domain of the BRCA1 protein and its interacting partners, BRIP1 and CtIP. These also presented increased usage of longer MHs. Both CtIP [408] and BRIP1 [409] have previously been implicated in the process of resection, that is to say removal of nucleotides from the complementary strand so that it can align with the sister chromatide for “refilling” the nucleotides during HR or reveal microhomology used during A-EJ. We speculate that the function of BRCA1 in NHEJ is to inhibit resection of DNA ends, favoring NHEJ instead of A-EJ.

Overall, the CSR-junctions from BRCA1-deficient cells presented higher frequencies of intra-switch deletions (ISDs) and very unusual insertions containing inverted S region sequences, compared to controls. These were also found at elevated frequency in BRCA2-deficient cells. We thus propose that BRCA1 and BRCA2 could be involved in preventing inversions.

Given the known tumor suppression functions of BRCA1 in several cancers, including breast but also, for example, colorectal cancer [410], we wondered if defects in BRCA1 could confer an increased risk of lymphoma. In animal models, mice with truncated BRCA1 and additional p53-deficiency developed lymphoma earlier than they did sarcomas or carcinomas [411]. In humans, previously published data found an increased risk of hematological cancers in carriers of mutations within the BRCA1/2 pathways [412]. Consistently, we found a number of somatic and germline mutations in BRCA1 in exome sequencing data from 31 diffuse large B cell lymphoma patients [413].

Cumulatively, we propose that BRCA1 and its interacting proteins maintain genome stability by performing key functions both during HR and NHEJ and thus serve to prevent tumorigenesis in mature B lymphocytes.

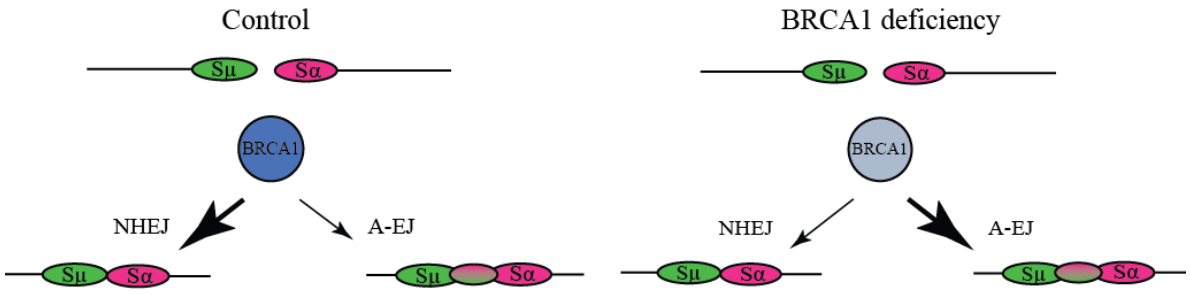

Figure 17. Summary of key finding of study V. In BRCA1-deficient patients, alternative end joining, with increased microhomologies (shaded green-pink in figure) predominates during repair of double strand breaks between switch junctions. Proposed mechanisms are discussed in the text but not shown.

4 PERSPECTIVES AND CONCLUDING REMARKS

"From this point forth, we shall be leaving the firm foundation of fact and journeying together […] into thickets of wildest guesswork"
-Albus Dumbledore

The recognition of the immune system as the determinant of tumor fate reached its apex with the announcement of 2018 Nobel prize in physiology or medicine. While T cells get the credit in this context, discussion throughout this text has highlighted the close crosstalk between all cells in the tumor microenvironment, a crosstalk that can amplify or attenuate the T cell response. Indeed, several experimental models have shown improved efficiencies when checkpoint inhibitors and TAMs are targeted in combination [112] [414]. To fully engage the immune system against the tumor – the anti-cancer potential of which is now so well illustrated – we need to understand the finer nuances of this crosstalk.

To this end, we also need a better understanding of the many-layered mechanisms shaping the behavior of the individual cell. Two of the studies presented in this thesis highlighted the discrepancy between what we think a cell does, based on its transcriptome, to what is more likely that a cell actually does, based on its translatome. Many of the approximately 1000 genes we identified as changing on the level of translational efficiency in TAMs during tumor growth in **study III**, would not have been picked up as important for macrophage function on the basis of transcriptome data alone. While this adds to the known complexity governing cell behavior, this also opens new possibilities for its manipulation, as we proceed to show.

The work presented in this thesis – much like the cell types it describes – is heterogeneous, shifting focus between cells, techniques and pathways. What is lost in detailed understanding of one cell type or one pathway, is gained in increased awareness of the interconnectedness between the cells in the tumor microenvironment. **Study I** and **study II** exemplified how one molecule – SEMA3A - affected two different cell types in the tumor microenvironment in different ways but with the combined outcome of impeded tumor growth. It is possible that the effects of SEMA3A on the cells we studied build upon each other. For example, altered tumor microenvironment as a consequence of increased anti-tumor TAM proliferation generated a milieu that prevented recruitment, expansion and/or activation of PMN-MDSCs, which in turn amplified the tumor cytotoxicity of recruited T cells. Based on the discussion of the reciprocity of tumor-infiltrating myeloid cells, it is furthermore plausible that the insights into TAMs we gained by studying regulation of gene expression on the level of translation in **study III** are applicable across the broader range of TIMs.

Existence as a multicellular organism entails cooperation between constituent cells. Cancer manipulates this cooperation, but it remains dependent on it nonetheless. The work presented in this thesis has built upon, but also extended, existing knowledge of how this dependency can be targeted to obstruct tumor growth.

5 ACKNOWLEDGEMENTS

No man is an island, as they say. Long gone is the time of the solitary hobby scientist accidentally stumbling upon fundamental principles of life as we know it while planting peas in his backyard. Or I am simply in the wrong field..? Anyway, this thesis may have my name on it, but it represents the toil and dedication of many great people it has been my privilege to work with. If there is one thing from this thesis the reader should take away (…since I am pretty sure it won't be the translation initiation pathway, despite the time it took to make that figure :-P), it is my gratitude to the people who helped to shape it.

First and foremost, I have been lucky to have two supervisors who are true supervisors in the full sense of this word and whose personalities and areas of expertise and supervising styles harmoniously complement each other. **Charlotte Rolny** and **Ola Larsson** – I am very grateful for having you as my supervisors. You look out for me, in obvious and less obvious ways and I notice and I don't take it for granted. This thesis is the product of team work and you assembled the team. P.S. Charlotte, you are great ;-) P.S.S. And you, Ola ☺

I would like to thank my opponent, **Raúl Méndez**, and my examination board: **Lisa Westerberg, Ola Söderberg** and **Guillem Genové** for taking time to read my thesis and discuss my work. I hope it was worth your time.

I owe a debt to professors **Qiang Pan-Hammarström** and **Lennart Hammarström,** to whom I am sincerely grateful. You taught me the importance of hard work and determination, which, I know, if I learn to apply it properly, will be the foundation of whatever I choose to do in the future.

To my colleagues, my co-authors and my friends: **Laia, Majken, Tatjana**: I couldn't have asked for better people to share a PhD experience with. **Majken**, I don't know if you remember it, but we first met a long, long time ago, back when my knowledge of macrophages comprised that they were, ehm, cells that – *probably* - were large and – *perhaps* – ate things. You seemed so happy with your project and your lab - how funny that a couple years later, by a complete coincidence, I were to join you. Somehow when we discussed science you always seemed to ask me critical questions that forced me to think a step further. **Tatjana**, you are a rock. I trust you fully and implicitly. If I ever asked you for help – and I did, oh so many times – I would bet my right arm you would do it and do it well. As I said, and meant, at your defense, I couldn't imagine a better companion to be stranded on a desert island with. Oh, and without your sharp-eyed corrections this thesis would have (even more!) sloppy inconsistencies. Thank you so much! **Laia**, the great optimist among us :-P I miss our evenings out with artsy movies and good food. In fact, I don't think I have been to an artsy movie since you left, so you see, my intellectual development has greatly suffered by your absence. Ah, and the trip to Gotland – definitely in my all-time top list… It's time for a sequel, don't you think?

Julie, you were the person to take care of me, when I arrived on my first day in Ola's lab, dazed and disoriented, and you continued to take care of me ever since. I long suspected that I probably abuse your helpfulness – okay, let's drop the pretense, I totally do abuse it – but I do appreciate so immensely the lengths you go to help out. I wouldn't understand half the things in this thesis if it weren't for you! Whenever we had one of the (*many…*) sit-downs with you, I left feeling like stuff finally made sense. I also know that I definitely took advantage of your non-scientific listening skills over the years too... I promise I can do more than whine and complain and I hope I will have a chance to demonstrate this hitherto dormant ability in our conversations henceforth. ☺

Andrea, the fact that I can write this at this very moment and the fact that my plans after PhD are still what I want them to be is because of you. Thank you! P.S. Sansa says thank you too ☺ P.P.S. You're a great writer ☺

Hui, you are a joy to work with ☺ I am so impressed by your inquisitive nature, critical thinking and fast learning. You will do well in your PhD, there is no doubt! **Dongmei**, you arrived at a chaotic time, but I am impressed by how you have risen to the challenge. Thank you for all your help so far and all the work you will carry on.

All the members of **Ola Larsson lab**: **Johannes, Christian, Baila, Shuo, Vincent, Shan** – you create a great scientific environment and the lab meeting discussions are excellent. I learned so much since the first disastrous one. Whenever I stop by the office, somehow we often end up talking for hours and it's such a pleasure to discuss science with more knowledgeable colleagues ☺ All the previous members of **Charlotte Rolny's lab: Emma, Jeanette, Pradeepa, Dennis, Laura, Stina** – your work laid the foundation of this thesis. Thank you! And you were a blast to eat lunch with ☺

Anne-Laure and **Christina**, how annoying you must find me when I stroll in an hour later than I said I would – *oh sorry, the dissociation took longer than expected* – and then stay for hours later than I promised – *but I just want to sort another tumor, please?* Well, if you did, it never showed. Your helpfulness and flexibility is the stuff of legends. And talking to you makes boring sorting so much more fun ☺ In fact, we should do it more often (talking, not sorting ☺) **John**, thank you too ☺

Kristina, you teach me the importance of a positive outlook. You are right, everything works out in the end ☺ It is a pleasure to collaborate with you even if, so far, it hasn't been on anything scientific. Your true interest in and knowledge of science is inspiring, I try to emulate you ☺ **Andreas Lundqvist**: in a very concrete way, I am *here* because of you. Thank you! Other members of **Andreas Lundqvist group**: **Chen, Neo** – thank you so much for helping me out, whenever asked! **Ying, Veronika** - thank you for great company. Everyone at CCK, floor 1, in particular **Jeroen, Linnéa, Yago, Stina,** thank you for a great work atmosphere, company during late evenings and weekends and occasional chats ☺ **Marianne Farnebo's lab: Stefanie**, I learned a lot interviewing you ☺ **Soniya** – always a pleasure to

meet by the coffee machine ☺ **Jonas Bergh** and all the members of **Bergh lab**, in particular **Ioannis** and **Nick**: thank you for excellent scientific collaboration and fruitful meetings. **Elin** and **Arne Östman**, thank you for including me in your project. My mammary fat pad injection skills wouldn't be what they are if it weren't for the practice I got from this! All the support stuff at CCK – **Elle, Elisabeth, Sören, Jobel**: thank you for making what we do easier and for going above and beyond to help. You are appreciated.

Our delightful roomies (well, sadly, not anymore) – **Smaranda, Susanne, Sara, Anette, Sue-Li** and other members of **Pär Nordlund's** lab, in particular **Henriette** – honestly, did I abuse your generosity with "Oh, can I just borrow this"? I know I did :-P My personal record – eight 26-well gels in a day – would not be possible without your generous gift. Thank you so much!

