DEPARTMENT OF LABORATORY MEDICINE

Karolinska Institutet, Stockholm, Sweden

MECHANISMS OF MULTICELLULAR DRUG RESISTANCE AND NOVEL APPROACHES FOR TARGETED THERAPY IN CANCER

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Karolinska Institutet

Stockholm 2019

Published by Karolinska Institutet
Printed by E-print AB 2019

ISBN 978-91-7831-318-1

Mechanisms of Multicellular Drug Resistance and Novel Approaches for Targeted Therapy in Cancer
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Dysregulated gene expression, due to genetic and epigenetic aberrations, as well as cancer cell-stroma interactions underlie both tumorigenesis and resistance to anti-cancer therapy. Although governed by common mechanisms, these features are unique to each individual tumor and therefore a personalized treatment regimen needs to be composed for each patient. At the same time, novel therapeutic targets for cancer treatment are warranted to be able to match these individual variations, along with identification of biomarkers for their effective use. STAT3 is a transcriptional regulator involved in both cancer development and therapy resistance. In this thesis we have explored the role of STAT3 as well as interferon-related gene signature in multicellular drug resistance. The second part of this thesis explores the use of novel siRNN prodrugs for silencing of Plk1, a cell cycle kinase, in acute lymphoblastic leukemia (ALL) patient samples.

In **Paper I** we used multicellular spheroids (MCS) as a model to study genes associated with drug resistance. A subset of interferon-stimulated genes (ISGs), that belong the interferon-related DNA damage resistance signature (IRDS), was enriched in MCS compared to monolayer culture. We found that a panel of IRDS genes was expressed in cell lines of different origin when grown as MCS or as confluent monolayer culture. The induction of these ISGs depended on increased expression of IRF9 and STAT2. Overexpression of IRF9 alone was sufficient to induce the ISGs and confer resistance to chemotherapeutic agents. In **Paper II** STAT3 was found to be activated in MCS, downstream of gp130-JAK signaling. STAT3 activity was required for the induced expression of IRF9 and the panel of IRDS genes in MCS. We identified a potential STAT3 binding site in the IRF9 promoter and confirmed that STAT3 was enriched at this site in MCS compared to non-confluent monolayer culture. Together, our data suggest that STAT3 is activated in conditions of high cellular density and drives the transcription of IRF9, which in turn induces the expression of a subset of ISGs that confer resistance to chemotherapeutic drugs.

In **Paper III** we attempted to identify novel STAT3-interacting proteins that affect transcription of STAT3-target genes. In order to achieve this, we combined chromatin immunoprecipitation using anti-STAT3 antibodies with biotinylation and pull down of DNA, and finally mass spectrometry to identify STAT3 interactors. Among the hits were previously described STAT3-binding proteins, as well as new potential interacting partners.

In **Paper IV** we analyzed the effect of novel self-delivering siRNN prodrugs, targeting cell cycle kinase Plk1, in pediatric ALL. We used CD3/IL-2 to stimulate ALL patient samples in order to induce proliferation and Plk1 expression. Our data demonstrates that the siRNN prodrugs successfully enter cycling ALL cells and induce RNAi mediated knockdown of Plk1, which leads to cell cycle arrest and apoptosis.

LIST OF SCIENTIFIC PAPERS

I. Kolosenko I, Fryknäs M, Forsberg S, Johnsson P, Cheon H, Holvey-Bates EG, **Edsbäcker E**, Pellegrini P, Rassoolzadeh H, Brnjic S, Larsson R, Stark GR, Grandér D, Linder S, Pokrovskaja Tamm K*, and De Milito A*. Cell Crowding induces interferon regulatory factor 9, which confers resistance to chemotherapeutic drugs. *International journal of cancer*. 136, E51-61 (2015).

II. **Edsbäcker E**, Serviss JT, Kolosenko I, Palm-Apergi C, De Milito A, and Pokrovskaja Tamm K. STAT3 is activated in multicellular spheroids of colon carcinoma cells and mediates expression of IRF9 and interferon stimulated genes. *Accepted for publication in Scientific Reports.*

III. **Edsbäcker E***, Kolosenko I*, Busker S, Lerner M, Grandér D, Palm-Apergi C, Lehtiö J, Johansson H, and Pokrovskaja Tamm K. STAT3 interacting proteins identified in a complex with DNA affect expression of STAT3-dependent genes. *Manuscript*.

IV. Kolosenko I*, **Edsbäcker E***, Björklund A-C*, Hamil AS, Goroshchuk O, Grandér D, Dowdy SF, and Palm-Apergi C. RNAi prodrugs targeting Plk1 induce specific gene silencing in primary cells from pediatric T-acute lymphoblastic leukemia patients. *Journal of controlled release*. 261, 199-206 (2017).

*Equal contribution

Publication not included in the thesis:

Virkki MT, Agrawal N, **Edsbäcker E**, Cristobal S, Elofsson A, and Kauko A. Folding of Aquaporin 1: multiple evidence that helix 3 can shift out of the membrane core. *Protein Science*, 23, 981-992 (2014).

CONTENTS

LIST OF ABBREVIATIONS

2D	Two-dimensional
3D	Three-dimensional
5-FU	Fluorouracil
Ago2	Argonaute 2
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
APC	Adenomatous polyposis coli
APRE	Acute-phase response element
APRF	Acute-phase response factor
ATP	Adenosine triphosphate
AxV	Annexin V
BCL2	B-cell lymphoma 2
BMMC	Bone marrow mononuclear cell
CBX3	Chromobox 3
CCL2	C-C motif chemokine ligand 2
CDE/CHR	Cell cycle-dependent element/cell cycle genes homology region
CDK	Cyclin-dependent kinase
ChIP	Chromatin Immunoprecipitation
CM	Condition medium
CML	Chronic myelogenous leukemia
CMV	Cytomegalovirus
CRC	Colorectal cancer
CTEN	C-terminal tensin-like
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
EGF	Epidermal growth factor
EMT	Epithelial-mesenchymal transition
EpCAM	Epithelial cell adhesion molecule
FAP	Familial adenomatous polyposis
FGF	Fibroblast growth factor
GAS	Gamma-IFN-activation site
gp130	Glycoprotein 130

GSEA	Gene set enrichment analysis
GTSE1	G2 and S-phase-expressed-1
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HIF1	Hypoxia-inducible factor
HNPCC	Hereditary nonpolyposis colorectal cancer
HSP	Heat shock protein
IFI	Interferon alpha-induced protein
IFITM1	Interferon-induced transmembrane protein 1
IFN	Interferon
IFNAR	Interferon-α receptor
IFNGR	Interferon-γ receptor
IL	Interleukin
IP	Immunoprecipitation
IRF	Interferon regulatory factor
IRDS	Interferon-related DNA damage resistance signature
ISGF3	Interferon-stimulated gene factor 3
ISGs	Interferon-stimulated genes
ISRE	Interferon-stimulated response element
JAK	Janus kinase
MCR	Multicellular resistance
MCS	Multicellular spheroids
MDR	Multidrug resistance
MHC	Major histocompability complex
MLH1	MutL homolog 1
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
MS	Mass spectrometry
MYPT1	Myosin phosphatase target subunit 1
NF-κB	Nuclear factor-κB
NHDF	Normal human dermal fibroblasts
NK-cell	Natural killer cells
OAS1	2'-5'-oligoadenylate synthetase 1
OSM	Oncostatin M
PARP	Poly (ADP ribose) polymerase

PBD	Polo-box domain
PD-L1	Programmed death-ligand 1
pH3	Phosphorylated histone H3
PI	Propidium Iodide
PLK	Polo-like kinase
PP1	Protein phosphatase 1
P-STAT	Phosphorylated STAT
PTD	Peptide transduction domain
Rb	Retinoblastoma protein
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Reactive oxygen species
RT-qPCR	Real-time quantitative polymerase chain reaction
SCC	Squamous cell carcinoma
SH2-domain	Src homology 2-domain
SIE	STAT-inducible element
siRNA	Small interfering RNA
siRNN	Short interfering ribonucleic neutral
SOCS	Suppressor of cytokine signaling
ssDNA	Single stranded DNA
SSP	Sessile serrated polyps
STAT	Signal transducer and activator of transcription
TGF	Transforming growth factor
THRAP3	Thyroid hormone receptor-associated protein 3
TNF	Tumor necrosis factor
TOPORS	Topo-1 binding protein
TYK2	Tyrosine-protein kinase 2
U-ISGF3	Unphosphorylated ISGF3
U-STAT	Unphosphorylated STAT
VEGF	Vascular endothelial growth factor

1 INTRODUCTION

Cancer is one of the major causes of death worldwide. Despite the progress in the oncology field over the past decades, incidence and cancer deaths are on the rise. The World Health Organization (WHO) predicts global cancer deaths to increase by 45% between 2008 and 2030. Longevity and an increased exposure to risk factors are believed to contribute to this trend.

Carcinogenesis is a multistep process where normal cells acquire malignant potential over time through genetic and epigenetic changes. Cancer is a heterogeneous disease, and a collective term for over 100 different malignancies. Tumors are broadly divided into subgroups based on the site of origin. Irrespective of the origin, most malignant tumors share a number of common features (Figure 1), which were summarized by Hannahan and Wienberg, and are referred to as the hallmarks of cancer. The hallmarks include the ability to sustain proliferation, evade growth suppressors, resist cell death, enable replicative immortality, induce angiogenesis, activate invasion and metastasis, evade immune destruction, and reprogram energy metabolism (Hanahan and Weinberg 2000, 2011). Two additional hallmarks have been described; genomic instability, and tumor promoting inflammation (Hanahan and Weinberg 2011). These features contribute to tumorigenesis by enabling the acquisition of the other hallmarks.

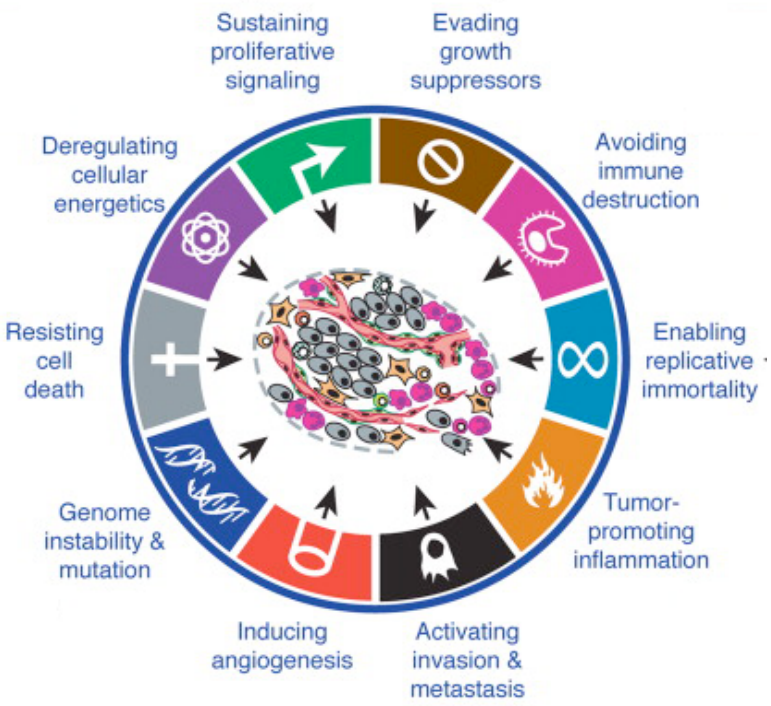

Figure 1. The hallmarks of cancer. Overview of common characteristics of malignant neoplasms. Adapted from Hanahan, D., Weinberg R.A., 2011. *Hallmarks of cancer: the next generation*. Cell 144, 646-674. Reprinted with permission from Elsevier.