To my first lab teachers: M**argaret Rush, Aino Ruusala, Tobias Bergström, Romain Guyot, Frédéric Flamant, Jens Schuster, Maria Sobol, Likun Du** and my first lab students: **Eric, Mareike** – thank you for bearing with me. I know it can't have been easy when I spilled ethidium bromide for the umpteenth time, when I ran PCR without closing the tube lids properly, when I got an infection in my precious reprogrammed iPSCs, when I made you do westerns I was too lazy to do myself…. I hope I taught you something, because you taught me lots.

Lena Ekström and **Anna Falk** I don't think I can thank you enough for your help and support during a difficult time. You really made a difference in my PhD experience. Thank you.

Natalie von der Lehr and **Lisa Beste** – thank you for showing me that there is life after…hum…PhD. My internship with you was one of the highlights of my PhD, and my CV is a skill richer because of you. I admire your courage and grit! Whatever I do from now on, the lessons I learned from you will be a big part of it.

Members of **Sonia del Rincón's** lab at Lady Davis Institute, especially **Sonia, Christophe Audrey, Vivienne**: I am so grateful for the excellent welcome, support, and assistance during my crazy 3-week experiment. 300 RNA samples and 300 protein samples collected, 600 FACS samples run, 18h working days, minus 30 °C outside and yet all I remember is how fun it was. It wouldn't be possible without your immense help! (Say "I am sorry" to your cats from me for keeping you away from them, Christophe ☺.)

To the people of the past that I want to have more of in the present: **Konstantinos**, do you remember the muffin you brought to an uncomfortable PhD student candidate on her first day in an intimidating lab? I loved our out-of-lab adventures; Stockholm was more fun when you were in it. Heck, a lot of things were more fun when you were a part of them. I hope we get to travel together as we discussed. **Radhika**, ah, you are such a ray of sunshine! Your positive outlook on things, no matter what live throws at you is amazing and leaves me in awe. If I could adopt one personality, it would totally be yours.

Kerstin Bergman, When I no longer had you to rely on, I discovered all the things you did for us students and postdocs that we never noticed that made our lives easier. Thank you. **Rosa, Nina, Hassan, Omar, Yin, Che Kang, Moa, Pernilla, Georgia, Harold, Kasper, Rozina, Noel, Kiki, Gökce, Weicheng** – you made work - and afterwork - fun ☺

Dorothea, it is a rare – in my case, unique - experience to meet a person with whom you resonate in phase. With whom you just can't stop talking because each new topic sidetracks you and sidetracks you again since there are so many of them that you wish to cover. You were with me for half of this journey – thank you for the scientific and private example you set - and your absence in the second half was felt. **Katrin**, your integrity sets an example. One day I hope to be as strong-willed as you.

To my wonderful family-in-law: **Lars** and **Cecilia, Sofia and Matthias, John** och **Simon**, **Eva** thank you for your interest in what I do and for your support during the ebb of my PhD experience, for all those cosy and delicious family dinners and the warm-even-if-chilly Christmases. **Matthias**, the creator of the most epic cover illustration in the history of Karolinska! Thank you so much for the crash course in illustrator that you gave me! That was probably some of the most fruitful few hours of my PhD career and all the figures in this thesis are dedicated to you. Oh, and have I mentioned how *epic* the cover illustration you made is? ;) To my "friends-in-law" **Fredrik, Fanny, Micke, Gustav** – thank you for great midsummers and other get-togethers – an awesome way for a tired PhD student to recharge her batteries ☺

To **my parents** and **Mark**: The unconditional love and support I receive from you means everything to me. Your opinion of me – that I am "the best" – may not always be the most objective, but it's so welcome at times, when no one else thinks so. Thank you, mom, especially, for making sure I always have an awesome dress to wear to a PhD defense party, thank you, dad, for reading some of the books quoted here for me when I was a child, and thank you, Mark, for letting me torture your precious "Saaben" (*poor thing…*) whenever I ask. I don't deserve you and I don't tell you this enough. You are amazing. **Бабушкам и дедушкам** - спасибо. Я скучаю по вам.

Johan, you accompanied me throughout (almost) all of this time. If you were bothered by a partner who has to work every weekend, including February 14th, a Sunday, you didn't let it show. Instead, you met me with dinner on the table, when I returned, tired, grumpy and hungry. You provided me with the most inspiring thesis writing environment and the best thesis writing companion (who, for some reason, thinks my keyboard is the ultimate place to nap on). You made sure I could get to where I needed to be, even at uncomfortable times and days. You did all those little things that made this experience easier for me. But above all, you gave me a hug when I curled up in a fetal position on the floor, needing it. I don't take you for granted. *Thank you*.

6 REFERENCES

1. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer.* Cell, 2000. **100**(1): p. 57-70.
2. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* Cell, 2011. **144**(5): p. 646-74.
3. Weinberg, R.A., *The biology of cancer*. Second edition. ed. 2014, New York: Garland Science, Taylor & Francis Group. xx, 876, A 6, G 30, I 28 pages.
4. Hanahan, D. and L.M. Coussens, *Accessories to the crime: functions of cells recruited to the tumor microenvironment.* Cancer Cell, 2012. **21**(3): p. 309-22.
5. Grivennikov, S.I. and M. Karin, *Inflammation and oncogenesis: a vicious connection.* Curr Opin Genet Dev, 2010. **20**(1): p. 65-71.
6. Horimoto, Y., et al., *Emerging roles of the tumor-associated stroma in promoting tumor metastasis.* Cell Adh Migr, 2012. **6**(3): p. 193-202.
7. Dunn, G.P., L.J. Old, and R.D. Schreiber, *The three Es of cancer immunoediting.* Annu Rev Immunol, 2004. **22**: p. 329-60.
8. Thomas, L., *On immunosurveillance in human cancer.* Yale J Biol Med, 1982. **55**(3-4): p. 329-33.
9. Dunn, G.P., et al., *Cancer immunoediting: from immunosurveillance to tumor escape.* Nat Immunol, 2002. **3**(11): p. 991-8.
10. Grivennikov, S.I., F.R. Greten, and M. Karin, *Immunity, inflammation, and cancer.* Cell, 2010. **140**(6): p. 883-99.
11. Gonzalez, H., C. Hagerling, and Z. Werb, *Roles of the immune system in cancer: from tumor initiation to metastatic progression.* Genes Dev, 2018. **32**(19-20): p. 1267-1284.
12. Stefater, J.A., 3rd, et al., *Metchnikoff's policemen: macrophages in development, homeostasis and regeneration.* Trends Mol Med, 2011. **17**(12): p. 743-52.
13. Wood, W. and P. Martin, *Macrophage Functions in Tissue Patterning and Disease: New Insights from the Fly.* Dev Cell, 2017. **40**(3): p. 221-233.
14. Wynn, T.A., A. Chawla, and J.W. Pollard, *Macrophage biology in development, homeostasis and disease.* Nature, 2013. **496**(7446): p. 445-55.
15. Xiong, M., et al., *Production of vascular endothelial growth factor by murine macrophages: regulation by hypoxia, lactate, and the inducible nitric oxide synthase pathway.* Am J Pathol, 1998. **153**(2): p. 587-98.
16. Machnik, A., et al., *Macrophages regulate salt-dependent volume and blood pressure by a vascular endothelial growth factor-C-dependent buffering mechanism.* Nat Med, 2009. **15**(5): p. 545-52.
17. Chawla, A., K.D. Nguyen, and Y.P. Goh, *Macrophage-mediated inflammation in metabolic disease.* Nat Rev Immunol, 2011. **11**(11): p. 738-49.
18. Odegaard, J.I., K. Ganeshan, and A. Chawla, *Adipose tissue macrophages: Amicus adipem?* Cell Metab, 2013. **18**(6): p. 767-8.

19. Ginhoux, F., et al., *Fate mapping analysis reveals that adult microglia derive from primitive macrophages.* Science, 2010. **330**(6005): p. 841-5.

20. Stremmel, C., et al., *Yolk sac macrophage progenitors traffic to the embryo during defined stages of development.* Nat Commun, 2018. **9**(1): p. 75.

21. Sheng, J., C. Ruedl, and K. Karjalainen, *Fetal HSCs versus EMP2s.* Immunity, 2015. **43**(6): p. 1025.

22. Perdiguero, E.G., et al., *The Origin of Tissue-Resident Macrophages: When an Erythro-myeloid Progenitor Is an Erythro-myeloid Progenitor.* Immunity, 2015. **43**(6): p. 1023-4.

23. Gomez Perdiguero, E., et al., *Tissue-resident macrophages originate from yolk-sac-derived erythro-myeloid progenitors.* Nature, 2015. **518**(7540): p. 547-51.

24. Mass, E., et al., *Specification of tissue-resident macrophages during organogenesis.* Science, 2016. **353**(6304).

25. Hoeffel, G., et al., *Adult Langerhans cells derive predominantly from embryonic fetal liver monocytes with a minor contribution of yolk sac-derived macrophages.* J Exp Med, 2012. **209**(6): p. 1167-81.

26. Geissmann, F., S. Jung, and D.R. Littman, *Blood monocytes consist of two principal subsets with distinct migratory properties.* Immunity, 2003. **19**(1): p. 71-82.

27. Yona, S., et al., *Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis.* Immunity, 2013. **38**(1): p. 79-91.

28. Mildner, A., S. Yona, and S. Jung, *A close encounter of the third kind: monocyte-derived cells.* Adv Immunol, 2013. **120**: p. 69-103.

29. Gautier, E.L., et al., *Gene-expression profiles and transcriptional regulatory pathways that underlie the identity and diversity of mouse tissue macrophages.* Nat Immunol, 2012. **13**(11): p. 1118-28.

30. Schmidt, S.V., et al., *The transcriptional regulator network of human inflammatory macrophages is defined by open chromatin.* Cell Res, 2016. **26**(2): p. 151-70.

31. Gosselin, D., et al., *Environment drives selection and function of enhancers controlling tissue-specific macrophage identities.* Cell, 2014. **159**(6): p. 1327-40.

32. Lavin, Y., et al., *Tissue-resident macrophage enhancer landscapes are shaped by the local microenvironment.* Cell, 2014. **159**(6): p. 1312-26.

33. Suzuki, T., et al., *Pulmonary macrophage transplantation therapy.* Nature, 2014. **514**(7523): p. 450-4.

34. Daems, W.T. and J.M. de Bakker, *Do resident macrophages proliferate?* Immunobiology, 1982. **161**(3-4): p. 204-11.

35. Aziz, A., et al., *MafB/c-Maf deficiency enables self-renewal of differentiated functional macrophages.* Science, 2009. **326**(5954): p. 867-71.

36. Jenkins, S.J., et al., *Local macrophage proliferation, rather than recruitment from the blood, is a signature of TH2 inflammation.* Science, 2011. **332**(6035): p. 1284-8.

37. Hashimoto, D., et al., *Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes.* Immunity, 2013. **38**(4): p. 792-804.

38. Sieweke, M.H. and J.E. Allen, *Beyond stem cells: self-renewal of differentiated macrophages.* Science, 2013. **342**(6161): p. 1242974.

39. Wallerius, M., et al., *Guidance Molecule SEMA3A Restricts Tumor Growth by Differentially Regulating the Proliferation of Tumor-Associated Macrophages.* Cancer Res, 2016. **76**(11): p. 3166-78.

40. Stein, M., et al., *Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation.* J Exp Med, 1992. **176**(1): p. 287-92.

41. Pace, J.L., et al., *Recombinant mouse gamma interferon induces the priming step in macrophage activation for tumor cell killing.* J Immunol, 1983. **130**(5): p. 2011-3.

42. Mills, C.D., et al., *M-1/M-2 macrophages and the Th1/Th2 paradigm.* J Immunol, 2000. **164**(12): p. 6166-73.

43. Sica, A. and A. Mantovani, *Macrophage plasticity and polarization: in vivo veritas.* J Clin Invest, 2012. **122**(3): p. 787-95.

44. Biswas, S.K. and A. Mantovani, *Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm.* Nat Immunol, 2010. **11**(10): p. 889-96.

45. Martinez, F.O. and S. Gordon, *The M1 and M2 paradigm of macrophage activation: time for reassessment.* F1000Prime Rep, 2014. **6**: p. 13.