Resistance to chemo- and radiotherapy is a major hurdle in the clinical management of cancer patients. As our understanding of the molecular mechanisms of cancer has increased, drug development has shifted towards a new therapeutic approach where the drugs interfere with specific proteins or pathways that are altered in tumors. Targeted therapies confer cytotoxicity in a more-cell specific manner and are generally well tolerated compared to conventional chemotherapeutic drugs. There are targeted agents that have had great success in improving progression free and overall survival for certain cancers, for example Imatinib in the treatment of chronic myleogenous leukemia (CML) (Palumbo et al. 2013). However, as with chemotherapeutic agents, the effect of a targeted drug can be compromised by resistance through genetic instability and/or tumor heterogeneity as well as de novo mutations in the target protein or its signaling pathway. The success of cancer therapy thus depends on further genetic characterization of tumors and validation of novel targets, as well as understanding of the mechanisms of resistance and identification of predictive markers in order to determine the optimal combination of drugs for each individual patient.

1.1 INTERFERON SIGNALING

1.1.1 Interferons

Interferons (IFNs) are a group of cytokines secreted by cells in response to danger-sensing patterns and exert antiviral, antiproliferative and immunomodulatory effects. The IFN family consists of type I, II and III. Type I includes IFN$\alpha_{1\text{-}12}$, IFNβ, IFNε, IFNκ and IFNω. IFNγ is the only type II IFN and type III consists of IFN$\lambda_{1\text{-}4}$.

1.1.2 Interferon-induced signaling

Type I IFNs are secreted by most cell types in the body in response to infection. Non-immune cells predominantly produce IFNβ while immune cells, such as dendritic cells, produce IFNα (Ivashkiv and Donlin 2014). Type I IFNs signal through the IFNα receptor, a hetero-dimeric transmembrane receptor consisting of the subunits IFNAR1 and IFNAR2. Binding of type I IFN to the receptor triggers activation of the receptor-associated Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2) which in turn phosphorylate signal transducers and activator of transcription (STATs). Phosphorylated STATs form homo- or hetero-dimers and translocate to the nucleus where they induce transcription of interferon-stimulated genes (ISGs) (Platanias 2005). Type I IFN signaling induce phosphorylation of different STAT proteins (STAT1-5), depending on the nature of the stimuli and cellular context. Canonical type I IFN signaling results in phosphorylation and dimerization of STAT1 and STAT2 that together with interferon regulatory factor (IRF) 9 form a complex termed interferon-stimulated gene factor 3 (ISGF3). The ISGF3 complex binds to a consensus DNA sequence called interferon-stimulated response element (ISRE) in the promoters of ISGs inducing their transcription (Figure 2). In addition, type I IFN signaling can also give rise to homo- and hetero-dimers of phosphorylated STATs that induce a different set of ISGs through the gamma-interferon-activation site (GAS) element (Ivashkiv and Donlin 2014). Type I IFNs have also been shown to induce other signaling pathways such as PI3K and RAS/MAPK (Uddin et al. 1995; Li et al. 2004).

IFNγ binds to the type II IFN receptor, which consists of subunits IFGR1 and IFGR2 whose cytoplasmic domains are associated with JAK1 and JAK2. In contrast to type I IFNs, IFNγ expression is induced mainly by mitogens or cytokines, for example IL-12 and IL-18, which are typically expressed by T- and NK-cells (Parker, Rautela, and Hertzog 2016). The main transcription factor induced by IFNγ signaling is the STAT1 homodimer that induce transcription of ISGs controlled by the GAS element (Figure 2) (Darnell 1997). There is an overlap between IFN type I and II induced genes since both can trigger transcription through the GAS element, furthermore, there are ISG that contain both GAS and ISRE in their promoters (Platanias 2005). There are about 2000 identified ISGs whose induction depends on cell type, dose, duration, and nature of the stimuli (Hertzog, Forster, and Samarajiwa 2011). Certain ISGs can also be transcriptionally regulated by other cytokines such as IL-6 through phosphorylation and homo-dimerization of STAT3, a complex that can induce their transcription through the GAS element (Peter C. Heinrich et al. 2003).

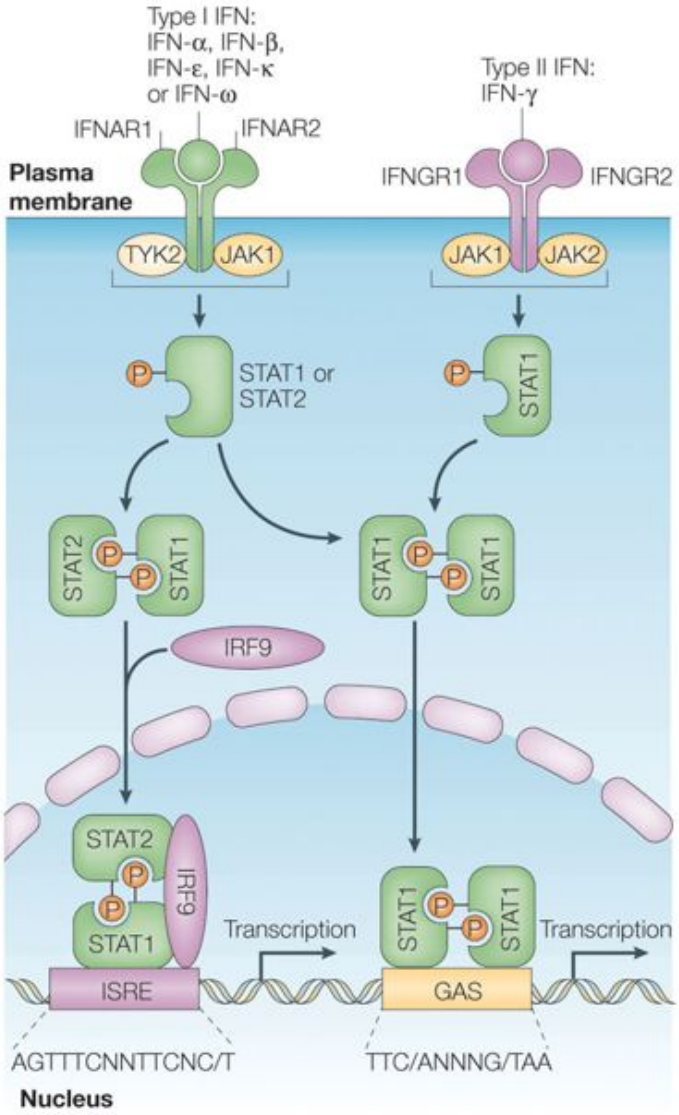

Figure 2. Canonical IFN signaling. Planatias, L.C., 2005. *Mechanisms of type-I-and type-II-interferon-mediated signalling.* Nature Reviews Immunology. Reprinted with permission from Springer Nature.

1.1.3 Interferon signaling in cancer

In addition to the antiviral effect, anti-proliferative and tumor-suppressive roles of IFNs have also been described. The anti-tumor effect of IFNs was discovered decades ago, when administration of IFNα was shown to inhibit tumor growth in mice (Gresser, Maury, and Brouty-Boyé 1972). A number of studies have since then demonstrated the important anti-tumor functions of IFN signaling. Mice lacking a functional IFNGR or STAT1 develop tumors faster and to a greater extent when subjected to a carcinogen, compared with wild-type mice (Kaplan et al. 1998). Type I IFNs have been shown to exert a direct anti-proliferative effect in cancer cell lines by prolonging the phases of the cell cycle and upregulating inhibitors of cyclin dependent kinases, for example p21 (Balkwill, Watling, and Taylor-Papadimitriou 1978; Hobeika, Subramaniam, and Johnson 1997). IFNs have also been demonstrated to induce apoptosis in cancer cell lines, both through the death-receptor mediated pathway and the mitochondrial pathway (Thyrell et al. 2002; Bernardo et al. 2013). In addition, type I IFNs regulate the activity of immune cells and are an important part of the anti-tumor immune response. For example, IFN signaling can lead to upregulation of major histocompatibility complex (MHC) class I, which enhances tumor antigen presentation (Boyer et al. 1989) and activates dendritic cells to cross-present tumor antigen to CD8+ T-cells, thus inducing tumor specific cytotoxic T-cell responses (Schiavoni, Mattei, and Gabriele 2013). Furthermore, IFNs have been reported to suppress the proliferation of T-helper cells and myeloid-derived suppressor cells, both which contribute to the suppression of cytotoxic T-cell activity (Pace et al. 2010; Zoglmeier et al. 2011). Natural killer (NK) cells play an important role in anti-tumor immunity and are regulated by type I IFNs (Swann et al. 2007).

Resistance to the anti-tumor effects of IFNs can be achieved by deletion of IFN genes, downregulation of receptors, or silencing of important mediators of the IFN signaling pathway (Parker, Rautela, and Hertzog 2016). Furthermore, mouse models of breast cancer with genetically impaired type I IFN signaling display an accelerated development of bone metastasis (Rautela et al. 2015). Bidwell et al. identified a specific signature of ISGs, all target genes of IRF7, that were downregulated in bone metastasis compared to the primary tumors in a mouse model of breast cancer (Bidwell et al. 2012). Enforcing expression of IRF7 in the tumor cells resulted in a drastic reduction of bone metastasis and enhanced IFN signaling. Moreover, high expression of the identified IRF7-target genes in primary tumors from breast cancer patients correlated to bone metastasis-free survival. The authors suggest that downregulation of this pathway enables metastasis by restricting immune cell activation (Bidwell et al. 2012).

1.1.3.1 IFN-related DNA damage resistance signature

The above-mentioned studies demonstrate the anti-tumor effects of IFN signaling. Paradoxically, Khodarev et al. published a study in 2004 linking the IFN-signaling pathway to resistance to radiation therapy in a human tumor xenograft model. A radiosensitive squamous cell carcinoma (SCC) xenograft was made resistant by subjection to repeated cycles of radiation. STAT1 and a subset of 31 IFN-stimulated genes were found to be

upregulated in the resistant tumors compared to the parental (N. N. Khodarev et al. 2004). This set of genes, termed IFN-related DNA damage resistance signature (IRDS), was later found to be induced in various types of cancer cell lines in response to chemo-and radiotherapy (N. N. Khodarev et al. 2007; Tsai et al. 2007). The signature has also been identified in samples from patients with glioma, head and neck, prostate, lung and breast cancer and can be correlated to resistance to chemo- and radiotherapy in the latter (Weichselbaum et al. 2008; Duarte et al. 2012).

STAT1 has been suggested to be the main driver of IRDS expression and resistance. Overexpression of STAT1 in a SCC cell line conferred resistance to irradiation, while suppression of STAT1 expression resulted in increased sensitivity (N. N. Khodarev et al. 2007). STAT1 expression has also been shown to be increased in a docetaxel-resistant prostate cancer cell line compared to the sensitive parental cells. Knockdown of STAT1 re-sensitized the cells to docetaxel (Patterson et al. 2006). Similar observations were made in cell lines of different solid cancers (Roberts et al. 2005; Luszczek et al. 2010).

Erdal et al. have suggested a possible mechanism for the induction of IRDS by chemo- and radio-therapy. DNA damage inflicted by the treatment causes single stranded DNA (ssDNA) to be released in the cytosol, which triggers the activation of anti-viral IFN signaling. There was a positive correlation between the abundance of ssDNA in the cytosol and phosphorylation of STAT1. Knockdown of BLM and EXO1, factors involved in end resection upon double strand breaks, reduced the amount of both cytosolic ssDNA and pSTAT1 by irradiation. In addition, knockdown of Trex1, which degrades cytosolic ssDNA, increased the expression of ISGs following irradiation treatment. Lastly, low mRNA expression of Trex1 and high expression of BLM and EXO1 in breast tumors correlates with poor prognosis. Thus, BLM and EXO1 are potential targets for circumventing IRDS induced by irradiation (Erdal et al. 2017).

Infliction of DNA damage by drugs or irradiation is not the only way to induce the IRDS. A study by Cheon et al. showed that chronic stimulation by low doses of IFNβ results in rising levels of unphosphorylated STAT1, STAT2 and IRF9. They form a complex termed unphosphorylated-ISGF3 (U-ISGF3), which is capable of sustaining expression of specific antiviral genes for at least 12 days, much longer than the classical ISGF3. The limited set of ISGs induced by U-ISGF3 closely resembles the IRDS. The same study also showed a correlation between STAT1, STAT2 and IRF9 levels with decreased sensitivity to DNA damage. The authors propose that the function of the ISGs induced by U-ISGF3 is to provide a prolonged antiviral state without the pro-apoptotic effects seen with ISGF3-induced genes (Cheon et al. 2013). The function of the individual IRDS genes in cancer and how they contribute to DNA damage resistance is still poorly understood.

1.2 SIGNAL TRANSDUCERS AND ACTIVATORS OF TRANSCRIPTION

The family of signal transducers and activators of transcription (STAT) consists of seven members in humans, STAT1-4, STAT5A and STAT5B, and STAT6. They were discovered in the 1990s as factors that mediate cellular responses to cytokines and growth factors (Sadowski et al. 1993). Structurally, all STAT proteins share the same five domains; an

amino-terminal domain, a coil-coiled domain, an Src homology 2 (SH2)-domain, and a carboxy-terminal transactivation domain (Figure 3). The transactivation domain contains specific amino acids whose phosphorylation determines the transcriptional activity of the STATs. Phosphorylation of tyrosine induces dimerization in all STATs, while phosphorylation of serine increases the transcriptional activity of STAT1, STAT3, STAT5A, and STAT5B (Lim and Cao 2006). Unphosphorylated STATs reside in the cytoplasm as monomers or inactive dimers until activated. Activation of STATs occurs upon binding of a cytokine or growth factor, to its receptor, which leads to trans-phosphorylation of receptor-associated JAKs. The JAKs in turn phosphorylate tyrosine residues on the cytoplasmic part of the receptor, leading to the recruitment of STATs via interaction with their SH2-domain. JAKs phosphorylate STATs on a tyrosine residue in the transactivation domain, which triggers homo- or heterodimerization and translocation to the nucleus. In the nucleus, STAT dimers bind to specific response elements in the promoters of genes to induce or repress transcription (Rawlings, Rosler, and Harrison 2004). STATs released from DNA are inactivated by nuclear phosphatases, for example TC45, and transported back to the cytoplasm (Reich 2013). STAT activity is regulated by cytoplasmic phosphatases and suppressors of cytokine signaling (SOCS), whose expression is induced by STATs, thus creating a negative feedback mechanism (Alexander 2002).

Despite the structural similarities, each of the STATs has distinct, and sometimes opposing, functions. The specific roles of STAT1, STAT2 and STAT3 in cancer are briefly described below.

1.2.1 STAT1

STAT1 is an important mediator of IFN signaling and regulates multiple cellular functions, such as apoptosis, differentiation, and stimulation of the immune system. STAT1 deficient mice are highly susceptible to infections and are prone to spontaneous tumor development (Leopold Wager et al. 2014; Chan et al. 2012). Activation of STAT1 by tyrosine phosphorylation leads to the formation of two transcriptional complexes; the STAT1 homodimer, which binds to the GAS element, or ISGF3, which binds to the ISRE sequence. Other complexes with STAT1 have been described, for example the STAT1-STAT2 or STAT1-STAT3 heterodimers, although the functions of these complexes are not well understood (Delgoffe and Vignali 2013). STAT1 has been demonstrated to exhibit both tumor-suppressive and tumor-promoting functions. This controversy may in part be explained by the diverse functions of STAT1 and the cell- and context-dependent outcome of STAT1 activation. Expression of STAT1 has been correlated with good prognosis in a number of solid malignancies, including breast cancer (Meissl et al. 2015). However, other studies in breast cancer have reported a correlation between STAT1 expression and poor prognosis (Widschwendter et al. 2002; N. Khodarev et al. 2010). These discrepancies could in part stem from differences in the parameters used, whether mRNA, total protein, or phosphorylated STAT1 (P-STAT1) was analyzed.

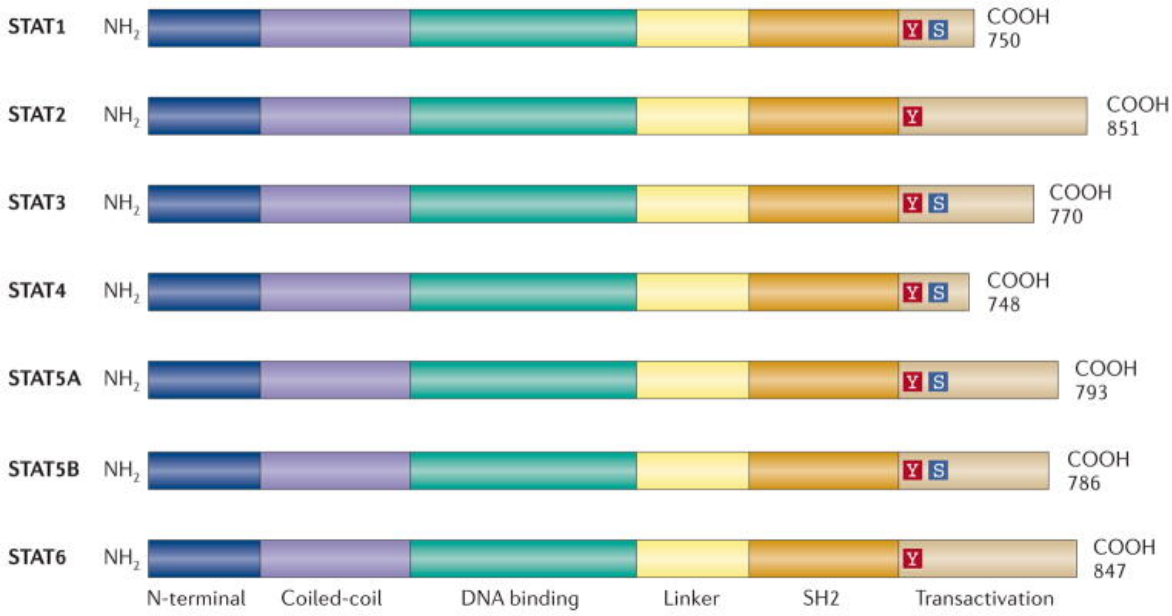

Figure 3. A schematic representation of the structures of the STAT proteins. Miklossy, G., et al., 2013. *Therapeutic modulators of STAT signaling for human diseases.* Nature Reviews Drug Discovery. Reprinted with permission from Springer Nature.

The mechanisms of STAT1 in tumor suppression includes control of tumor growth, activation of anti-tumor immune responses, and inhibition of angiogenesis (Meissl et al. 2015). STAT1 induces expression of cell cycle regulators, e.g. p21 and p27, and mediates degradation of cyclin D1 (Chin et al. 1996; Dimco et al. 2010). Expression of pro-apoptotic factors, such as Bak and caspase 1, and death receptor FAS and its ligand FASL are all induced by STAT1 (Putz et al. 2013; Fallarino and Gajewski 1999). STAT1 is also important for anti-tumor immunity. It is a central mediator of NK- and T-cell cytotoxicity (Putz et al. 2013; Fallarino and Gajewski 1999) and induces the upregulation of MHC class I expression on tumor cells (Shankaran et al. 2001). STAT1 has also been reported to interfere with angiogenesis by blocking the expression of pro-angiogenic factors and hypoxia-inducible factor 1 α (HIF1α) (Meissl et al. 2015).

The tumor-promoting function of STAT1 has been reported in several different types of cancer. Elevated levels of P-STAT1 were identified in samples from breast tumors compared to healthy controls (Watson and Miller 1995). High expression of unphosphorylated STAT1 (U-STAT1) can be correlated to poor prognosis in soft tissue sarcoma, while high levels of P-STAT1 were linked to longer survival. It appears that U-STAT1 induce anti-apoptotic signaling in these cells, in contrast to the pro-apoptotic actions of P-STAT1 (M. A. Zimmerman et al. 2012). Oncoproteins mucin (MUC) 1 and MUC4 are regulated by IFNγ/STAT1 signaling and can be correlated with poor prognosis in breast cancer (N. Khodarev et al. 2010; Andrianifahanana et al. 2007). STAT1 has also been proposed to contribute to suppression of anti-tumor immunity, for example by inducing the expression of the programmed death ligand 1 (PD-L1) (Romberg et al. 2013; Bellucci et al. 2015). Furthermore, numerous studies report that STAT1 is involved in therapy resistance. The proposed mechanisms of STAT1 in resistance include regulation of metabolic pathways and induction of autophagy, upregulation of multidrug resistance (MDR) 1 gene expression, and

regulation of mRNA translation (Meissl et al. 2015). The connection between STAT1 and the IRDS was described above in the corresponding section.

To summarize, STAT1 signaling in cancer is highly complex, exhibiting both tumor-suppressive and promoting roles, affecting both the tumor cells and immune cells in the tumor microenvironment. Thus, it is likely that the outcome of STAT1 signaling depends on the cellular context, as well as the nature and duration of the stimuli.

1.2.2 STAT2

STAT2 was identified as a mediator of type I IFN signaling as a part of the ISGF3 complex and, for a long time, it was believed to be the only function of STAT2. However, over the years, a number of studies have suggested that STAT2 exists in alternative complexes and that it regulates specific type I IFN signaling beyond the canonical ISGF3 (Blaszczyk et al. 2016).

STAT2 has been identified in heterodimers with STAT1, STAT3 and STAT6 upon type I IFN stimulation. However, not much is known of the function of these dimers since very few ISGs have been identified as target genes (Blaszczyk et al. 2016). On the other hand, an alternative ISGF3 complex, consisting of STAT2/IRF9, has been reported to be able to propagate antiviral signaling in the absence of STAT1 (Abdul-Sater et al. 2015; Blaszczyk et al. 2015). The affinity for ISRE was considerably lower for this complex compared to ISGF3 and the transcription of ISGs was delayed. The STAT2/IRF9 complex has so far only been identified in STAT2 overexpressing or STAT1 null cells, thus, it is difficult to draw conclusions about its biological function. However, it is possible that STAT2/IRF9 is responsible for a prolonged antiviral response (Blaszczyk et al. 2016).

Not much is known about the specific role of STAT2 in cancer. It has been suggested to contribute to colorectal and skin carcinogenesis by promoting expression of pro-inflammatory cytokines IL-6 and C-C Motif Chemokine Ligand 2 (CCL2) (Gamero et al. 2010). Furthermore, increased STAT2 expression was correlated with cervical cancer progression (Liang et al. 2012). STAT2 may also be implicated in the resistance to DNA damaging agents by regulating the expression of ISGs, as is described above in the section about IRDS.

1.2.3 STAT3

STAT3 is activated by the cytokines that signal through the glycoprotein 130 (gp130) receptor, i.e. the IL-6 family, as well as a variety of growth factors, such as EGF or FGF, and IFNs. STAT3 has diverse biological functions in normal cells including migration, proliferation, apoptosis, and survival, and is required for the development of various tissues. Furthermore, STAT3 knock out in mice is embryonically lethal (Takeda et al. 1997).

STAT3 has been found to be constitutively activated in a variety of solid tumors and hematological malignancies (Yu and Jove 2004). Several mechanisms have been shown to contribute to this abnormal activation; loss of negative regulation (e.g. silencing or repression of SOCS and tyrosine phosphatases), excessive stimulation of STAT3 (high expression of

cytokines, growth factors and their receptors, and oncogenic protein tyrosine kinases e.g. Src), positive feedback loops (e.g. STAT3 induced transcription leads to activation of NF-κB, which in turn increases the production of IL-6, thus sustaining STAT3 activation), and somatic mutations rendering STAT3 constitutively active (H.-F. Zhang and Lai 2014).