46. Hibbs, J.B., Jr., Z. Vavrin, and R.R. Taintor, *L-arginine is required for expression of the activated macrophage effector mechanism causing selective metabolic inhibition in target cells.* J Immunol, 1987. **138**(2): p. 550-65.

47. Xue, J., et al., *Transcriptome-based network analysis reveals a spectrum model of human macrophage activation.* Immunity, 2014. **40**(2): p. 274-88.

48. Murray, P.J., et al., *Macrophage activation and polarization: nomenclature and experimental guidelines.* Immunity, 2014. **41**(1): p. 14-20.

49. Nahrendorf, M. and F.K. Swirski, *Abandoning M1/M2 for a Network Model of Macrophage Function.* Circ Res, 2016. **119**(3): p. 414-7.

50. Lahmar, Q., et al., *Tissue-resident versus monocyte-derived macrophages in the tumor microenvironment.* Biochim Biophys Acta, 2016. **1865**(1): p. 23-34.

51. Movahedi, K., et al., *Different tumor microenvironments contain functionally distinct subsets of macrophages derived from Ly6C(high) monocytes.* Cancer Res, 2010. **70**(14): p. 5728-39.

52. Qian, B.Z. and J.W. Pollard, *Macrophage diversity enhances tumor progression and metastasis.* Cell, 2010. **141**(1): p. 39-51.

53. Yang, M., et al., *Diverse Functions of Macrophages in Different Tumor Microenvironments.* Cancer Res, 2018. **78**(19): p. 5492-5503.

54. Biswas, S.K., A. Sica, and C.E. Lewis, *Plasticity of macrophage function during tumor progression: regulation by distinct molecular mechanisms.* J Immunol, 2008. **180**(4): p. 2011-7.

55. Mantovani, A., et al., *Tumour-associated macrophages as treatment targets in oncology.* Nat Rev Clin Oncol, 2017. **14**(7): p. 399-416.

56. Lin, E.Y., et al., *Macrophages regulate the angiogenic switch in a mouse model of breast cancer.* Cancer Res, 2006. **66**(23): p. 11238-46.

57. Schoppmann, S.F., et al., *Tumor-associated macrophages express lymphatic endothelial growth factors and are related to peritumoral lymphangiogenesis.* Am J Pathol, 2002. **161**(3): p. 947-56.

58. Carmeliet, P. and R.K. Jain, *Molecular mechanisms and clinical applications of angiogenesis.* Nature, 2011. **473**(7347): p. 298-307.

59. Stockmann, C., et al., *Deletion of vascular endothelial growth factor in myeloid cells accelerates tumorigenesis.* Nature, 2008. **456**(7223): p. 814-8.

60. Jain, R.K., *Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy.* Science, 2005. **307**(5706): p. 58-62.

61. Rolny, C., et al., *HRG inhibits tumor growth and metastasis by inducing macrophage polarization and vessel normalization through downregulation of PlGF.* Cancer Cell, 2011. **19**(1): p. 31-44.

62. Lee, S., et al., *Processing of VEGF-A by matrix metalloproteinases regulates bioavailability and vascular patterning in tumors.* J Cell Biol, 2005. **169**(4): p. 681-91.

63. Sangaletti, S., et al., *Macrophage-derived SPARC bridges tumor cell-extracellular matrix interactions toward metastasis.* Cancer Res, 2008. **68**(21): p. 9050-9.

64. Hagemann, T., et al., *Macrophages induce invasiveness of epithelial cancer cells via NF-kappa B and JNK.* J Immunol, 2005. **175**(2): p. 1197-205.

65. Hiratsuka, S., et al., *MMP9 induction by vascular endothelial growth factor receptor-1 is involved in lung-specific metastasis.* Cancer Cell, 2002. **2**(4): p. 289-300.

66. Hagemann, T., et al., *Enhanced invasiveness of breast cancer cell lines upon co-cultivation with macrophages is due to TNF-alpha dependent up-regulation of matrix metalloproteases.* Carcinogenesis, 2004. **25**(8): p. 1543-9.

67. Wyckoff, J., et al., *A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors.* Cancer Res, 2004. **64**(19): p. 7022-9.

68. Wyckoff, J.B., et al., *Direct visualization of macrophage-assisted tumor cell intravasation in mammary tumors.* Cancer Res, 2007. **67**(6): p. 2649-56.

69. Harney, A.S., et al., *Real-Time Imaging Reveals Local, Transient Vascular Permeability, and Tumor Cell Intravasation Stimulated by TIE2hi Macrophage-Derived VEGFA.* Cancer Discov, 2015. **5**(9): p. 932-43.

70. Abbas, A.K. and A.H. Lichtman, *Basic immunology : functions and disorders of the immune system*. 3rd ed. 2009, Philadelphia, PA: Saunders/Elsevier. viii, 312 p.

71. Fidler, I.J. and A.J. Schroit, *Recognition and destruction of neoplastic cells by activated macrophages: discrimination of altered self.* Biochim Biophys Acta, 1988. **948**(2): p. 151-73.

72. Hagemann, T., et al., *"Re-educating" tumor-associated macrophages by targeting NF-kappaB.* J Exp Med, 2008. **205**(6): p. 1261-8.

73. Orlikowsky, T.W., et al., *Expression and regulation of B7 family molecules on macrophages (MΦ) in preterm and term neonatal cord blood and peripheral blood of adults.* Cytometry Part B: Clinical Cytometry, 2003. **53B**(1): p. 40-47.

74. DeNardo, D.G., et al., *CD4(+) T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages.* Cancer Cell, 2009. **16**(2): p. 91-102.

75. Biswas, S.K., et al., *A distinct and unique transcriptional program expressed by tumor-associated macrophages (defective NF-kappaB and enhanced IRF-3/STAT1 activation).* Blood, 2006. **107**(5): p. 2112-22.

76. Bronte, V. and P. Zanovello, *Regulation of immune responses by L-arginine metabolism.* Nat Rev Immunol, 2005. **5**(8): p. 641-54.

77. Rodriguez, P.C., D.G. Quiceno, and A.C. Ochoa, *L-arginine availability regulates T-lymphocyte cell-cycle progression.* Blood, 2007. **109**(4): p. 1568-73.

78. Kuang, D.M., et al., *Activated monocytes in peritumoral stroma of hepatocellular carcinoma foster immune privilege and disease progression through PD-L1.* J Exp Med, 2009. **206**(6): p. 1327-37.

79. Curiel, T.J., et al., *Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival.* Nat Med, 2004. **10**(9): p. 942-9.

80. Yang, L., Y. Pang, and H.L. Moses, *TGF-beta and immune cells: an important regulatory axis in the tumor microenvironment and progression.* Trends Immunol, 2010. **31**(6): p. 220-7.

81. Mitchem, J.B., et al., *Targeting tumor-infiltrating macrophages decreases tumor-initiating cells, relieves immunosuppression, and improves chemotherapeutic responses.* Cancer Res, 2013. **73**(3): p. 1128-41.

82. DeNardo, D.G., et al., *Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy.* Cancer Discov, 2011. **1**(1): p. 54-67.

83. Poh, A.R. and M. Ernst, *Targeting Macrophages in Cancer: From Bench to Bedside.* Front Oncol, 2018. **8**: p. 49.

84. Qian, B.Z., et al., *CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis.* Nature, 2011. **475**(7355): p. 222-5.

85. Leek, R.D., et al., *Macrophage infiltration is associated with VEGF and EGFR expression in breast cancer.* J Pathol, 2000. **190**(4): p. 430-6.

86. Sidibe, A., et al., *Angiogenic factor-driven inflammation promotes extravasation of human proangiogenic monocytes to tumours.* Nat Commun, 2018. **9**(1): p. 355.

87. Van Overmeire, E., et al., *M-CSF and GM-CSF Receptor Signaling Differentially Regulate Monocyte Maturation and Macrophage Polarization in the Tumor Microenvironment.* Cancer Res, 2016. **76**(1): p. 35-42.

88. Sanchez-Martin, L., et al., *The chemokine CXCL12 regulates monocyte-macrophage differentiation and RUNX3 expression.* Blood, 2011. **117**(1): p. 88-97.

89. Zhu, Y., et al., *Tissue-Resident Macrophages in Pancreatic Ductal Adenocarcinoma Originate from Embryonic Hematopoiesis and Promote Tumor Progression.* Immunity, 2017. **47**(3): p. 597.

90. Chen, Z., et al., *Cellular and Molecular Identity of Tumor-Associated Macrophages in Glioblastoma.* Cancer Res, 2017. **77**(9): p. 2266-2278.

91. Franklin, R.A., et al., *The cellular and molecular origin of tumor-associated macrophages.* Science, 2014. **344**(6186): p. 921-5.

92. Scholzen, T. and J. Gerdes, *The Ki-67 protein: from the known and the unknown.* J Cell Physiol, 2000. **182**(3): p. 311-22.

93. Tymoszuk, P., et al., *In situ proliferation contributes to accumulation of tumor-associated macrophages in spontaneous mammary tumors.* Eur J Immunol, 2014. **44**(8): p. 2247-62.

94. Ruffell, B., N.I. Affara, and L.M. Coussens, *Differential macrophage programming in the tumor microenvironment.* Trends Immunol, 2012. **33**(3): p. 119-26.

95. Komohara, Y., M. Jinushi, and M. Takeya, *Clinical significance of macrophage heterogeneity in human malignant tumors.* Cancer Sci, 2014. **105**(1): p. 1-8.

96. Ruffell, B. and L.M. Coussens, *Macrophages and therapeutic resistance in cancer.* Cancer Cell, 2015. **27**(4): p. 462-72.

97. Mahmoud, S.M., et al., *Tumour-infiltrating macrophages and clinical outcome in breast cancer.* J Clin Pathol, 2012. **65**(2): p. 159-63.

98. Yang, M., et al., *Stromal Infiltration of Tumor-Associated Macrophages Conferring Poor Prognosis of Patients with Basal-Like Breast Carcinoma.* J Cancer, 2018. **9**(13): p. 2308-2316.

99. Mantovani, A. and P. Allavena, *The interaction of anticancer therapies with tumor-associated macrophages.* J Exp Med, 2015. **212**(4): p. 435-45.

100. Casares, N., et al., *Caspase-dependent immunogenicity of doxorubicin-induced tumor cell death.* J Exp Med, 2005. **202**(12): p. 1691-701.

101. Obeid, M., et al., *Calreticulin exposure dictates the immunogenicity of cancer cell death.* Nat Med, 2007. **13**(1): p. 54-61.

102. Ruffell, B., et al., *Macrophage IL-10 blocks CD8+ T cell-dependent responses to chemotherapy by suppressing IL-12 expression in intratumoral dendritic cells.* Cancer Cell, 2014. **26**(5): p. 623-37.

103. Panni, R.Z., D.C. Linehan, and D.G. DeNardo, *Targeting tumor-infiltrating macrophages to combat cancer.* Immunotherapy, 2013. **5**(10): p. 1075-87.

104. Loberg, R.D., et al., *Targeting CCL2 with systemic delivery of neutralizing antibodies induces prostate cancer tumor regression in vivo.* Cancer Res, 2007. **67**(19): p. 9417-24.

105. Lu, X. and Y. Kang, *Chemokine (C-C motif) ligand 2 engages CCR2+ stromal cells of monocytic origin to promote breast cancer metastasis to lung and bone.* J Biol Chem, 2009. **284**(42): p. 29087-96.

106. Bonapace, L., et al., *Cessation of CCL2 inhibition accelerates breast cancer metastasis by promoting angiogenesis.* Nature, 2014. **515**(7525): p. 130-3.

107. Sandhu, S.K., et al., *A first-in-human, first-in-class, phase I study of carlumab (CNTO 888), a human monoclonal antibody against CC-chemokine ligand 2 in patients with solid tumors.* Cancer Chemother Pharmacol, 2013. **71**(4): p. 1041-50.