STAT3 regulates the transcription of a wide variety of genes that are involved in oncogenesis and play key roles in several of the hallmarks of cancer. Non-canonical functions of STAT3, independent of transcription, have also been described in tumorigenesis. A few of these mechanisms are summarized below and depicted in Figure 4.

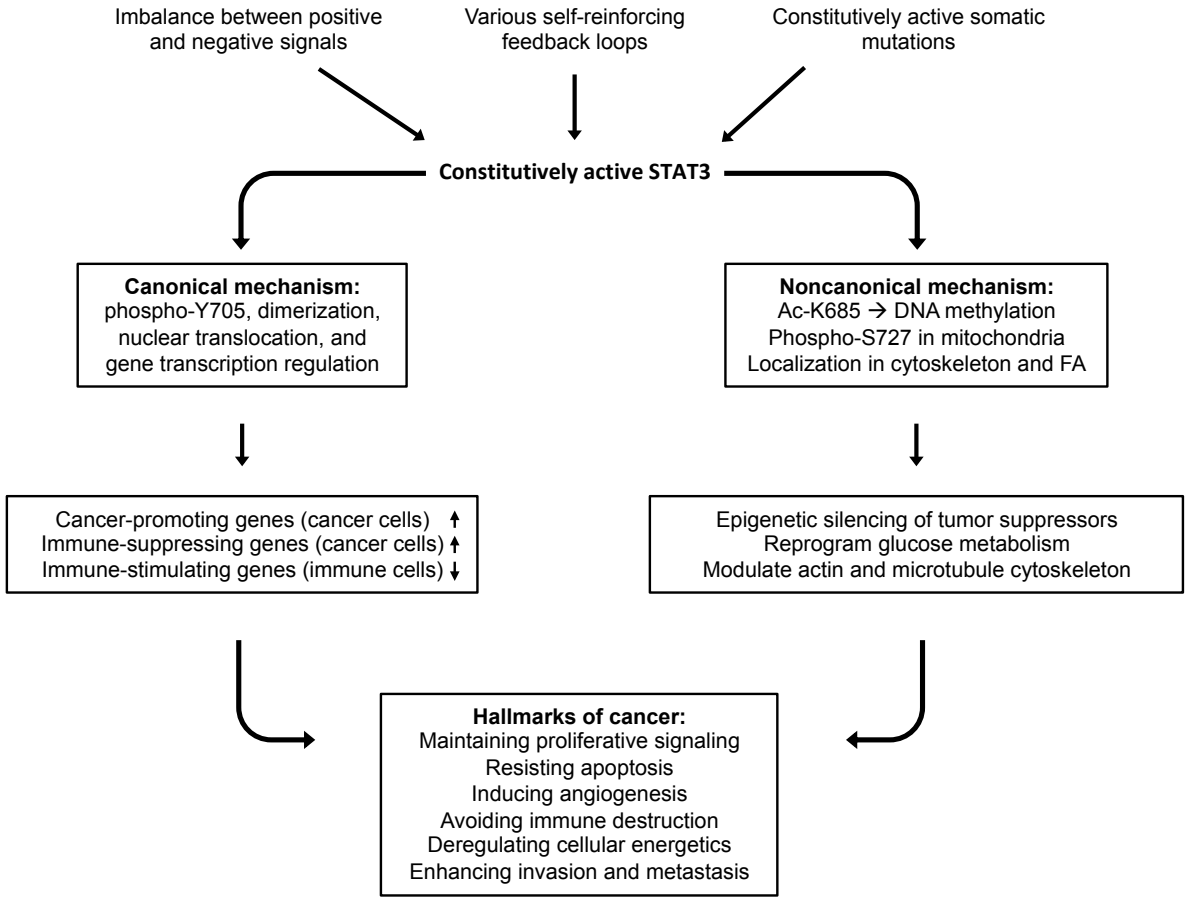

Figure 4. Mechanisms underlying the tumorigenic function of constitutively active STAT3 in cancer. Adapted from Zhang, H-F., Lai, R., 2014. *STAT3 in cancer – Friend or Foe?* Cancers. Reprinted with permission from MDPI (http://creativecommons.org/licenses/by/3.0/).

STAT3 regulates the expression of several genes involved in cell cycle progression e.g cyclin D1, Polo-like kinase (Plk) 1, and c-Myc. Inhibition or knock down of STAT3 results in reduced proliferation and tumor growth in both cancer cell lines and mouse models (H.-F. Zhang and Lai 2014). STAT3 contributes to apoptosis-resistance by suppressing both the intrinsic- and extrinsic apoptotic pathway. Several anti-apoptotic proteins regulating intrinsic apoptosis, such as survivin and the members of the Bcl-2 family, are positively regulated by STAT3 (Catlett-Falcone et al. 1999; Aoki, Feldman, and Tosato 2003). Extrinsic apoptosis is inhibited through suppression of FAS expression by STAT3 and c-Jun (Ivanov et al. 2001). Furthermore, STAT3 has been shown to repress p53 expression through binding to its

promoter. p53 expression was restored and apoptosis induced in melanoma cells upon STAT3 inhibition (Niu et al. 2005). STAT3 has been shown to directly induce angiogenesis by triggering expression of the vascular endothelial growth factor (VEGF) (Niu et al. 2002). Additionally, STAT3 can indirectly promote VEGF expression. p53 promotes proteasomal degradation of the HIF1α subunit of the pro-angiogenic factor HIF1, which induces VEGF expression (Ravi et al. 2000). As mentioned above, STAT3 inhibits p53 expression and thus, contributes to angiogenesis by increasing HIF1α stability (Yu and Jove 2004). STAT3 has been proposed to contribute to invasion and metastasis by multiple mechanisms, for example by inducing the expression of several regulators of epithelial-mesenchymal transition (EMT) (e.g Twist-1 and Snail) (Yadav et al. 2011; Lo et al. 2007). Moreover, expression of dominant negative STAT3 blocked EMT induced by transforming growth factor (TGF) -β1 in hepatocytes (Y. Yang et al. 2006). Several matrix metalloproteinases (MMPs), which degrade extracellular matrix proteins and thus facilitate tumor invasiveness, are transcriptionally regulated by STAT3 (H.-F. Zhang and Lai 2014). STAT3 has also been proposed to regulate cell migration through cytoskeletal reorganization and expression of focal adhesion molecules, such as integrin β6 and C-terminal tensin-like (CTEN) (Azare et al. 2007; Barbieri et al. 2010). Furthermore, cytoplasmic un-phosphorylated STAT3 has been shown to increase microtubule stabilization, and thereby cell migration, by binding to and inhibiting the activity of the microtubule-destabilizing protein stathmin (Ng et al. 2006). Increasing evidence suggest that STAT3 is an important mediator of tumor-associated immunosuppression (Y. Wang et al. 2018). Constitutive STAT3 activity in immune cells, such as dendritic cells, have been shown to inhibit their maturation thus impairing antigen presentation and T-cell responses (Cheng et al. 2003). Additionally, STAT3 signaling in tumor cells leads to the secretion of immune-suppressing factors, such as IL-10, and VEGF, while simultaneously suppressing the expression of proinflammatory factors (T. Wang et al. 2004). STAT3 together with NF-κB were demonstrated to be key transcription factors regulating tumor-associated inflammation, thus actively contributing to tumor development in inflammatory-related cancers, e.g. colitis-associated colon cancer (Grivennikov and Karin 2010).

Apart from its transcriptional activity, additional functions of STAT3 have been described. It has been demonstrated that STAT3 is present in the mitochondria and involved in regulation of the electron transport chain. Mitochondrial STAT3 phosphorylated on serine 727 has been suggested to contribute to tumorigenesis by enhancing glycolytic and oxidative phosphorylation activities (H.-F. Zhang and Lai 2014; Q. Zhang et al. 2013). STAT3 activity is not only regulated by phosphorylation, but also by acetylation, which is controlled by the activity of histone acetyltransferases (HATs) and histone deacetylases (HDACs). Acetylation of STAT3 on lysine 685 increased the DNA binding affinity, transcriptional activity, protein-protein interactions, and modulates dimerization (Zhuang 2013). K685-acetylated STAT3 was found to interact with DNA methyltransferase (DNMT) 1 to silence several tumor suppressor genes by CpG island methylation (Lee et al. 2012).

To conclude, STAT3 is an important regulator of diverse cellular events, and a point of convergence for many oncogenic signaling pathways. Considering this, and the fact that

STAT3 is constitutively activated in numerous different cancers, makes STAT3 an attractive target for cancer therapy.

1.3 3D CULTURE SYSTEMS

The majority of cell-based assays use conventional two-dimension (2D) culture, growing the cells in monolayer on a flat surface. However, this system lacks the natural three-dimensional (3D) architecture, characterized by cell-cell and cell-matrix interactions of cells in vivo. In fact, 2D culture results in cytoskeletal rearrangements and creates an unnatural polarity (Nath and Devi 2016). Cells in monolayer culture are exposed to a homogenous concentration of nutrients and excreted factors, lacking the natural gradient that exists in vivo. A number of cellular processes are affected by the 2D conditions, such as proliferation, apoptosis, differentiation, and gene expression (Tibbitt and Anseth 2009). Cells grown in 3D systems form aggregates or spheroids and better mimic the cell morphology as well as cell-cell or cell-matrix interactions that occur in vivo. Cancer cells in 3D can be co-cultured with other types of cells, for example fibroblasts, in order to study the role of stromal cells in tumors. Similarly to solid tumors, the structure of the spheroids gives rise to a heterogeneous population, created by a gradient of the supply with oxygen, nutrients, growth factors and cytokines. Generally, cells in the outer layers of the sphere are proliferating, cells in the hypoxic core are necrotic and, in between them, there is a layer of quiescent cells (Edmondson et al. 2014). Importantly, cells grown in 3D exhibit decreased sensitivity to anti-cancer drugs compared to 2D cultures and are considered to better predict the effect of these drugs in vivo (Horning et al. 2008; David et al. 2008). For example, colorectal cancer cell line HCT116 cultured in 3D were more resistant to four different chemotherapeutic drugs, with different mechanism of action, compared to 2D culture. Furthermore, spheres grown for 6 days were less sensitive to the drugs compared to those cultured for only 3 days (Karlsson et al. 2012). A number of factors may contribute to the difference in drug sensitivity between 3D and 2D cultures, which is described further below in the section about multicellular drug resistance. Together, these studies demonstrate the advantages of using 3D culture for studies of tumor biology and drug discovery, producing more physiologically relevant data compared to the 2D system.

1.3.1 Techniques for generating multicellular spheroids

Multicellular spheroids (MCS) are constructed from tumor cells alone or in co-culture with other types of cells, with or without scaffolds. The morphology of the spheres depends on the cell line and on the 3D culturing technique used. In scaffold-based techniques the spheres are seeded in or on top of biologically active hydrogels that promote cell-cell and cell-matrix interactions. Scaffold-free techniques can be used for cell lines that self-aggregate and form tissue-like structures. One way to generate scaffold-free MCS is to seed cells in ultra-low attachment plates. These plates are coated with polystyrene which blocks attachment to the plate, causing cells in suspension to aggregate and form spheres. Another way is to allow the cells to aggregate hanging in a droplet of culture media, called the hanging drop technique (Nath and Devi 2016). The hanging-drop technique, which is used to generate MCS in this thesis, has several advantages. The setup is simple, and the droplet eliminates surface

interactions. The absence of a scaffold simplifies experimental procedures, such as drug treatments. Furthermore, the hanging drop technique produces one spheroid per droplet that are of homogenous size, which makes the process highly reproducible. The hanging-drop technique has successfully been applied to a number of cell lines of different origin (Timmins and Nielsen 2007).

1.3.2 JAK-STAT signaling in MCS

Several studies have demonstrated that culturing cells in 3D results in alterations in gene and protein expression compared to 2D culture (Edmondson et al. 2014). Cancer cell lines cultured in 2D or 3D often differ in expression of genes involved in proliferation, angiogenesis, migration, invasion and drug sensitivity, where the expression profile of 3D cultures are more similar to those observed in tumor samples (L'Espérance et al. 2008; Zietarska et al. 2007; Oloumi et al. 2002). In an attempt to identify signaling pathways that correlate to spheroid structure, Park et al cultured 100 different cancer cell lines as MCS and classified them in four different groups according to morphology; round, mass, and aggregate-type and those who did not form spheres (Park et al. 2016). The round-type spheroids displayed increased hypoxia and decreased drug permeability compared to the other types, probably due to the tight cell-cell interactions. In line with this, these spheres were also less sensitive to the chemotherapeutic agent Fluorouracil (5-FU). JAK-STAT was one of the signature pathways identified in the round-type spheroids of 30 different cancer cells lines. Additionally, phosphorylation and total expression of STAT3 was higher in the round-type spheres compared to the mass- or aggregate-type. Blocking STAT3 phosphorylation, using the JAK-inhibitor AG490, resulted in a morphological change in the compact round-type spheres into a visibly looser structure. The reduced density was also confirmed by evaluating cell adhesion markers E-cadherin and epithelial cell adhesion molecule (EpCAM). Furthermore, AG490 treatment greatly increased the sensitivity of the spheres to 5-FU, likely by improving the drug penetration in the spheres (Park et al. 2016).

1.4 DRUG RESISTANCE

Resistance to therapy continues to be a major hurdle in medical oncology. It can be divided in two categories, intrinsic and acquired resistance. Intrinsic resistance indicates that the cells possess some means of resistance prior to subjection to treatment, while acquired resistance is the result of additional mutations or other adaptive responses that arise during the treatment. The heterogeneous nature of tumors is an important factor in resistance, where therapy-induced selection can enrich for a resistant subpopulation of cells. A number of different mechanisms have been implicated in drug resistance including increased efflux, decreased drug uptake, altered drug metabolism, modification of drug target, dysregulation of apoptotic pathways and an enhanced DNA repair. Many of these mechanisms contribute to multidrug resistance (MDR) where the cancer cells can escape the toxicity of a variety of drugs, irrespective of their different chemical structures and mechanisms of action (Holohan et al. 2013). A specific type of intrinsic resistance, termed multicellular drug resistance, is studied in this thesis and outlined below.

1.4.1 Multicellular resistance

Cancer cell lines cultured as MCS display enhanced resistance to anti-cancer drugs and irradiation, a mechanism known as multicellular drug resistance (MCR). This resistance is contact-dependent and thus disappears when the cell contact is disrupted (Desoize and Jardillier 2000). The phenomenon of MCR is likely to contribute to the drug resistance in tumors in vivo (Kobayashi et al. 1993; Desoize and Jardillier 2000) and studying the mechanisms underlying MCR could identify novel predictive markers and anti-cancer treatments. MCR can be divided in two types that are described below; contact resistance, which is also observed in confluent 2D cultures, and resistance related to the inherent structure of the sphere.

1.4.1.1 Contact resistance

One factor that contributes to contact resistance is the slowdown of proliferation that occurs in confluent cells. Similarly to the quiescent cells present in spheres, the confluent mono-layer cells are less sensitive to drugs that require proliferation to be effective, compared to non-confluent exponentially-growing cells (Desoize and Jardillier 2000). Protein expression also changes between confluent and non-confluent cultures and affects drug sensitivity. Cyclin-dependent kinase inhibitor p27Kip1 (p27) expression was elevated in confluent HT29 colorectal cancer cells as well as a panel of carcinoma cell lines cultured in 3D (Dimanche-Boitrel et al. 1998; St Croix et al. 1996). Treating mouse mammary carcinoma cell line EMT-6 spheroids with a p27 antisense oligonucleotide resulted in increased proliferation and sensitized the cells to chemotherapeutic agents (St Croix et al. 1996). Furthermore, overexpression of p27 reduced the toxicity of cisplatin, 5-FU, and doxorubicin in non-confluent HT29 cells (Dimanche-Boitrel et al. 1998). Confluent cells express lower levels of topoisomerase II, an enzyme involved in DNA repair, and are thus less sensitive to topoisomerase II inhibitors compared to non-confluent cells (Garrido et al. 1995). NAD(P)H: quinone acceptor oxireductase expression is higher in MCS and confluent cultures compared to non-confluent, and has been suggested to be involved in drug inactivation (Phillips et al. 1994). Expression and phosphorylation of stress protein HSP27 was shown to be increased in two colorectal cancer cell lines cultured to confluence (Garrido et al. 1997). HSP27 expression protects cells from apoptosis induced by tumor necrosis factor α (TNFα) by reducing the levels of reactive oxygen species (ROS) (Mehlen et al. 1995). Expressing HSP27 in non-confluent cells, at a similar level as in confluent cells, lead to a similar resistance to doxorubicin and cisplatin as observed in confluent cells (Garrido et al. 1997). Confluency has also been shown to reduce the diffusion of compounds across the cell membrane, thus contributing to drug insensitivity by a decrease in uptake. However, confluent cells with the same intracellular concentration of a drug, were still less sensitive to that drug, compared to non-confluent cells (Pelletier et al. 1990). The cell-matrix contact is believed to be an important mechanism of MCR through inhibition of apoptosis. A similar phenomenon can be observed in non-transformed cells. Disruption of cell-matrix interactions in epithelial cells lead to induction of apoptosis, a process known as anoikis (Dimanche-Boitrel et al. 1998; St Croix et al. 1996). High expression of bcl-2 protects cells against this type of apoptosis. It has been suggested that interactions between integrins and extracellular

matrix proteins induce the expression of bcl-2, thus suppressing anoikis (Frisch et al. 1996; Frisch and Ruoslahti 1997).

1.4.1.2 Resistance related to spheroid structure

Many of the mechanism known to be involved in MCR are common between confluent 2D and 3D culture, while others are observed in spheres alone and are related to the 3D structure. While drugs are equally diffused to cells in monolayer culture, this is not always the case in spheroids. For large compounds, for example vincristine, penetration into the sphere is inefficient (Nederman and Carlsson 1984). Furthermore, the hypoxic environment in the spheres leads to a decrease in pH, which has been shown to reduce the uptake of weakly basic chemotherapeutic drugs (Swietach et al. 2012). Differences in the expression and spatial distribution of cell surface receptors between 2D and 3D cultures may also affect the response to drugs targeting these receptors (Luca et al. 2013). As mentioned above, interaction between tumor cell integrins with components of the extracellular matrix are believed to contribute to inhibition of apoptosis in both confluent monolayer and 3D culture. Cells cultured in 3D excrete more extracellular matrix components, for example proteoglycans and fibronectin, compared to their monolayer counterparts (Glimelius et al. 1988). Expression of DNA mismatch repair proteins PMS2 and MutL homolog 1 (MLH1) were found to be downregulated in MCS of several human breast cancer and melanoma cell lines compared to their respective monolayer culture (Francia et al. 2005). The absence of a functional mismatch repair system has been suggested to allow cells to avoid detection of lesions in the DNA and contribute to resistance to alkylating agents and irradiation (Fritzell et al. 1997; Francia et al. 2005).

1.5 COLORECTAL CANCER

Colorectal cancer (CRC) is the second most common type of cancer in women and third most common in men. It is the fourth leading cause of cancer death in the world. Risk factors include advanced age, genetic factors, intestinal inflammatory disease, obesity, diet and other lifestyle factors. Sporadic cases, due to somatic mutations, constitute about 70% of all CRC cases. There are several hereditary CRC syndromes, the most common are familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC) (De Rosa et al. 2015).

Typically, CRCs begin as a benign intestinal polyp that slowly develops into a carcinoma. There are two main types of polyps involved in CRC, adenomas and sessile serrated polyps (SSPs). (Simon 2016). CRCs, as all cancers, develop in a stepwise manner, accumulating mutations as the disease progresses. Molecularly, CRCs are very heterogenous, due to loss of genomic stability. Different mechanisms of genomic instability have been described in CRC oncogenesis; chromosome instability, microsatellite instability, and aberrant DNA methylation and DNA repair (Tariq and Ghias 2016). The classical adenoma pathway was first described in 1990 by Fearon and Vogelstein, and begins with loss of function of the adenomatous polyposis coli (APC) gene (Fearon and Vogelstein 1990). APC negatively regulates β-catenin, which is a crucial mediator of the Wnt signaling pathway. Wnt signaling induces proliferation and inhibits differentiation, and it is the most commonly dysregulated

pathway in sporadic CRCs (De Rosa et al. 2015). The mutation of APC is typically followed by activating mutations in KRAS and loss or inactivation of p53. In contrast, the initiating event in the development of SSPs is typically mutations in the BRAF gene, leading to dysregulated proliferation and inhibition of apoptosis (Simon 2016). Other commonly dysregulated pathways in CRCs include PI3K/AKT, NF-κB, and GSK-3β signaling (De Rosa et al. 2015).

Staging of CRCs is done using the TNM classification, where T describes the local invasion depth, N the lymph node involvement, and M describes the presences of distant metastasis. The treatment depends on the stage of the tumor. Low stage disease can be treated with surgery alone, while higher stages with systemic spread will also be treated with chemo and/or radiotherapy. The main chemotherapeutic agents used are 5-FU, leucovorin, oxaliplatin, and capecitabine. (De Rosa et al. 2015). Antibodies targeting the EGF-receptor and VEGF or the VEGF-receptor are used to treat advanced CRCs (Moriarity et al. 2016).

1.5.1 Inflammation and IL6 signaling in CRC

In addition to genetic and life style factors, inflammation has also been shown to be an important risk factor for the development of cancer. This is clearly demonstrated in patients with inflammatory bowel diseases, such as ulcerative colitis and Crohn's disease, who are at a much higher risk of developing CRC (Ullman and Itzkowitz 2011). Chronic inflammation can contribute to carcinogenesis by inducing excessive tissue remodeling, loss of tissue architecture, and modifications of proteins and DNA through the production of ROS (de Visser, Eichten, and Coussens 2006). In addition, several pro-inflammatory cytokines, for example IL6, have also been shown to contribute to tumor progression (Waldner, Foersch, and Neurath 2012).

IL6 is a pleiotropic cytokine and an important regulator of immune and inflammatory responses. IL6 is expressed mainly by immune cells, such as monocytes, macrophages and lymphocytes, but also by fibroblasts, endothelial cells and tumor cells (Vendramini-Costa and Carvalho 2012). Transcription of IL6 is controlled by several transcription factors, e.g NF-κB and AP-1, and its expression is induced by inflammatory stimuli such as TNFα or IL1 as well as lipopolysaccharide (LPS) or viral infections (Dendorfer, Oettgen, and Libermann 1994). The IL6 receptor exists in two forms, the transmembrane form and the soluble form. Expression of the membrane-bound receptor is restricted to hepatocytes and immune cells, while the soluble form is expressed by most cell types. IL6 signals through the gp130 receptor, which is also expressed by most cells. The cytokine binds to the IL6 receptor, which then dimerizes with gp130 resulting in activation of receptor-associated JAKs. This leads to the activation of STAT3 and STAT1, as well as the Ras-MAPK- and PI3K-AKT-mTor pathways (P C Heinrich et al. 1998).