108. Brana, I., et al., *Carlumab, an anti-C-C chemokine ligand 2 monoclonal antibody, in combination with four chemotherapy regimens for the treatment of patients with solid tumors: an open-label, multicenter phase 1b study.* Target Oncol, 2015. **10**(1): p. 111-23.

109. Ries, C.H., et al., *Targeting tumor-associated macrophages with anti-CSF-1R antibody reveals a strategy for cancer therapy.* Cancer Cell, 2014. **25**(6): p. 846-59.

110. Pradel, L.P., et al., *Macrophage Susceptibility to Emactuzumab (RG7155) Treatment.* Mol Cancer Ther, 2016. **15**(12): p. 3077-3086.

111. Pyonteck, S.M., et al., *CSF-1R inhibition alters macrophage polarization and blocks glioma progression.* Nat Med, 2013. **19**(10): p. 1264-72.

112. Guerriero, J.L., et al., *Class IIa HDAC inhibition reduces breast tumours and metastases through anti-tumour macrophages.* Nature, 2017. **543**(7645): p. 428-432.

113. Wanderley, C.W., et al., *Paclitaxel Reduces Tumor Growth by Reprogramming Tumor-Associated Macrophages to an M1 Profile in a TLR4-Dependent Manner.* Cancer Res, 2018. **78**(20): p. 5891-5900.

114. Chen, D., et al., *Chloroquine modulates antitumor immune response by resetting tumor-associated macrophages toward M1 phenotype.* Nat Commun, 2018. **9**(1): p. 873.

115. Gabrilovich, D.I. and S. Nagaraj, *Myeloid-derived suppressor cells as regulators of the immune system.* Nat Rev Immunol, 2009. **9**(3): p. 162-74.

116. Newton, K. and V.M. Dixit, *Signaling in innate immunity and inflammation.* Cold Spring Harb Perspect Biol, 2012. **4**(3).

117. Gabrilovich, D.I., *Myeloid-Derived Suppressor Cells.* Cancer Immunol Res, 2017. **5**(1): p. 3-8.

118. Millrud, C.R., C. Bergenfelz, and K. Leandersson, *On the origin of myeloid-derived suppressor cells.* Oncotarget, 2017. **8**(2): p. 3649-3665.

119. Gabrilovich, D.I., et al., *The terminology issue for myeloid-derived suppressor cells.* Cancer Res, 2007. **67**(1): p. 425; author reply 426.

120. Fleming, T.J., M.L. Fleming, and T.R. Malek, *Selective expression of Ly-6G on myeloid lineage cells in mouse bone marrow. RB6-8C5 mAb to granulocyte-differentiation antigen (Gr-1) detects members of the Ly-6 family.* J Immunol, 1993. **151**(5): p. 2399-408.

121. Youn, J.I., et al., *Subsets of myeloid-derived suppressor cells in tumor-bearing mice.* J Immunol, 2008. **181**(8): p. 5791-802.

122. Bruger, A.M., et al., *How to measure the immunosuppressive activity of MDSC: assays, problems and potential solutions.* Cancer Immunol Immunother, 2018.

123. Brandau, S., et al., *Myeloid-derived suppressor cells in the peripheral blood of cancer patients contain a subset of immature neutrophils with impaired migratory properties.* J Leukoc Biol, 2011. **89**(2): p. 311-7.

124. Mandruzzato, S., et al., *Toward harmonized phenotyping of human myeloid-derived suppressor cells by flow cytometry: results from an interim study.* Cancer Immunol Immunother, 2016. **65**(2): p. 161-9.

125. Condamine, T., et al., *Lectin-type oxidized LDL receptor-1 distinguishes population of human polymorphonuclear myeloid-derived suppressor cells in cancer patients.* Sci Immunol, 2016. **1**(2).

126. Condamine, T. and D.I. Gabrilovich, *Molecular mechanisms regulating myeloid-derived suppressor cell differentiation and function.* Trends Immunol, 2011. **32**(1): p. 19-25.

127. Yu, H., et al., *SOCS3 Deficiency in Myeloid Cells Promotes Tumor Development: Involvement of STAT3 Activation and Myeloid-Derived Suppressor Cells.* Cancer Immunol Res, 2015. **3**(7): p. 727-40.

128. Nefedova, Y., et al., *Hyperactivation of STAT3 is involved in abnormal differentiation of dendritic cells in cancer.* J Immunol, 2004. **172**(1): p. 464-74.

129. Movahedi, K., et al., *Identification of discrete tumor-induced myeloid-derived suppressor cell subpopulations with distinct T cell-suppressive activity.* Blood, 2008. **111**(8): p. 4233-44.

130. Delano, M.J., et al., *MyD88-dependent expansion of an immature GR-1(+)CD11b(+) population induces T cell suppression and Th2 polarization in sepsis.* J Exp Med, 2007. **204**(6): p. 1463-74.

131. Bergenfelz, C., et al., *Systemic Monocytic-MDSCs Are Generated from Monocytes and Correlate with Disease Progression in Breast Cancer Patients.* PLoS One, 2015. **10**(5): p. e0127028.

132. Gabrilovich, D.I., S. Ostrand-Rosenberg, and V. Bronte, *Coordinated regulation of myeloid cells by tumours.* Nat Rev Immunol, 2012. **12**(4): p. 253-68.

133. Heuvers, M.E., et al., *Arginase-1 mRNA expression correlates with myeloid-derived suppressor cell levels in peripheral blood of NSCLC patients.* Lung Cancer, 2013. **81**(3): p. 468-74.

134. Srivastava, M.K., et al., *Myeloid-derived suppressor cells inhibit T-cell activation by depleting cystine and cysteine.* Cancer Res, 2010. **70**(1): p. 68-77.

135. Nagaraj, S., et al., *Altered recognition of antigen is a mechanism of CD8+ T cell tolerance in cancer.* Nat Med, 2007. **13**(7): p. 828-35.

136. Hanson, E.M., et al., *Myeloid-derived suppressor cells down-regulate L-selectin expression on CD4+ and CD8+ T cells.* J Immunol, 2009. **183**(2): p. 937-44.

137. Molon, B., et al., *Chemokine nitration prevents intratumoral infiltration of antigen-specific T cells.* J Exp Med, 2011. **208**(10): p. 1949-62.

138. Huang, B., et al., *Gr-1+CD115+ immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell anergy in tumor-bearing host.* Cancer Res, 2006. **66**(2): p. 1123-31.

139. Umansky, V., et al., *The Role of Myeloid-Derived Suppressor Cells (MDSC) in Cancer Progression.* Vaccines (Basel), 2016. **4**(4).

140. Zhang, S., et al., *The Role of Myeloid-Derived Suppressor Cells in Patients with Solid Tumors: A Meta-Analysis.* PLoS One, 2016. **11**(10): p. e0164514.

141. Yang, L., et al., *Expansion of myeloid immune suppressor Gr+CD11b+ cells in tumor-bearing host directly promotes tumor angiogenesis.* Cancer Cell, 2004. **6**(4): p. 409-21.

142. Yang, L., et al., *Abrogation of TGF beta signaling in mammary carcinomas recruits Gr-1+CD11b+ myeloid cells that promote metastasis.* Cancer Cell, 2008. **13**(1): p. 23-35.

143. Liu, Y., et al., *Targeting myeloid-derived suppressor cells for cancer immunotherapy.* Cancer Immunol Immunother, 2018. **67**(8): p. 1181-1195.

144. Kumar, V., et al., *The Nature of Myeloid-Derived Suppressor Cells in the Tumor Microenvironment.* Trends Immunol, 2016. **37**(3): p. 208-220.

145. Corzo, C.A., et al., *HIF-1alpha regulates function and differentiation of myeloid-derived suppressor cells in the tumor microenvironment.* J Exp Med, 2010. **207**(11): p. 2439-53.

146. Kumar, V., et al., *CD45 Phosphatase Inhibits STAT3 Transcription Factor Activity in Myeloid Cells and Promotes Tumor-Associated Macrophage Differentiation.* Immunity, 2016. **44**(2): p. 303-15.

147. Qin, H., et al., *Generation of a new therapeutic peptide that depletes myeloid-derived suppressor cells in tumor-bearing mice.* Nat Med, 2014. **20**(6): p. 676-81.

148. Condamine, T., et al., *ER stress regulates myeloid-derived suppressor cell fate through TRAIL-R-mediated apoptosis.* J Clin Invest, 2014. **124**(6): p. 2626-39.

149. Dominguez, G.A., et al., *Selective Targeting of Myeloid-Derived Suppressor Cells in Cancer Patients Using DS-8273a, an Agonistic TRAIL-R2 Antibody.* Clin Cancer Res, 2017. **23**(12): p. 2942-2950.

150. Hossain, D.M., et al., *TLR9-Targeted STAT3 Silencing Abrogates Immunosuppressive Activity of Myeloid-Derived Suppressor Cells from Prostate Cancer Patients.* Clin Cancer Res, 2015. **21**(16): p. 3771-82.

151. Awad, R.M., et al., *Turn Back the TIMe: Targeting Tumor Infiltrating Myeloid Cells to Revert Cancer Progression.* Front Immunol, 2018. **9**: p. 1977.

152. Bonelli, S., et al., *Beyond the M-CSF receptor - novel therapeutic targets in tumor-associated macrophages.* FEBS J, 2018. **285**(4): p. 777-787.

153. Kumar, V., et al., *Cancer-Associated Fibroblasts Neutralize the Anti-tumor Effect of CSF1 Receptor Blockade by Inducing PMN-MDSC Infiltration of Tumors.* Cancer Cell, 2017. **32**(5): p. 654-668 e5.

154. Kortylewski, M. and H. Yu, *Role of Stat3 in suppressing anti-tumor immunity.* Curr Opin Immunol, 2008. **20**(2): p. 228-33.

155. Su, Y.L., et al., *STAT3 in Tumor-Associated Myeloid Cells: Multitasking to Disrupt Immunity.* Int J Mol Sci, 2018. **19**(6).

156. Manfroi, B., et al., *Tumor-associated neutrophils correlate with poor prognosis in diffuse large B-cell lymphoma patients.* Blood Cancer J, 2018. **8**(7): p. 66.

157. Liu, Q., et al., *Tumor-educated CD11bhighIalow regulatory dendritic cells suppress T cell response through arginase I.* J Immunol, 2009. **182**(10): p. 6207-16.

158. Bhowmick, N.A., E.G. Neilson, and H.L. Moses, *Stromal fibroblasts in cancer initiation and progression.* Nature, 2004. **432**(7015): p. 332-7.

159. Bremnes, R.M., et al., *The role of tumor stroma in cancer progression and prognosis: emphasis on carcinoma-associated fibroblasts and non-small cell lung cancer.* J Thorac Oncol, 2011. **6**(1): p. 209-17.

160. Erdogan, B. and D.J. Webb, *Cancer-associated fibroblasts modulate growth factor signaling and extracellular matrix remodeling to regulate tumor metastasis.* Biochem Soc Trans, 2017. **45**(1): p. 229-236.

161. Chang, H.Y., et al., *Gene expression signature of fibroblast serum response predicts human cancer progression: similarities between tumors and wounds.* PLoS Biol, 2004. **2**(2): p. E7.

162. Liao, D., et al., *Cancer associated fibroblasts promote tumor growth and metastasis by modulating the tumor immune microenvironment in a 4T1 murine breast cancer model.* PLoS One, 2009. **4**(11): p. e7965.

163. Cho, H., et al., *Cancer-Stimulated CAFs Enhance Monocyte Differentiation and Protumoral TAM Activation via IL6 and GM-CSF Secretion.* Clin Cancer Res, 2018.

164. Hashimoto, O., et al., *Collaboration of cancer-associated fibroblasts and tumour-associated macrophages for neuroblastoma development.* J Pathol, 2016. **240**(2): p. 211-23.

165. Krtolica, A., et al., *Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging.* Proc Natl Acad Sci U S A, 2001. **98**(21): p. 12072-7.

166. Trimboli, A.J., et al., *Pten in stromal fibroblasts suppresses mammary epithelial tumours.* Nature, 2009. **461**(7267): p. 1084-91.

167. Watson, J.D. and F.H. Crick, *Genetical implications of the structure of deoxyribonucleic acid.* Nature, 1953. **171**(4361): p. 964-7.

168. Dahm, R., *Friedrich Miescher and the discovery of DNA.* Dev Biol, 2005. **278**(2): p. 274-88.

169. Jacob, F. and J. Monod, *Genetic regulatory mechanisms in the synthesis of proteins.* J Mol Biol, 1961. **3**: p. 318-56.

170. Kollmar, R. and P.J. Farnham, *Site-specific initiation of transcription by RNA polymerase II.* Proc Soc Exp Biol Med, 1993. **203**(2): p. 127-39.

171. Kornberg, R.D., *Chromatin structure: a repeating unit of histones and DNA.* Science, 1974. **184**(4139): p. 868-71.

172. Allfrey, V.G., R. Faulkner, and A.E. Mirsky, *Acetylation and Methylation of Histones and Their Possible Role in the Regulation of Rna Synthesis.* Proc Natl Acad Sci U S A, 1964. **51**: p. 786-94.

173. Heinzel, T., et al., *A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression.* Nature, 1997. **387**(6628): p. 43-8.

174. Sainsbury, S., C. Bernecky, and P. Cramer, *Structural basis of transcription initiation by RNA polymerase II.* Nat Rev Mol Cell Biol, 2015. **16**(3): p. 129-43.

175. Kim, Y.J., et al., *A multiprotein mediator of transcriptional activation and its interaction with the C-terminal repeat domain of RNA polymerase II.* Cell, 1994. **77**(4): p. 599-608.

176. Johnson, P.F. and S.L. McKnight, *Eukaryotic transcriptional regulatory proteins.* Annu Rev Biochem, 1989. **58**: p. 799-839.

177. Furuichi, Y., et al., *Blocked, methylated 5'-terminal sequence in avian sarcoma virus RNA.* Nature, 1975. **257**(5527): p. 618-20.

178. Darnell, J.E., R. Wall, and R.J. Tushinski, *An adenylic acid-rich sequence in messenger RNA of HeLa cells and its possible relationship to reiterated sites in DNA.* Proc Natl Acad Sci U S A, 1971. **68**(6): p. 1321-5.

179. Berget, S.M., C. Moore, and P.A. Sharp, *Spliced segments at the 5' terminus of adenovirus 2 late mRNA.* Proc Natl Acad Sci U S A, 1977. **74**(8): p. 3171-5.

180. Schoenberg, D.R. and L.E. Maquat, *Regulation of cytoplasmic mRNA decay.* Nat Rev Genet, 2012. **13**(4): p. 246-59.

181. Sonenberg, N. and A.G. Hinnebusch, *Regulation of translation initiation in eukaryotes: mechanisms and biological targets.* Cell, 2009. **136**(4): p. 731-45.

182. Englander, S.W. and L. Mayne, *The nature of protein folding pathways.* Proc Natl Acad Sci U S A, 2014. **111**(45): p. 15873-80.

183. Knorre, D.G., N.V. Kudryashova, and T.S. Godovikova, *Chemical and functional aspects of posttranslational modification of proteins.* Acta Naturae, 2009. **1**(3): p. 29-51.

184. Amm, I., T. Sommer, and D.H. Wolf, *Protein quality control and elimination of protein waste: the role of the ubiquitin-proteasome system.* Biochim Biophys Acta, 2014. **1843**(1): p. 182-96.

185. Meyer, S., C. Temme, and E. Wahle, *Messenger RNA turnover in eukaryotes: pathways and enzymes.* Crit Rev Biochem Mol Biol, 2004. **39**(4): p. 197-216.

186. Sorrentino, S., *The eight human "canonical" ribonucleases: molecular diversity, catalytic properties, and special biological actions of the enzyme proteins.* FEBS Lett, 2010. **584**(11): p. 2194-200.

187. Tubbs, R.R. and M.H. Stoler, *Cell and tissue based molecular pathology*. 1st ed. Foundations in diagnostic pathology. 2009, Philadelphia: Churchill Livingstone/Elsevier. x, 441 p.

188. Pennington, S.R., et al., *Proteome analysis: from protein characterization to biological function.* Trends Cell Biol, 1997. **7**(4): p. 168-73.

189. Vogel, C. and E.M. Marcotte, *Insights into the regulation of protein abundance from proteomic and transcriptomic analyses.* Nat Rev Genet, 2012. **13**(4): p. 227-32.

190. Vogel, C., *Translation's coming of age.* Mol Syst Biol, 2011. **7**: p. 498.

191. Gygi, S.P., et al., *Correlation between protein and mRNA abundance in yeast.* Mol Cell Biol, 1999. **19**(3): p. 1720-30.

192. Chen, G., et al., *Discordant protein and mRNA expression in lung adenocarcinomas.* Mol Cell Proteomics, 2002. **1**(4): p. 304-13.

193. Orntoft, T.F., et al., *Genome-wide study of gene copy numbers, transcripts, and protein levels in pairs of non-invasive and invasive human transitional cell carcinomas.* Mol Cell Proteomics, 2002. **1**(1): p. 37-45.

194. Anderson, L. and J. Seilhamer, *A comparison of selected mRNA and protein abundances in human liver.* Electrophoresis, 1997. **18**(3-4): p. 533-7.

195. Lichtinghagen, R., et al., *Different mRNA and protein expression of matrix metalloproteinases 2 and 9 and tissue inhibitor of metalloproteinases 1 in benign and malignant prostate tissue.* Eur Urol, 2002. **42**(4): p. 398-406.

196. Schwanhausser, B., et al., *Global quantification of mammalian gene expression control.* Nature, 2011. **473**(7347): p. 337-42.

197. Li, J.J. and M.D. Biggin, *Gene expression. Statistics requantitates the central dogma.* Science, 2015. **347**(6226): p. 1066-7.

198. Li, J.J., P.J. Bickel, and M.D. Biggin, *System wide analyses have underestimated protein abundances and the importance of transcription in mammals.* PeerJ, 2014. **2**: p. e270.

199. Csardi, G., et al., *Accounting for experimental noise reveals that mRNA levels, amplified by post-transcriptional processes, largely determine steady-state protein levels in yeast.* PLoS Genet, 2015. **11**(5): p. e1005206.

200. Liu, Y., A. Beyer, and R. Aebersold, *On the Dependency of Cellular Protein Levels on mRNA Abundance.* Cell, 2016. **165**(3): p. 535-50.

201. Li, J.J., G.L. Chew, and M.D. Biggin, *Quantitating translational control: mRNA abundance-dependent and independent contributions and the mRNA sequences that specify them.* Nucleic Acids Res, 2017. **45**(20): p. 11821-11836.

202. Bhat, M., et al., *Targeting the translation machinery in cancer.* Nat Rev Drug Discov, 2015. **14**(4): p. 261-78.

203. Wu, B., et al., *Translation dynamics of single mRNAs in live cells and neurons.* Science, 2016. **352**(6292): p. 1430-5.

204. Yan, X., et al., *Dynamics of Translation of Single mRNA Molecules In Vivo.* Cell, 2016. **165**(4): p. 976-89.

205. Wang, C., et al., *Real-Time Imaging of Translation on Single mRNA Transcripts in Live Cells.* Cell, 2016. **165**(4): p. 990-1001.

206. Morisaki, T., et al., *Real-time quantification of single RNA translation dynamics in living cells.* Science, 2016. **352**(6292): p. 1425-9.

207. Ingolia, N.T., L.F. Lareau, and J.S. Weissman, *Ribosome profiling of mouse embryonic stem cells reveals the complexity and dynamics of mammalian proteomes.* Cell, 2011. **147**(4): p. 789-802.

208. Hornstein, E., H. Tang, and O. Meyuhas, *Mitogenic and nutritional signals are transduced into translational efficiency of TOP mRNAs.* Cold Spring Harb Symp Quant Biol, 2001. **66**: p. 477-84.

209. Larsson, O., B. Tian, and N. Sonenberg, *Toward a genome-wide landscape of translational control.* Cold Spring Harb Perspect Biol, 2013. **5**(1): p. a012302.

210. Gandin, V., et al., *nanoCAGE reveals 5' UTR features that define specific modes of translation of functionally related MTOR-sensitive mRNAs.* Genome Res, 2016. **26**(5): p. 636-48.

211. King, H.A. and A.P. Gerber, *Translatome profiling: methods for genome-scale analysis of mRNA translation.* Brief Funct Genomics, 2016. **15**(1): p. 22-31.

212. Liang, S., et al., *Polysome-profiling in small tissue samples.* Nucleic Acids Res, 2018. **46**(1): p. e3.

213. Ingolia, N.T., et al., *Genome-wide analysis in vivo of translation with nucleotide resolution using ribosome profiling.* Science, 2009. **324**(5924): p. 218-23.

214. Ingolia, N.T., J.A. Hussmann, and J.S. Weissman, *Ribosome Profiling: Global Views of Translation.* Cold Spring Harb Perspect Biol, 2018.

215. Masvidal, L., et al., *mTOR-sensitive translation: Cleared fog reveals more trees.* RNA Biol, 2017. **14**(10): p. 1299-1305.

216. Floor, S.N. and J.A. Doudna, *Tunable protein synthesis by transcript isoforms in human cells.* Elife, 2016. **5**.

217. Yoshikawa, H., et al., *Efficient analysis of mammalian polysomes in cells and tissues using Ribo Mega-SEC.* Elife, 2018. **7**.

218. Heiman, M., et al., *A translational profiling approach for the molecular characterization of CNS cell types.* Cell, 2008. **135**(4): p. 738-48.

219. Larsson, O., N. Sonenberg, and R. Nadon, *Identification of differential translation in genome wide studies.* Proc Natl Acad Sci U S A, 2010. **107**(50): p. 21487-92.

220. Larsson, O., N. Sonenberg, and R. Nadon, *anota: Analysis of differential translation in genome-wide studies.* Bioinformatics, 2011. **27**(10): p. 1440-1.

221. Mathews, M., N. Sonenberg, and J.W.B. Hershey, *Translational control in biology and medicine*. 3rd ed. Cold Spring Harbor monograph series. 2007, Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press. x, 934 p.

222. Duncan, R., S.C. Milburn, and J.W. Hershey, *Regulated phosphorylation and low abundance of HeLa cell initiation factor eIF-4F suggest a role in translational control. Heat shock effects on eIF-4F.* J Biol Chem, 1987. **262**(1): p. 380-8.

223. Heikkinen, T., et al., *Eukaryotic translation initiation factor 4E (eIF4E) expression is associated with breast cancer tumor phenotype and predicts survival after anthracycline chemotherapy treatment.* Breast Cancer Res Treat, 2013. **141**(1): p. 79-88.

224. Salehi, Z., F. Mashayekhi, and F. Shahosseini, *Significance of eIF4E expression in skin squamous cell carcinoma.* Cell Biol Int, 2007. **31**(11): p. 1400-4.

225. Graff, J.R., et al., *eIF4E activation is commonly elevated in advanced human prostate cancers and significantly related to reduced patient survival.* Cancer Res, 2009. **69**(9): p. 3866-73.

226. Lazaris-Karatzas, A., K.S. Montine, and N. Sonenberg, *Malignant transformation by a eukaryotic initiation factor subunit that binds to mRNA 5' cap.* Nature, 1990. **345**(6275): p. 544-7.

227. Ruggero, D., et al., *The translation factor eIF-4E promotes tumor formation and cooperates with c-Myc in lymphomagenesis.* Nat Med, 2004. **10**(5): p. 484-6.

228. Truitt, M.L., et al., *Differential Requirements for eIF4E Dose in Normal Development and Cancer.* Cell, 2015. **162**(1): p. 59-71.

229. Lejbkowicz, F., et al., *A fraction of the mRNA 5' cap-binding protein, eukaryotic initiation factor 4E, localizes to the nucleus.* Proc Natl Acad Sci U S A, 1992. **89**(20): p. 9612-6.