Elevated levels of IL6 in serum and tumor tissue has been detected in several different types of solid malignancies, including CRC, where it can be correlated to poor prognosis (Belluco et al. 2000; Knüpfer and Preiss 2010). As mentioned above, IL6 signaling leads to activation of downstream oncogenic pathways, such as STAT3. Through these signaling pathways, IL6

has been reported to regulate multiple tumorigenic processes such as proliferation, survival, angiogenesis, EMT, and therapy resistance (Kumari et al. 2016). IL6 is also important in regulating inflammation and interactions with the tumor microenvironment. Both pro- and anti-inflammatory functions of IL6 have been reported. It is a crucial mediator of the acute inflammatory response as well as the resolution of inflammation through controlling the expression of both pro- and anti-inflammatory molecules thus maintaining host-tumor homeostasis (Xing et al. 1998; Kumari et al. 2016). STAT3 activation is one of the key pathways for IL6-mediated pro-tumorigenic effects, making it an attractive target for anti-cancer therapy. The oncogenic functions of STAT3 are described above in the corresponding section.

1.6 PEDIATRIC ACUTE LYMPHOBLASTIC LEUKEMIA

Pediatric acute lymphoblastic leukemia (ALL) is the most common malignancy in children and is one of the leading causes of death in children in developed countries (Cheok and Evans 2006). Yearly about 70-90 new ALL cases are diagnosed in Sweden and the incidence peaks in children around 2-5 years of age (Gustafsson, Kogner, and Heyman 2013). The disease is characterized by an expansion of immature B or T lymphocytes. Thus, pediatric ALL is generally divided into two immunological subtypes: preB-ALL and T-ALL. The disease is heterogeneous and the subtypes are further divided into more than 10 different genetic subgroups characterized by genetic abnormalities that include chromosomal translocations, gene amplifications, and mutations (Downing et al. 2012). Interestingly, although used for stratification of ALL, the genetic abnormalities are insufficient to fully explain ALL pathogenesis as they fail to induce leukemia in in vivo models (Inaba, Greaves, and Mullighan 2013). This finding indicates that additional, yet uncovered factors are involved. Nevertheless, the genetic abnormalities as well as other factors (e.g. immunological subtype and age at diagnosis) underlie the current stratification of patients into high risk, intermediate to high risk and low risk groups, which determine the treatment regimen. The treatment includes high doses of glucocorticoids dexamethasone or prednisolone in combination with multi-agent chemotherapy. The combination of risk-based stratification and the multi-agent chemotherapy has significantly increased the survival rate of pediatric ALL to approximately 90%. However, lifelong adverse effects due to treatment are common in survivors (Inaba, Greaves, and Mullighan 2013).

1.7 POLO-LIKE KINASES

The Polo-like kinase (Plk) family of serine/threonine protein kinases is key regulators of mitosis in eukaryotic cells. All five members of the family contain at least two polo-box domains (PBD) in the carboxyl-terminal, which regulates localization and function (Figure 5). The catalytic kinase domain of the N-terminal is very similar between Plk1, 2, and 3, while Plk4 has a unique sequence and Plk5 lacks a functional kinase domain (Zitouni et al. 2014).

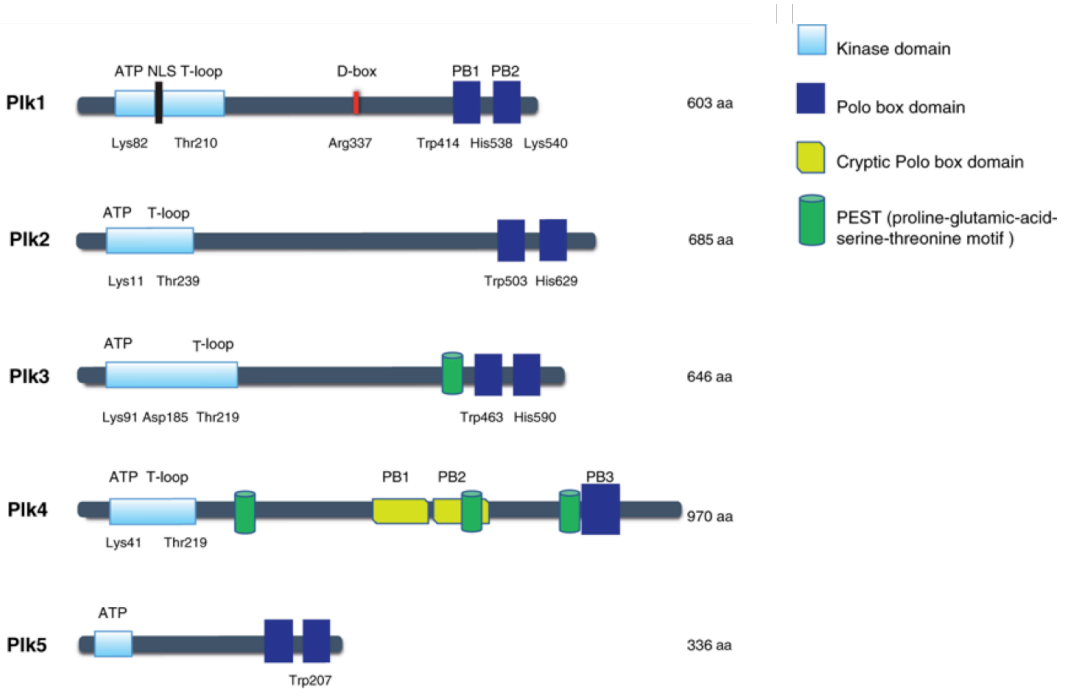

Figure 5. Structure of the Plk family. Goroshchuk, O., et al., 2018. *Polo-like kinases and acute leukemia.* Oncogene. Reprinted with permission from Springer Nature.

1.7.1 Plk1

1.7.1.1 Plk1 function

The best-characterized member of the Plk family, Plk1, plays an important role in multiple aspects of the cell cycle in humans (Figure 6). Cells deficient for this kinase show spindle defects and late mitotic abnormalities (Strebhardt and Ullrich 2006). The expression of Plk1 begins in late S phase, continues to rise throughout G2 and then peaks in M phase (Golsteyn et al. 1995). Plk1 is essential for G2/M transition as it phosphorylates CDC25C which in turn activates cyclin-dependent kinase (CDK) 1-Cyclin B1 to trigger mitotic entry (Toyoshima-Morimoto et al. 2001). Plk1 also inactivate inhibitors of CDK1, such as Wee1 and Myt1 (Watanabe et al. 2004; Inoue and Sagata 2005). In case of DNA damage, CDK1-Cyclin B1 is inhibited by activation of checkpoint kinases, causing the cell to arrest at G2/M (Bartek and Lukas 2007). The ability to complete the cell cycle after the damage has been repaired, is dependent on Plk1 by inducing degradation of the CDK1-Cyclin B1 inhibitors (van Vugt, Brás, and Medema 2004). Plk1 is also involved in centrosome maturation (Lane and Nigg 1996), bipolar spindle formation (Ohkura, Hagan, and Glover 1995), cytokinesis (Carmena et al. 1998) and mitotic exit (Descombes and Nigg 1998). To be able to exert all of these functions, Plk1 changes its subcellular location between different components of the mitotic spindle as mitosis progresses (Takaki et al. 2008). Plk1 contains an N-terminal catalytic domain and two PBDs in the C-terminal (Figure 5). The PBD is an evolutionary conserved sequence that functions as a peptide-binding domain and is believed to be important for substrate selection and proper subcellular localization of Plk1 (Elia, Cantley, and Yaffe 2003).

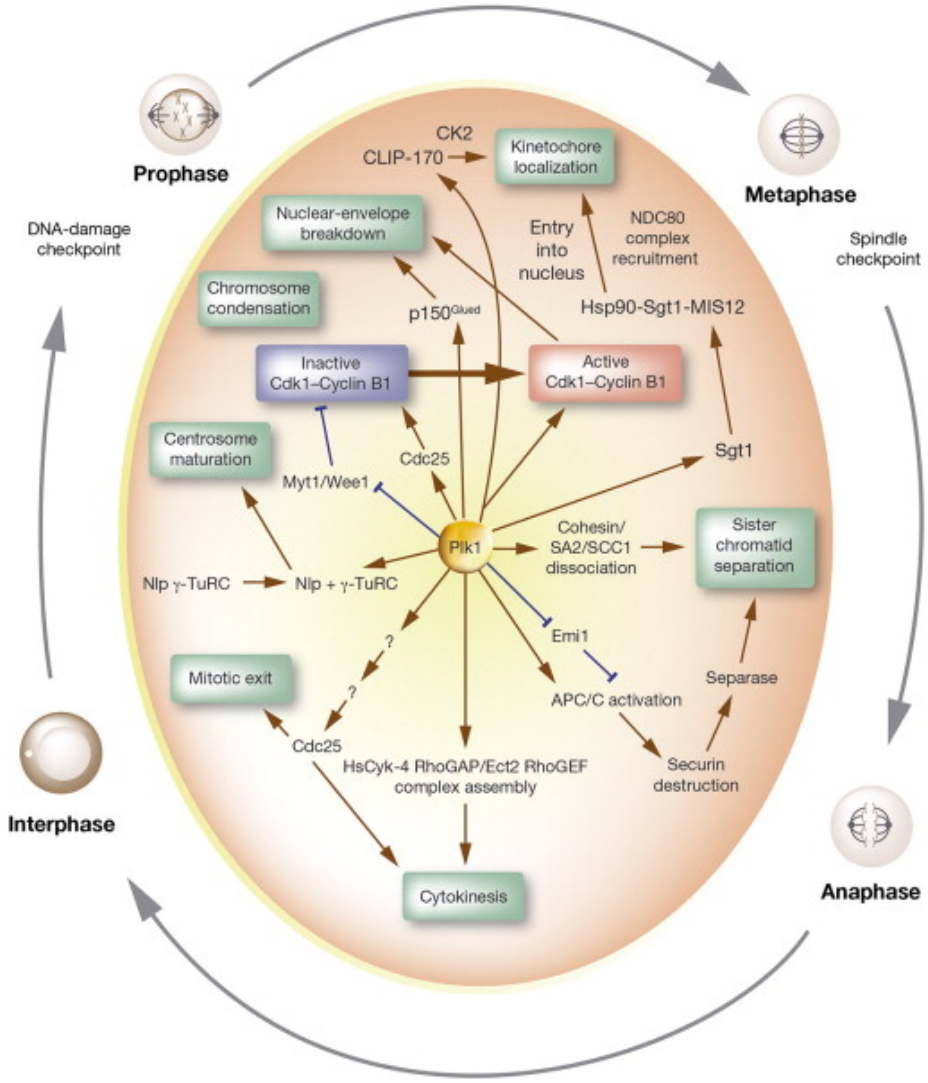

Figure 6. Functional roles of Plk1 in cell cycle progression. Liu, X., 2015. *Targeting Polo-Like Kinases: A Promising Therapeutic Approach for Cancer Treatment.* Translational Oncology. Reprinted with permission from Elsevier (https://creativecommons.org/licenses/by-nc-nd/4.0/).

1.7.1.2 Regulation of Plk1

The activity of Plk1 is tightly controlled during the cell cycle. In the inactive state, the PBD interacts with the kinase domain and suppresses its activity (Jang et al. 2002). In mitosis the G2 induced Bora protein binds to Plk1 and changes its conformation, allowing for activation by phosphorylation of the kinase domain on threonine 210 by Aurora A kinase (Seki, Coppinger, Jang, et al. 2008). Active Plk1 promotes ubiquitination and proteasomal degradation of Bora which if not degraded would interfere with PBD function (Seki, Coppinger, Du, et al. 2008). Inactivation of Plk1 is achieved by dephosphorylation of Thr210 by Protein phosphatase 1 (PP1) and its adaptor Myosin phosphatase-targeting subunit 1 (MYPT1) (Yamashiro et al. 2008). Plk1 is also tightly regulated on the transcriptional level. p53 and p21 have been shown to inhibit transcription during G1 through interaction with a repressor element called the cell-cycle-dependent element/cell cycle gene homology region (CDE/CHR) present in the Plk1 promoter (St Clair and Manfredi 2006; Zhu et al. 2002). Activated retinoblastoma (Rb) protein inhibits Plk1 transcription in a CDE/CHR independent

fashion by recruiting a chromatin-remodeling complex that represses Plk1 transcription through histone deacetylation (Gunawardena et al. 2004).

1.7.1.3 Plk1 in cancer

Considering the pivotal role in cell division, it is not surprising that aberrant expression of Plk1 have been observed in several types of human cancers including solid tumors and hematological malignancies as well as in a number of cancer cell lines (Eckerdt, Yuan, and Strebhardt 2005; Ikezoe et al. 2009; Simizu and Osada 2000). There are also several studies showing a correlation between tumor metastasis and poor patient outcome with elevated Plk1 expression (Kneisel et al. 2002; Wolf et al. 1997; J. Yuan et al. 1997; Takai et al. 2001). Furthermore, a study by Smith et al revealed that constitutive expression of Plk1 in mouse embryonic fibroblast cell line NIH 3T3 results in oncogenic transformation of these cells with the ability to form colonies in soft agar and grow tumors when injected into nude mice (Smith et al. 1997). This indicates that Plk1 overexpression might not be a consequence, but a cause of malignant transformation. In addition, expression of hyperactive Plk1 mutant resulted in reversion of the DNA damage checkpoint G2 arrest induced by doxorubicin treatment in U2OS cells (Smits et al. 2000). The ability to carry out mitosis with damaged DNA leads to accumulation of mutations and subsequent transformation. Moreover, Plk1 has been shown to bind to and inhibit the pro-apoptotic actions of p53 (Ando et al. 2004). Plk1 activates G2 and S-phase-expressed-1 protein (GTSE1), a negative regulator of p53, and Topo-1 binding protein (TOPORS) that promotes ubiquitylation and degradation of p53 (X. S. Liu et al. 2010; X. Yang et al. 2009).

1.7.1.4 Plk1 in ALL

Plk1 mRNA levels have been shown to be significantly higher in samples from adult patients with B-ALL compared to bone marrow mononuclear cells (BMMCs) from healthy donors, while the expression in T-ALL samples were not significantly elevated (Renner et al. 2009; Ikezoe et al. 2009). The expression of Plk1 mRNA was not found to be differentially expressed in samples from 65 children with ALL (B-ALL=58, T-ALL=7) compared to normal bone marrow (Oliveira et al. 2014). However, in a study with 172 pediatric ALL patient samples, Plk1 protein and Thr210 phosphorylation levels were significantly increased compared to normal bone marrow mononuclear cells in both B- and T-ALL samples (Hartsink-Segers et al. 2013). A number of ALL cell lines have also been found to overexpress Plk1 on mRNA and protein level (Ikezoe et al. 2009; Oliveira et al. 2014).

A number of studies have shown that targeting Plk1 using different inhibitors or siRNA effectively induces cell cycle arrest and apoptosis in a variety of ALL cell lines (Renner et al. 2009; Ikezoe et al. 2009; Oliveira et al. 2014; Hartsink-Segers et al. 2013; Spartà et al. 2014). Additionally, Plk1 inhibitor BI 2536 was shown to drastically reduce the proliferation and clonogenic potential of primary AML patient samples, while normal hematopoietic CD34+ progenitors were unaffected (Renner et al. 2009). Treatment with Plk1 inhibitor NMS-P937 significantly reduced cell survival in 15 ALL patient samples, where the samples with high Plk1 expression were the most sensitive to the inhibitor. Furthermore, normal bone marrow

mononuclear cells were resistant to NMS-P397 up to a concentration of 3.3μM (Hartsink-Segers et al. 2013).

1.7.1.5 Plk1 as a target for cancer therapy

The key role of Plk1 in mitosis, its ability to trigger malignant transformation and the fact that it is commonly overexpressed in human cancers makes Plk1 an attractive target in cancer therapy. Studies have shown that inhibiting Plk1 function, by injecting antibodies or viral expression of dominant-negative Plk1, led to the induction of mitotic catastrophe and subsequent cell death in various cancer cell lines while non-transformed cells were unharmed (Lane and Nigg 1996; Cogswell et al. 2000). Thus, targeting Plk1 could be an effective weapon against cancer cells without causing damage to the healthy tissues. Several small molecules targeting Plk1 have already been developed, such as Poloxin (Juping Yuan et al. 2011), which targets the PBD, and BI2536, an adenosine triphosphate (ATP)-competitor that inhibits the kinase activity (Steegmaier et al. 2007). However, the PBD is common for all Plks and the similarity between the kinase domains in Plk1-3 makes it difficult to develop an inhibitor that specifically targets Plk1. Both Poloxin and BI2536 show activity against Plk2-3 and while they effectively inhibit proliferation and induce apoptosis in malignant cells in vitro, they also affect normal non-transformed cells (Juping Yuan et al. 2011; Steegmaier et al. 2007). In contrast, specific depletion of Plk1 using RNA interference (RNAi) did not affect non-transformed cells MCF10A and hTERT-RPE1, while inducing G2 arrest and apoptosis in malignant cells (X. Liu, Lei, and Erikson 2006).

There are a number of ongoing clinical trials with different Plk1 inhibitors. BI6727 (Volasertib) is an ATP-competitor inhibitor with IC50 for Plk1/2/3 of 0.85, 5.0 and 56 nM, respectively (Rudolph et al. 2009). This compound showed promise in a phase I clinical trial with advanced solid tumors, side effects include neutropenia and thrombocytopenia (Lin et al. 2014). BI6727 is currently the Plk1 inhibitor that has progressed the furthest in clinical trials with several ongoing phase II trials in solid tumors and phase III trial in adults with acute myeloid leukemia (AML) (Kumar and Kim 2015; Brandwein 2015).

Another promising ATP-competitive inhibitor is NMS-P937. It effectively inhibits Plk1 with an IC_{50} of 2 nM and displayed high selectivity when tested on a panel of about 300 protein kinases. NMS-P397 showed 5000-fold greater inhibition selectivity towards Plk1 compared to Plk2-3 and reduced toxicity towards normal human dermal fibroblasts (NHDF) compared to ovarian carcinoma cell line A2780 (Valsasina et al. 2012). A phase I dose-escalation study has successfully been completed in patients with advanced solid tumors (Kumar and Kim 2015).

TKM-080301 is a lipid nanoparticle formulation of Plk1-targeting siRNA (X. Liu 2015). One advantage with using RNAi is the high specificity, and TKM-080301 does not target any of the other members of the Plk family. It showed tolerable side effects in a phase I dose escalation study in patients with neuroendocrine tumors and adrenocortical carcinoma (Kumar and Kim 2015).

1.7.2 Plk2-5

The other members of the Plk family are not as well studied as Plk1. Plk2 is involved in centriole duplication that occurs shortly before G1-S transition (Warnke et al. 2004). It is also a direct target of p53 and contributes to triggering a G2 checkpoint following DNA damage (Burns et al. 2003). Epigenetic silencing of Plk2 is a frequent event in B-cell malignancies and ovarian carcinoma and it is correlated to resistance to therapy and poor outcome in the latter (Syed et al. 2006, 2011). Plk3 is a positive regulator of cyclin E and it has been found to be required for G1-S transition (W. C. Zimmerman and Erikson 2007). It is also activated by DNA damage and is involve in triggering cell cycle arrest and/or apoptosis through the ATM/p53 pathway (S. Xie et al. 2001). The role of Plk3 in cancer is unclear, it has been found to be downregulated in lung, liver, head and neck cancer and upregulated in ovarian and breast cancer (Helmke, Becker, and Strebhardt 2015). Plk4 localizes to the centrosome and is essential for centriole duplication (Habedanck et al. 2005). It is also involved in regulating G2-M transition and cytokinesis through phosphorylation of both CDC25C44 and the RhoA guanine nucleotide exchange factor Ect2 (Rosario et al. 2010). Plk4 and Plk1 are both exclusively expressed in proliferating cells, while Plk2 and Plk3 are expressed in both cycling- and non-dividing differentiated cells (van de Weerdt and Medema 2006). Plk5 is the most recently identified member of the Plk family. It is frequently epigenetically silenced in glioblastoma and has been proposed to have tumor suppressive functions (de Cárcer et al. 2011).

1.8 RNAI-BASED THERAPEUTICS

Since specific targeting of Plk1 appears to reduce the toxicity towards healthy cells, RNAi could be the preferable strategy to use (Raab et al. 2011). RNAi is the process where double stranded RNA (dsRNA) induces sequence-specific gene silencing through targeting and cleavage of complementary mRNA (Fire et al. 1998). Members of the evolutionary conserved RNase III ribonuclease family, called the Dicer enzymes, induce the RNAi process. Dicer cleaves dsRNA into small interfering RNA (siRNA); 21-23 nucleotide long fragments with 5’ phosphate groups and 2-nucleotide 3’ overhang (Bernstein et al. 2001). The siRNA is then unwound and the antisense strand is loaded into a multicomponent nuclease, the RNA-induced silencing complex (RISC) (Nykänen, Haley, and Zamore 2001), while Argonaute 2 (Ago2), an endonuclease recruited by RISC, cleaves the complementary strand (Frank, Sonenberg, and Nagar 2010). RISC then repeatedly cleaves mRNA molecules using the antisense strand as a guide to substrate selection, making the RNAi process very effective since one single antisense strand can degrade a high number of complementary mRNAs (Hammond et al. 2000).

The high specificity and efficacy make RNAi an exiting therapeutic option. A number of RNAi-based therapies are in clinical trials for treatment of viral diseases, neurodegenerative disorders and cancers (Kacsinta and Dowdy 2016). The U.S Food and Drug Administation (FDA) recently approved the first siRNA-based drug, encased in a lipid nanoparticle, to be used in patients suffering from hereditary polyneuropathy (FDA 2018). siRNAs that are to be used in therapeutic settings should be designed in order to induce a specific and potent RNAi

response, without triggering any unwanted side effects, for example interferon signaling. One of the main challenges of RNAi-therapeutics is how to safely and effectively deliver the siRNA to target cells and tissues. The negatively charged backbone and the large size prevent siRNAs from crossing the cell membrane. There are various delivery strategies, for example nanoparticles, aptamers, cholesterol and antibodies (Tiemann and Rossi 2009). The advantages and disadvantages of the different delivery approaches are summarized in Table 1.

Almost thirty years ago it was found that certain peptides, termed peptide transduction domains (PTDs), are able to enter cells without the help of any delivery vehicle (Frankel and Pabo 1988). Over the years more than 20 Phase I and II clinical trials have been performed with PTDs coupled to cargo (van den Berg and Dowdy 2011). These peptides have been used as transporters to deliver siRNA into cells in vivo and may be the way forward in RNAi therapeutics (Meade et al. 2014).

Delivery strategies	**Advantages**	**Disadvantages**
Naked siRNA	Chemically modified siRNAs are stable, potentially non-immunogenic, easy to manufacture.	Poor cellular uptake, activation of toll-like receptors, not targeted to specific cell types.
Aptamer	Highly target-specific, large scale manufacturing possible, can be backbone modified for stability *in vivo*.	Large relative to siRNA, repeated treatment might be necessary, need to be modified for enhanced circulation and pharmacodynamics, costly to manufacture.
Antibodies	Highly target-specific, can use monoclonals or antibody fragments.	Costly to produce, repeated treatments might be necessary, possibly immunogenic.
Cholesterol	Non-immunogenic, proven *in vivo* delivery in non-human primates, potentially low cost.	Possible liver toxicity if used repeatedly, not useful for delivery to most organs other than liver, very large doses required for efficacy in animal model testing.
Synthetic nanoparticles	Specific targeting possible, can be synthesized in large scale, able to accommodate large amounts of siRNAs and can be engineered to escape endosome.	Need to be conjugated to specific ligands for tissue specific delivery, costly manufacturing, repeated administration necessary.
Viral vectors for shRNAs	Infect target cells with high affinity, long-lasting expression, possible to combine multiple RNAi triggers in a single vector.	Possible immune response to vector envelope, integrating vectors can cause gene disruption, potential toxicity from continuous expression of shRNA, costly to manufacture.