230. Iborra, F.J., D.A. Jackson, and P.R. Cook, *Coupled transcription and translation within nuclei of mammalian cells.* Science, 2001. **293**(5532): p. 1139-42.

231. Sonenberg, N., et al., *A polypeptide in eukaryotic initiation factors that crosslinks specifically to the 5'-terminal cap in mRNA.* Proc Natl Acad Sci U S A, 1978. **75**(10): p. 4843-7.

232. Shatkin, A.J., *Capping of eucaryotic mRNAs.* Cell, 1976. **9**(4 PT 2): p. 645-53.

233. Tarun, S.Z., Jr., et al., *Translation initiation factor eIF4G mediates in vitro poly(A) tail-dependent translation.* Proc Natl Acad Sci U S A, 1997. **94**(17): p. 9046-51.

234. Mazumder, B., et al., *Translational silencing of ceruloplasmin requires the essential elements of mRNA circularization: poly(A) tail, poly(A)-binding protein, and eukaryotic translation initiation factor 4G.* Mol Cell Biol, 2001. **21**(19): p. 6440-9.

235. Korneeva, N.L., et al., *Mutually cooperative binding of eukaryotic translation initiation factor (eIF) 3 and eIF4A to human eIF4G-1.* J Biol Chem, 2000. **275**(52): p. 41369-76.

236. LeFebvre, A.K., et al., *Translation initiation factor eIF4G-1 binds to eIF3 through the eIF3e subunit.* J Biol Chem, 2006. **281**(32): p. 22917-32.

237. Trachsel, H., et al., *Initiation of mammalian protein synthesis. II. The assembly of the initiation complex with purified initiation factors.* J Mol Biol, 1977. **116**(4): p. 755-67.

238. Chakrabarti, A. and U. Maitra, *Function of eukaryotic initiation factor 5 in the formation of an 80 S ribosomal polypeptide chain initiation complex.* J Biol Chem, 1991. **266**(21): p. 14039-45.

239. de la Parra, C., et al., *A widespread alternate form of cap-dependent mRNA translation initiation.* Nat Commun, 2018. **9**(1): p. 3068.

240. Imataka, H., H.S. Olsen, and N. Sonenberg, *A new translational regulator with homology to eukaryotic translation initiation factor 4G.* EMBO J, 1997. **16**(4): p. 817-25.

241. Levy-Strumpf, N., et al., *DAP-5, a novel homolog of eukaryotic translation initiation factor 4G isolated as a putative modulator of gamma interferon-induced programmed cell death.* Mol Cell Biol, 1997. **17**(3): p. 1615-25.

242. Lee, A.S., et al., *eIF3d is an mRNA cap-binding protein that is required for specialized translation initiation.* Nature, 2016. **536**(7614): p. 96-9.

243. Johannes, G., et al., *Identification of eukaryotic mRNAs that are translated at reduced cap binding complex eIF4F concentrations using a cDNA microarray.* Proc Natl Acad Sci U S A, 1999. **96**(23): p. 13118-23.

244. Pelletier, J. and N. Sonenberg, *Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA.* Nature, 1988. **334**(6180): p. 320-5.

245. Lacerda, R., J. Menezes, and L. Romao, *More than just scanning: the importance of cap-independent mRNA translation initiation for cellular stress response and cancer.* Cell Mol Life Sci, 2017. **74**(9): p. 1659-1680.

246. Holcik, M. and N. Sonenberg, *Translational control in stress and apoptosis.* Nat Rev Mol Cell Biol, 2005. **6**(4): p. 318-27.

247. Gilbert, W.V., *Alternative ways to think about cellular internal ribosome entry.* J Biol Chem, 2010. **285**(38): p. 29033-8.

248. Terenin, I.M., et al., *A researcher's guide to the galaxy of IRESs.* Cell Mol Life Sci, 2017. **74**(8): p. 1431-1455.

249. Shatsky, I.N., et al., *Cap-Independent Translation: What's in a Name?* Trends Biochem Sci, 2018.

250. Brunn, G.J., et al., *Phosphorylation of the translational repressor PHAS-I by the mammalian target of rapamycin.* Science, 1997. **277**(5322): p. 99-101.

251. Laplante, M. and D.M. Sabatini, *mTOR signaling in growth control and disease.* Cell, 2012. **149**(2): p. 274-93.

252. Loewith, R., et al., *Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control.* Mol Cell, 2002. **10**(3): p. 457-68.

253. Jacinto, E., et al., *Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive.* Nat Cell Biol, 2004. **6**(11): p. 1122-8.

254. Oh, W.J., et al., *mTORC2 can associate with ribosomes to promote cotranslational phosphorylation and stability of nascent Akt polypeptide.* EMBO J, 2010. **29**(23): p. 3939-51.

255. Menon, S., et al., *Spatial control of the TSC complex integrates insulin and nutrient regulation of mTORC1 at the lysosome.* Cell, 2014. **156**(4): p. 771-85.

256. Gingras, A.C., et al., *Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism.* Genes Dev, 1999. **13**(11): p. 1422-37.

257. Magnuson, B., B. Ekim, and D.C. Fingar, *Regulation and function of ribosomal protein S6 kinase (S6K) within mTOR signalling networks.* Biochem J, 2012. **441**(1): p. 1-21.

258. Jones, S.W., et al., *A Xenopus ribosomal protein S6 kinase has two apparent kinase domains that are each similar to distinct protein kinases.* Proc Natl Acad Sci U S A, 1988. **85**(10): p. 3377-81.

259. Wang, X., et al., *Regulation of elongation factor 2 kinase by p90(RSK1) and p70 S6 kinase.* EMBO J, 2001. **20**(16): p. 4370-9.

260. Carlberg, U., A. Nilsson, and O. Nygard, *Functional properties of phosphorylated elongation factor 2.* Eur J Biochem, 1990. **191**(3): p. 639-45.

261. Wang, X., et al., *Eukaryotic elongation factor 2 kinase activity is controlled by multiple inputs from oncogenic signaling.* Mol Cell Biol, 2014. **34**(22): p. 4088-103.

262. Faller, W.J., et al., *mTORC1-mediated translational elongation limits intestinal tumour initiation and growth.* Nature, 2015. **517**(7535): p. 497-500.

263. Shima, H., et al., *Disruption of the p70(s6k)/p85(s6k) gene reveals a small mouse phenotype and a new functional S6 kinase.* EMBO J, 1998. **17**(22): p. 6649-59.

264. Fingar, D.C., et al., *Mammalian cell size is controlled by mTOR and its downstream targets S6K1 and 4EBP1/eIF4E.* Genes Dev, 2002. **16**(12): p. 1472-87.

265. Raught, B., et al., *Phosphorylation of eucaryotic translation initiation factor 4B Ser422 is modulated by S6 kinases.* EMBO J, 2004. **23**(8): p. 1761-9.

266. Yang, H.S., et al., *The transformation suppressor Pdcd4 is a novel eukaryotic translation initiation factor 4A binding protein that inhibits translation.* Mol Cell Biol, 2003. **23**(1): p. 26-37.

267. Pause, A., et al., *Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5'-cap function.* Nature, 1994. **371**(6500): p. 762-7.

268. Dowling, R.J., et al., *mTORC1-mediated cell proliferation, but not cell growth, controlled by the 4E-BPs.* Science, 2010. **328**(5982): p. 1172-6.

269. Petroulakis, E., et al., *p53-dependent translational control of senescence and transformation via 4E-BPs.* Cancer Cell, 2009. **16**(5): p. 439-46.

270. Kim, Y.Y., et al., *Eukaryotic initiation factor 4E binding protein family of proteins: sentinels at a translational control checkpoint in lung tumor defense.* Cancer Res, 2009. **69**(21): p. 8455-62.

271. Avdulov, S., et al., *Activation of translation complex eIF4F is essential for the genesis and maintenance of the malignant phenotype in human mammary epithelial cells.* Cancer Cell, 2004. **5**(6): p. 553-63.

272. Carter, P.S., M. Jarquin-Pardo, and A. De Benedetti, *Differential expression of Myc1 and Myc2 isoforms in cells transformed by eIF4E: evidence for internal ribosome repositioning in the human c-myc 5'UTR.* Oncogene, 1999. **18**(30): p. 4326-35.

273. Rosenwald, I.B., et al., *Eukaryotic translation initiation factor 4E regulates expression of cyclin D1 at transcriptional and post-transcriptional levels.* J Biol Chem, 1995. **270**(36): p. 21176-80.

274. Hinnebusch, A.G., I.P. Ivanov, and N. Sonenberg, *Translational control by 5'-untranslated regions of eukaryotic mRNAs.* Science, 2016. **352**(6292): p. 1413-6.

275. Hsieh, A.C., et al., *The translational landscape of mTOR signalling steers cancer initiation and metastasis.* Nature, 2012. **485**(7396): p. 55-61.

276. Thoreen, C.C., et al., *A unifying model for mTORC1-mediated regulation of mRNA translation.* Nature, 2012. **485**(7396): p. 109-13.

277. Miloslavski, R., et al., *Oxygen sufficiency controls TOP mRNA translation via the TSC-Rheb-mTOR pathway in a 4E-BP-independent manner.* J Mol Cell Biol, 2014. **6**(3): p. 255-66.

278. Shama, S., et al., *Overexpression of initiation factor eIF-4E does not relieve the translational repression of ribosomal protein mRNAs in quiescent cells.* Gene Expr, 1995. **4**(4-5): p. 241-52.

279. Tcherkezian, J., et al., *Proteomic analysis of cap-dependent translation identifies LARP1 as a key regulator of 5'TOP mRNA translation.* Genes Dev, 2014. **28**(4): p. 357-71.

280. Lahr, R.M., et al., *La-related protein 1 (LARP1) binds the mRNA cap, blocking eIF4F assembly on TOP mRNAs.* Elife, 2017. **6**.

281. Fonseca, B.D., et al., *La-related Protein 1 (LARP1) Represses Terminal Oligopyrimidine (TOP) mRNA Translation Downstream of mTOR Complex 1 (mTORC1).* J Biol Chem, 2015. **290**(26): p. 15996-6020.

282. Rubio, C.A., et al., *Transcriptome-wide characterization of the eIF4A signature highlights plasticity in translation regulation.* Genome Biol, 2014. **15**(10): p. 476.

283. Ramirez-Valle, F., et al., *eIF4GI links nutrient sensing by mTOR to cell proliferation and inhibition of autophagy.* J Cell Biol, 2008. **181**(2): p. 293-307.

284. Furic, L., et al., *eIF4E phosphorylation promotes tumorigenesis and is associated with prostate cancer progression.* Proc Natl Acad Sci U S A, 2010. **107**(32): p. 14134-9.

285. Ueda, T., et al., *Mnk2 and Mnk1 are essential for constitutive and inducible phosphorylation of eukaryotic initiation factor 4E but not for cell growth or development.* Mol Cell Biol, 2004. **24**(15): p. 6539-49.

286. O'Loghlen, A., et al., *Identification and molecular characterization of Mnk1b, a splice variant of human MAP kinase-interacting kinase Mnk1.* Exp Cell Res, 2004. **299**(2): p. 343-55.

287. Scheper, G.C., et al., *The N and C termini of the splice variants of the human mitogen-activated protein kinase-interacting kinase Mnk2 determine activity and localization.* Mol Cell Biol, 2003. **23**(16): p. 5692-705.

288. Proud, C.G., *Mnks, eIF4E phosphorylation and cancer.* Biochim Biophys Acta, 2015. **1849**(7): p. 766-73.

289. Waskiewicz, A.J., et al., *Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2.* EMBO J, 1997. **16**(8): p. 1909-20.

290. Scheper, G.C., et al., *The mitogen-activated protein kinase signal-integrating kinase Mnk2 is a eukaryotic initiation factor 4E kinase with high levels of basal activity in mammalian cells.* Mol Cell Biol, 2001. **21**(3): p. 743-54.

291. Parra, J.L., M. Buxade, and C.G. Proud, *Features of the catalytic domains and C termini of the MAPK signal-integrating kinases Mnk1 and Mnk2 determine their differing activities and regulatory properties.* J Biol Chem, 2005. **280**(45): p. 37623-33.

292. Wang, X., et al., *The phosphorylation of eukaryotic initiation factor eIF4E in response to phorbol esters, cell stresses, and cytokines is mediated by distinct MAP kinase pathways.* J Biol Chem, 1998. **273**(16): p. 9373-7.

293. Pyronnet, S., et al., *Human eukaryotic translation initiation factor 4G (eIF4G) recruits mnk1 to phosphorylate eIF4E.* EMBO J, 1999. **18**(1): p. 270-9.

294. Topisirovic, I., M. Ruiz-Gutierrez, and K.L. Borden, *Phosphorylation of the eukaryotic translation initiation factor eIF4E contributes to its transformation and mRNA transport activities.* Cancer Res, 2004. **64**(23): p. 8639-42.

295. Ueda, T., et al., *Combined deficiency for MAP kinase-interacting kinase 1 and 2 (Mnk1 and Mnk2) delays tumor development.* Proc Natl Acad Sci U S A, 2010. **107**(32): p. 13984-90.

296. Robichaud, N., et al., *Phosphorylation of eIF4E promotes EMT and metastasis via translational control of SNAIL and MMP-3.* Oncogene, 2015. **34**(16): p. 2032-42.

297. Fan, S., et al., *Phosphorylated eukaryotic translation initiation factor 4 (eIF4E) is elevated in human cancer tissues.* Cancer Biol Ther, 2009. **8**(15): p. 1463-9.

298. Yoshizawa, A., et al., *Overexpression of phospho-eIF4E is associated with survival through AKT pathway in non-small cell lung cancer.* Clin Cancer Res, 2010. **16**(1): p. 240-8.

299. Carter, J.H., et al., *Phosphorylation of eIF4E serine 209 is associated with tumour progression and reduced survival in malignant melanoma.* Br J Cancer, 2016. **114**(4): p. 444-53.

300. Walsh, D. and I. Mohr, *Phosphorylation of eIF4E by Mnk-1 enhances HSV-1 translation and replication in quiescent cells.* Genes Dev, 2004. **18**(6): p. 660-72.

301. Knauf, U., C. Tschopp, and H. Gram, *Negative regulation of protein translation by mitogen-activated protein kinase-interacting kinases 1 and 2.* Mol Cell Biol, 2001. **21**(16): p. 5500-11.

302. Algire, M.A., D. Maag, and J.R. Lorsch, *Pi release from eIF2, not GTP hydrolysis, is the step controlled by start-site selection during eukaryotic translation initiation.* Mol Cell, 2005. **20**(2): p. 251-62.

303. Konieczny, A. and B. Safer, *Purification of the eukaryotic initiation factor 2-eukaryotic initiation factor 2B complex and characterization of its guanine nucleotide exchange activity during protein synthesis initiation.* J Biol Chem, 1983. **258**(5): p. 3402-8.

304. Clemens, M.J., et al., *Phosphorylation inhibits guanine nucleotide exchange on eukaryotic initiation factor 2.* Nature, 1982. **296**(5852): p. 93-5.

305. Kapp, L.D. and J.R. Lorsch, *GTP-dependent recognition of the methionine moiety on initiator tRNA by translation factor eIF2.* J Mol Biol, 2004. **335**(4): p. 923-36.

306. Sood, R., et al., *A mammalian homologue of GCN2 protein kinase important for translational control by phosphorylation of eukaryotic initiation factor-2alpha.* Genetics, 2000. **154**(2): p. 787-801.

307. Han, A.P., et al., *Heme-regulated eIF2alpha kinase (HRI) is required for translational regulation and survival of erythroid precursors in iron deficiency.* EMBO J, 2001. **20**(23): p. 6909-18.

308. Dauber, B. and T. Wolff, *Activation of the Antiviral Kinase PKR and Viral Countermeasures.* Viruses, 2009. **1**(3): p. 523-44.

309. Harding, H.P., Y. Zhang, and D. Ron, *Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase.* Nature, 1999. **397**(6716): p. 271-4.

310. Kaufman, R.J., *Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls.* Genes Dev, 1999. **13**(10): p. 1211-33.

311. Novoa, I., et al., *Stress-induced gene expression requires programmed recovery from translational repression.* EMBO J, 2003. **22**(5): p. 1180-7.

312. Novoa, I., et al., *Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2alpha.* J Cell Biol, 2001. **153**(5): p. 1011-22.

313. Guan, B.J., et al., *A Unique ISR Program Determines Cellular Responses to Chronic Stress.* Mol Cell, 2017. **68**(5): p. 885-900 e6.

314. Gebauer, F., T. Preiss, and M.W. Hentze, *From cis-regulatory elements to complex RNPs and back.* Cold Spring Harb Perspect Biol, 2012. **4**(7): p. a012245.

315. Mukhopadhyay, R., et al., *The GAIT system: a gatekeeper of inflammatory gene expression.* Trends Biochem Sci, 2009. **34**(7): p. 324-31.

316. Sampath, P., et al., *Transcript-selective translational silencing by gamma interferon is directed by a novel structural element in the ceruloplasmin mRNA 3' untranslated region.* Mol Cell Biol, 2003. **23**(5): p. 1509-19.

317. Klebanoff, S.J., *Bactericidal effect of Fe2+, ceruloplasmin, and phosphate.* Arch Biochem Biophys, 1992. **295**(2): p. 302-8.

318. Mazumder, B., et al., *Induction of ceruloplasmin synthesis by IFN-gamma in human monocytic cells.* J Immunol, 1997. **159**(4): p. 1938-44.

319. Ray, P.S. and P.L. Fox, *A post-transcriptional pathway represses monocyte VEGF-A expression and angiogenic activity.* EMBO J, 2007. **26**(14): p. 3360-72.

320. Vyas, K., et al., *Genome-wide polysome profiling reveals an inflammation-responsive posttranscriptional operon in gamma interferon-activated monocytes.* Mol Cell Biol, 2009. **29**(2): p. 458-70.

321. Liepelt, A., et al., *Translation control of TAK1 mRNA by hnRNP K modulates LPS-induced macrophage activation.* RNA, 2014. **20**(6): p. 899-911.

322. Ivshina, M., et al., *CPEB regulation of TAK1 synthesis mediates cytokine production and the inflammatory immune response.* Mol Cell Biol, 2015. **35**(3): p. 610-8.

323. Wang, C., et al., *TAK1 is a ubiquitin-dependent kinase of MKK and IKK.* Nature, 2001. **412**(6844): p. 346-51.

324. Pelletier, J. and N. Sonenberg, *Insertion mutagenesis to increase secondary structure within the 5' noncoding region of a eukaryotic mRNA reduces translational efficiency.* Cell, 1985. **40**(3): p. 515-26.

325. Dhamija, S., et al., *Interleukin-17 (IL-17) and IL-1 activate translation of overlapping sets of mRNAs, including that of the negative regulator of inflammation, MCPIP1.* J Biol Chem, 2013. **288**(26): p. 19250-9.

326. Liang, J., et al., *A novel CCCH-zinc finger protein family regulates proinflammatory activation of macrophages.* J Biol Chem, 2008. **283**(10): p. 6337-46.

327. Iwasaki, H., et al., *The IkappaB kinase complex regulates the stability of cytokine-encoding mRNA induced by TLR-IL-1R by controlling degradation of regnase-1.* Nat Immunol, 2011. **12**(12): p. 1167-75.

328. Huntzinger, E. and E. Izaurralde, *Gene silencing by microRNAs: contributions of translational repression and mRNA decay.* Nat Rev Genet, 2011. **12**(2): p. 99-110.

329. Iwakawa, H.O. and Y. Tomari, *The Functions of MicroRNAs: mRNA Decay and Translational Repression.* Trends Cell Biol, 2015. **25**(11): p. 651-665.

330. Zekri, L., D. Kuzuoglu-Ozturk, and E. Izaurralde, *GW182 proteins cause PABP dissociation from silenced miRNA targets in the absence of deadenylation.* EMBO J, 2013. **32**(7): p. 1052-65.

331. Braun, J.E., et al., *GW182 proteins directly recruit cytoplasmic deadenylase complexes to miRNA targets.* Mol Cell, 2011. **44**(1): p. 120-33.

332. Fukao, A., et al., *MicroRNAs trigger dissociation of eIF4AI and eIF4AII from target mRNAs in humans.* Mol Cell, 2014. **56**(1): p. 79-89.

333. Tsitsiou, E. and M.A. Lindsay, *microRNAs and the immune response.* Curr Opin Pharmacol, 2009. **9**(4): p. 514-20.

334. Taganov, K.D., et al., *NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses.* Proc Natl Acad Sci U S A, 2006. **103**(33): p. 12481-6.

335. Sakurai, A., C.L. Doci, and J.S. Gutkind, *Semaphorin signaling in angiogenesis, lymphangiogenesis and cancer.* Cell Res, 2012. **22**(1): p. 23-32.

336. Eissler, N. and C. Rolny, *The role of immune semaphorins in cancer progression.* Exp Cell Res, 2013. **319**(11): p. 1635-43.

337. Janssen, B.J., et al., *Neuropilins lock secreted semaphorins onto plexins in a ternary signaling complex.* Nat Struct Mol Biol, 2012. **19**(12): p. 1293-9.

338. Casazza, A., et al., *Systemic and targeted delivery of semaphorin 3A inhibits tumor angiogenesis and progression in mouse tumor models.* Arterioscler Thromb Vasc Biol, 2011. **31**(4): p. 741-9.

339. Carrer, A., et al., *Neuropilin-1 identifies a subset of bone marrow Gr1- monocytes that can induce tumor vessel normalization and inhibit tumor growth.* Cancer Res, 2012. **72**(24): p. 6371-81.

340. Casazza, A., et al., *Impeding macrophage entry into hypoxic tumor areas by Sema3A/Nrp1 signaling blockade inhibits angiogenesis and restores antitumor immunity.* Cancer Cell, 2013. **24**(6): p. 695-709.

341. Dejda, A., et al., *Neuropilin-1 mediates myeloid cell chemoattraction and influences retinal neuroimmune crosstalk.* J Clin Invest, 2014. **124**(11): p. 4807-22.

342. Laoui, D., et al., *Tumor hypoxia does not drive differentiation of tumor-associated macrophages but rather fine-tunes the M2-like macrophage population.* Cancer Res, 2014. **74**(1): p. 24-30.

343. Ji, J.D., K.H. Park-Min, and L.B. Ivashkiv, *Expression and function of semaphorin 3A and its receptors in human monocyte-derived macrophages.* Hum Immunol, 2009. **70**(4): p. 211-7.

344. Pulaski, B.A. and S. Ostrand-Rosenberg, *Mouse 4T1 breast tumor model.* Curr Protoc Immunol, 2001. **Chapter 20**: p. Unit 20 2.

345. Bronte, V., et al., *Recommendations for myeloid-derived suppressor cell nomenclature and characterization standards.* Nat Commun, 2016. **7**: p. 12150.

346. Marvel, D. and D.I. Gabrilovich, *Myeloid-derived suppressor cells in the tumor microenvironment: expect the unexpected.* J Clin Invest, 2015. **125**(9): p. 3356-64.

347. Dolcetti, L., et al., *Hierarchy of immunosuppressive strength among myeloid-derived suppressor cell subsets is determined by GM-CSF.* Eur J Immunol, 2010. **40**(1): p. 22-35.

348. Cheng, P., et al., *Inhibition of dendritic cell differentiation and accumulation of myeloid-derived suppressor cells in cancer is regulated by S100A9 protein.* J Exp Med, 2008. **205**(10): p. 2235-49.

349. Sinha, P., et al., *Proinflammatory S100 proteins regulate the accumulation of myeloid-derived suppressor cells.* J Immunol, 2008. **181**(7): p. 4666-75.

350. Mayadas, T.N., X. Cullere, and C.A. Lowell, *The multifaceted functions of neutrophils.* Annu Rev Pathol, 2014. **9**: p. 181-218.

351. Piccirillo, C.A., et al., *Translational control of immune responses: from transcripts to translatomes.* Nat Immunol, 2014. **15**(6): p. 503-11.

352. Lin, E.Y., et al., *Progression to malignancy in the polyoma middle T oncoprotein mouse breast cancer model provides a reliable model for human diseases.* Am J Pathol, 2003. **163**(5): p. 2113-26.

353. Lin, E.Y., et al., *Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy.* J Exp Med, 2001. **193**(6): p. 727-40.

354. Luo, W., et al., *GAGE: generally applicable gene set enrichment for pathway analysis.* BMC Bioinformatics, 2009. **10**: p. 161.

355. Covarrubias, A.J., H.I. Aksoylar, and T. Horng, *Control of macrophage metabolism and activation by mTOR and Akt signaling.* Semin Immunol, 2015. **27**(4): p. 286-96.

356. Geeraerts, X., et al., *Macrophage Metabolism As Therapeutic Target for Cancer, Atherosclerosis, and Obesity.* Front Immunol, 2017. **8**: p. 289.

357. Su, X., et al., *Interferon-gamma regulates cellular metabolism and mRNA translation to potentiate macrophage activation.* Nat Immunol, 2015. **16**(8): p. 838-849.

358. Bateman, R.L., et al., *Human carbonyl reductase 1 is an S-nitrosoglutathione reductase.* J Biol Chem, 2008. **283**(51): p. 35756-62.

359. Pompella, A., et al., *The changing faces of glutathione, a cellular protagonist.* Biochem Pharmacol, 2003. **66**(8): p. 1499-503.

360. Cummins, T.D., et al., *Quantitative mass spectrometry of diabetic kidney tubules identifies GRAP as a novel regulator of TGF-beta signaling.* Biochim Biophys Acta, 2010. **1804**(4): p. 653-61.

361. Konicek, B.W., et al., *Therapeutic inhibition of MAP kinase interacting kinase blocks eukaryotic initiation factor 4E phosphorylation and suppresses outgrowth of experimental lung metastases.* Cancer Res, 2011. **71**(5): p. 1849-57.

362. Murray, P.J., *The JAK-STAT signaling pathway: input and output integration.* J Immunol, 2007. **178**(5): p. 2623-9.

363. Welch, J.S., et al., *TH2 cytokines and allergic challenge induce Ym1 expression in macrophages by a STAT6-dependent mechanism.* J Biol Chem, 2002. **277**(45): p. 42821-9.

364. Loke, P., et al., *IL-4 dependent alternatively-activated macrophages have a distinctive in vivo gene expression phenotype.* BMC Immunol, 2002. **3**: p. 7.

365. Liao, X., et al., *Kruppel-like factor 4 regulates macrophage polarization.* J Clin Invest, 2011. **121**(7): p. 2736-49.

366. Kudlacz, E., et al., *The novel JAK-3 inhibitor CP-690550 is a potent immunosuppressive agent in various murine models.* Am J Transplant, 2004. **4**(1): p. 51-7.

367. Murray, P.J., *Understanding and exploiting the endogenous interleukin-10/STAT3-mediated anti-inflammatory response.* Curr Opin Pharmacol, 2006. **6**(4): p. 379-86.

368. Hutchins, A.P., D. Diez, and D. Miranda-Saavedra, *The IL-10/STAT3-mediated anti-inflammatory response: recent developments and future challenges.* Brief Funct Genomics, 2013. **12**(6): p. 489-98.

369. Lang, R., et al., *Shaping gene expression in activated and resting primary macrophages by IL-10.* J Immunol, 2002. **169**(5): p. 2253-63.

370. Yan, D., et al., *STAT3 and STAT6 Signaling Pathways Synergize to Promote Cathepsin Secretion from Macrophages via IRE1alpha Activation.* Cell Rep, 2016. **16**(11): p. 2914-2927.

371. Buxade, M., et al., *The Mnks are novel components in the control of TNF alpha biosynthesis and phosphorylate and regulate hnRNP A1.* Immunity, 2005. **23**(2): p. 177-89.

372. Buxade, M., et al., *The PSF.p54nrb complex is a novel Mnk substrate that binds the mRNA for tumor necrosis factor alpha.* J Biol Chem, 2008. **283**(1): p. 57-65.

373. Shav-Tal, Y. and D. Zipori, *PSF and p54(nrb)/NonO--multi-functional nuclear proteins.* FEBS Lett, 2002. **531**(2): p. 109-14.

374. Cabrita, M.A. and G. Christofori, *Sprouty proteins, masterminds of receptor tyrosine kinase signaling.* Angiogenesis, 2008. **11**(1): p. 53-62.

375. DaSilva, J., et al., *Regulation of sprouty stability by Mnk1-dependent phosphorylation.* Mol Cell Biol, 2006. **26**(5): p. 1898-907.

376. Edwin, F., K. Anderson, and T.B. Patel, *HECT domain-containing E3 ubiquitin ligase Nedd4 interacts with and ubiquitinates Sprouty2.* J Biol Chem, 2010. **285**(1): p. 255-64.

377. Hu, S.I., et al., *MNK2 inhibits eIF4G activation through a pathway involving serine-arginine-rich protein kinase in skeletal muscle.* Sci Signal, 2012. **5**(211): p. ra14.

378. Vary, T.C. and C.J. Lynch, *Nutrient signaling components controlling protein synthesis in striated muscle.* J Nutr, 2007. **137**(8): p. 1835-43.

379. Korneeva, N.L., et al., *Inhibition of Mitogen-activated Protein Kinase (MAPK)-interacting Kinase (MNK) Preferentially Affects Translation of mRNAs Containing Both a 5'-Terminal Cap and Hairpin.* J Biol Chem, 2016. **291**(7): p. 3455-67.

380. Robichaud, N., et al., *Translational control in the tumor microenvironment promotes lung metastasis: Phosphorylation of eIF4E in neutrophils.* Proc Natl Acad Sci U S A, 2018. **115**(10): p. E2202-E2209.

381. Rabe, K.F., et al., *Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary.* Am J Respir Crit Care Med, 2007. **176**(6): p. 532-55.

382. Mouronte-Roibas, C., et al., *COPD, emphysema and the onset of lung cancer. A systematic review.* Cancer Lett, 2016. **382**(2): p. 240-244.

383. Tockman, M.S., et al., *Airways obstruction and the risk for lung cancer.* Ann Intern Med, 1987. **106**(4): p. 512-8.

384. King, P.T., *Inflammation in chronic obstructive pulmonary disease and its role in cardiovascular disease and lung cancer.* Clin Transl Med, 2015. **4**(1): p. 68.

385. Hohberger, L.A., et al., *Correlation of regional emphysema and lung cancer: a lung tissue research consortium-based study.* J Thorac Oncol, 2014. **9**(5): p. 639-45.

386. Hernandez, M., et al., *Impact of using the new GOLD classification on the distribution of COPD severity in clinical practice.* Int J Chron Obstruct Pulmon Dis, 2018. **13**: p. 351-356.

387. Wiese, S., et al., *Protein labeling by iTRAQ: a new tool for quantitative mass spectrometry in proteome research.* Proteomics, 2007. **7**(3): p. 340-50.

388. Larsson, O., et al., *Distinct perturbation of the translatome by the antidiabetic drug metformin.* Proc Natl Acad Sci U S A, 2012. **109**(23): p. 8977-82.

389. Parker, M.W., et al., *Fibrotic extracellular matrix activates a profibrotic positive feedback loop.* J Clin Invest, 2014. **124**(4): p. 1622-35.

390. Bronisz, A., et al., *Reprogramming of the tumour microenvironment by stromal PTEN-regulated miR-320.* Nat Cell Biol, 2011. **14**(2): p. 159-67.

391. Coppe, J.P., et al., *Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor.* PLoS Biol, 2008. **6**(12): p. 2853-68.

392. Torres, S., et al., *Proteome profiling of cancer-associated fibroblasts identifies novel proinflammatory signatures and prognostic markers for colorectal cancer.* Clin Cancer Res, 2013. **19**(21): p. 6006-19.

393. De Boeck, A., et al., *Differential secretome analysis of cancer-associated fibroblasts and bone marrow-derived precursors to identify microenvironmental regulators of colon cancer progression.* Proteomics, 2013. **13**(2): p. 379-88.

394. Mombaerts, P., et al., *RAG-1-deficient mice have no mature B and T lymphocytes.* Cell, 1992. **68**(5): p. 869-77.

395. Shinkai, Y., et al., *RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement.* Cell, 1992. **68**(5): p. 855-67.

396. Shankaran, V., et al., *IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity.* Nature, 2001. **410**(6832): p. 1107-11.

397. Thompson, L.H. and D. Schild, *Recombinational DNA repair and human disease.* Mutat Res, 2002. **509**(1-2): p. 49-78.

398. Lieber, M.R., *The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway.* Annu Rev Biochem, 2010. **79**: p. 181-211.

399. Pannunzio, N.R., G. Watanabe, and M.R. Lieber, *Nonhomologous DNA end-joining for repair of DNA double-strand breaks.* J Biol Chem, 2018. **293**(27): p. 10512-10523.

400. de Miranda, N.F., A. Bjorkman, and Q. Pan-Hammarstrom, *DNA repair: the link between primary immunodeficiency and cancer.* Ann N Y Acad Sci, 2011. **1246**: p. 50-63.

401. Moynahan, M.E., et al., *Brca1 controls homology-directed DNA repair.* Mol Cell, 1999. **4**(4): p. 511-8.

402. Miki, Y., et al., *A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1.* Science, 1994. **266**(5182): p. 66-71.

403. Xu, C.F. and E. Solomon, *Mutations of the BRCA1 gene in human cancer.* Semin Cancer Biol, 1996. **7**(1): p. 33-40.

404. Zhong, Q., et al., *Deficient nonhomologous end-joining activity in cell-free extracts from Brca1-null fibroblasts.* Cancer Res, 2002. **62**(14): p. 3966-70.

405. Wang, H., et al., *Nonhomologous end-joining of ionizing radiation-induced DNA double-stranded breaks in human tumor cells deficient in BRCA1 or BRCA2.* Cancer Res, 2001. **61**(1): p. 270-7.

406. Pan, Q., et al., *Regulation of switching and production of IgA in human B cells in donors with duplicated alpha1 genes.* Eur J Immunol, 2001. **31**(12): p. 3622-30.

407. Pan, Q., et al., *Alternative end joining during switch recombination in patients with ataxia-telangiectasia.* Eur J Immunol, 2002. **32**(5): p. 1300-8.

408. Lee-Theilen, M., et al., *CtIP promotes microhomology-mediated alternative end joining during class-switch recombination.* Nat Struct Mol Biol, 2011. **18**(1): p. 75-9.

409. Dohrn, L., et al., *BRCA1-mediated repression of mutagenic end-joining of DNA double-strand breaks requires complex formation with BACH1.* Biochem J, 2012. **441**(3): p. 919-26.

410. Oh, M., et al., *BRCA1 and BRCA2 Gene Mutations and Colorectal Cancer Risk: Systematic Review and Meta-analysis.* J Natl Cancer Inst, 2018.

411. Ludwig, T., et al., *Tumorigenesis in mice carrying a truncating Brca1 mutation.* Genes Dev, 2001. **15**(10): p. 1188-93.

412. Friedenson, B., *The BRCA1/2 pathway prevents hematologic cancers in addition to breast and ovarian cancers.* BMC Cancer, 2007. **7**: p. 152.

413. de Miranda, N.F., et al., *DNA repair genes are selectively mutated in diffuse large B cell lymphomas.* J Exp Med, 2013. **210**(9): p. 1729-42.

414. Zhu, Y., et al., *CSF1/CSF1R blockade reprograms tumor-infiltrating macrophages and improves response to T-cell checkpoint immunotherapy in pancreatic cancer models.* Cancer Res, 2014. **74**(18): p. 5057-69.