Table 1. Pros and cons of delivery approaches. Tiemann, K., Rossi, J.J., 2009. *RNAi-based therapeutics-current status, challenges and prospects*. EMBO Molecular Medicine. Reprinted with permission from John Wiley and Sons.

2 AIMS OF THE THESIS

The overall aim of this thesis was to explore the role of STAT3 and interferon gene signature in multicellular drug resistance. Furthermore, we aimed to determine the effect of novel siRNN prodrugs targeting Plk1 in pediatric T-ALL.

The specific aims of each paper were:

Paper I: To investigate the connection between the expression of IFN-stimulated genes and drug resistance colorectal carcinoma.

Paper II: To elucidate the upstream signaling that lead to increased IRF9 and IRDS expression in conditions of high cell density.

Paper III: To identify novel interacting partners of STAT3 that affects the transcription of STAT3 target genes.

Paper IV: To evaluate a novel self-delivering RNAi prodrug targeting cell cycle kinase Plk1 in pediatric acute lymphoblastic leukemia.

3 RESULTS AND DISCUSSION

3.1 PAPER I AND II

Cell crowding induces interferon regulatory 9, which confers resistance to chemotherapeutic drugs

and

STAT3 is activated in multicellular spheroids of colon carcinoma cells and mediates expression of IRF9 and interferon stimulated genes

Therapy resistance remains a major challenge in the management of cancer patients. Conventional monolayer culture lacks many of the features that contribute to resistance in solid tumors and is therefore a poor experimental system for studying resistance mechanisms. 3D models, such as multicellular spheroids, have been shown to exhibit physiological features that reflect the conditions of *in vivo* tumors and display decreased sensitivity to anti-cancer drugs compared to 2D-cultured cells (Nath and Devi 2016). Culturing of cell in MCS alters signaling pathways resulting in vast changes in gene expression. We compared the gene expression profile of colorectal cancer cell line HCT116 cultured in 3D and 2D in an attempt to identify genes associated with drug resistance in MCS.

Microarray analysis revealed differential expression of over 3000 genes in cells cultured as MCS compared to monolayer. Among the top upregulated genes were a group of IFN-stimulated genes (ISGs) (**Paper I**, Supporting Information Table 1). In order to determine if expression of this group of ISGs were specific for the HCT116 cell line, we cultured cell lines of different origin (ovarian-, breast- and colon carcinomas) in 2D and 3D. The expression of three ISGs identified in the microarray and the members of the ISGF3 transcription complex (OAS1, IFITM1, IFI27, STAT1, STAT2, and IRF9), were analyzed by qRT-PCR and found to be upregulated in 3D across all cell lines, however to a varying degree (**Paper I**, Supplementary Figure S1). We conclude that the increased expression of this set of ISGs is a common phenomenon in MCS.

When attempting to identify the mechanism behind the induction of ISGs, we discovered that HCT116 cells cultured to confluence in 2D also upregulated the mRNA expression of these genes (**Paper I**, Figure 2c). Protein levels of the members of the ISGF3 complex (STAT1, STAT2, and IRF9) were also increased over time (**Paper I**, Figure 2a**)**. By comparing cells cultured for 24h (non-confluent) to cultures in different states of confluency, we reasoned that the induction of these ISGs depend on the density of the cells and, possibly, on cell-to-cell contact.

Transcription of ISGs are regulated by the ISGF3 complex (Fu et al. 1990). Hence, we used RNAi to knock down the expression of STAT1, STAT2 and IRF9 in 2D culture in order to investigate if this affected the induction of the ISGs (OAS1, IFI27, and IFITM1). STAT1 knockdown affected neither the mRNA expression of the ISGs, nor the protein or mRNA levels of IRF9 and STAT2 in confluent monolayer cells (**Paper I**, Supplementary Figure S3a-b, Figure 3a). On the other hand, knockdown of either IRF9 or STAT2, resulted in a

clear reduction in ISGs expression (**Paper I**, Figure 3c-d). This effect was also observed in HCT116 cells stably transduced with shIRF9 or shSTAT2 and cultured as MCS (**Paper I**, Figure 3e). Interestingly, knockdown of either IRF9 or STAT2 negatively affected the expression of STAT1, in both confluent monolayer culture and MCS (**Paper I**, Figure 3b-e). Furthermore, culturing of STAT1-negative cell line U3A (Müller et al. 1993) and parental line 2f-TGH demonstrated that STAT1 is not necessary for induction of ISGs expression in confluent monolayer culture, however it clearly augmented the effect (**Paper I**, Supplementary Figure S3d-e). Hence, IRF9 and STAT2 appear to drive the expression of a set of ISGs, independent of STAT1, under high cell density conditions. Indeed, it has been reported that IRF9 and STAT2 can form a complex, however, if this complex alone can induce the expression of ISGs remains to be investigated (Fink et al. 2013).

Expression of ISGs has previously been associated with therapy resistance. 17 out of the top 50 overexpressed genes in doxorubicin-resistant myeloma cells compared to parental cells were ISGs (Fryknäs et al. 2007). In HCT116-SN6 cells, which are resistant to the topoisomerase I inhibitor Irinotecan, ISGs constituted 29% of the most highly expressed genes (Gongora et al. 2008). Furthermore, a gene signature of 49 ISGs, termed the IFN-related DNA damage resistance signature (IRDS), has previously been associated with acquired resistance to radio- and/or chemotherapy in several different cancer cell lines and can be used as a predictive marker[1] in breast cancer (Weichselbaum et al. 2008). Gene set enrichment analysis (GSEA) revealed a high degree of similarity between the IRDS and the genes induced in our microarray (**Paper I**, Figure 1a), suggesting that the IRDS could be induced in conditions of high cellular density and contribute to the intrinsic drug resistance observed in MCS.

STAT1 has been proposed to be the main driver of IRDS expression and resistance (Weichselbaum et al. 2008; N. N. Khodarev et al. 2004). However, in our system, STAT1 was not required for the induced expression of IRF9, STAT2 or the ISGs. In a study by Luker et al. STAT1, STAT2 and IRF9 were found to be upregulated in paclitaxel resistant MCF7 cells. Interestingly, transient transfection of IRF9 alone, but not of STAT1 and STAT2, in the parental MCF7 cells significantly reduced the sensitivity to paclitaxel suggesting that IRF9 is the main driver of resistance in this system. Overexpression of IRF9 did induce transcription of STAT1 and STAT2, but expression of the genes in the IRDS was not investigated in this study (Luker et al. 2001). In order to investigate if IRF9 expression could trigger the expression of the ISGs and confer drug resistance in our system we stably transfected HCT116 cells with IRF9 (**Paper I**, Figure 4). mRNA expression of IFI27, IFITM1 and OAS1 were all increased in IRF9 overexpressing cells compared to mock transfected. Furthermore, IRF9 overexpressing cells were less sensitive to cisplatin, docetaxel, oxaliplatin, 5FU and etopside. Additionally, transient knockdown of IRF9 sensitized HCT116 cells to cisplatin.

[1] Indicates sensitivity or resistance to a specific therapeutic agent.

Hence, we concluded that increased IRF9 expression, induced in conditions of high cellular density, mediates expression of ISGs and confers resistance to chemotherapeutic agents.

Based on the finding that high cellular density leads to increased expression of IRF9 and, in turn, IRDS genes, we sought out to identify the upstream signaling pathway responsible for this induction in **Paper II**.

The results from our microarray of HCT116 cells showed an increase in STAT3 mRNA expression in MCS compared to cells in monolayer culture (**Paper I**, Supporting Information Table 1). Several studies have shown that STAT3 is activated in conditions of high cellular density (Steinman et al. 2003; Kreis et al. 2007). In agreement with this, we found that STAT3 is phosphorylated on tyrosine 705 in HCT116 cells cultured to confluence in 2D and in 3D (**Paper II**, Figure 1a). Phosphorylation of STAT1 and STAT2 was also observed, however only in 3D and not in confluent monolayer culture (**Paper II**, Supplementary Figure S1a).

In accordance with our previous results we found that RNAi mediated knockdown of IRF9 in HCT116 MCS resulted in a significant reduction of a panel of IRDS genes (OAS1, IFI6, IFI27, and IFI44). Phosphorylated- and total protein expression of STAT1 was also substantially reduced. On the other hand, STAT3 protein levels were not affected by IRF9 knockdown, suggesting that STAT3, if involved in this signaling pathway, is upstream of IRF9 (**Paper II**, Figure 1f-g). Knockdown of STAT3 in MCS completely abolished IRF9 protein expression and significantly reduced the mRNA expression of the IRDS genes (**Paper II**, Figure 3c-d), suggesting that STAT3 is upstream of IRF9 in the signaling pathway. However, STAT3 knockdown also altered the morphology of the sphere, making it difficult to distinguish if the effects on IRF9 and the IRDS genes were a direct cause of the STAT3 knockdown or a consequence the altered sphere-morphology (**Paper II**, Figure 3e). In order to determine this, we utilized another colorectal cancer cell line, DLD1, and its sub-lines A4 (STAT3-null) and A4wt (reconstituted with wt STAT3) (J. Yang et al. 2010). Despite the absence of STAT3, A4 cells cultured in 3D formed round-type spheres with a defined edge and were visibly indistinguishable from the A4wt spheres. However, IRF9 protein levels were clearly lower in A4 MCS compared to A4wt or parental DLD1, suggesting that STAT3 is involved in the upregulation of IRF9 in MCS (**Paper II**, Supplementary Figure 4c, Figure 3f). Additionally, we found a moderate, but significant, correlation between STAT3 and IRF9 protein abundance in 95 primary colorectal tumor patient samples and 44 colorectal cancer cell lines, further suggesting a connection between STAT3 and IRF9 in colorectal cancer (**Paper II**, Figure 4e).

Chromatin immunoprecipitation (ChIP) sequencing data from the University of California, Santa Cruz (UCSC) genome browser show that STAT3 binds to the IRF9 promoter, near the transcriptional start site, in several cell lines (**Paper II**, Figure 4a). Furthermore, a study by Lu et al. identified STAT3 at this site in the IRF9 promoter in two large B-cell lymphoma cells lines (Lu et al. 2018). However, in that system, STAT3 appeared to negatively regulate IRF9 transcription. We identified a possible STAT3 binding site located at position -12 in the IRF9 promoter. This exact sequence (TTCTGGGAA) has previously been identified as the

acute-phase response element (APRE). STAT3 is also known as the acute-phase response factor (APRF) and has been shown to bind to the APRE and induce transcription in response to IL6 stimulation (Wegenka et al. 1994; Ehret et al. 2001). In order to determine if STAT3 directly regulates IRF9 transcription in our system, we performed ChIP with an anti-STAT3 antibody and designed primers spanning the potential STAT3 binding site. We found STAT3 to be significantly enriched at the IRF9 promoter in MCS compared to non-confluent monolayer culture (**Paper II**, Figure 4c-d). Since we observed increased levels of IRF9 mRNA and protein in MCS, we conclude that STAT3 directly drives IRF9 transcription in this system.

Next, we wanted to investigate which upstream signaling pathway led to activation of STAT3 in high cell density conditions. STAT3 activation induced by high cell density in melanoma cells have been reported to be mediated by JAKs (Kreis et al. 2007). We cultured HCT116 cells in 3D for 6 days in the presence of two different JAK inhibitors and found that phosphorylation of STAT3, as well as protein expression of IRF9 was abolished. Furthermore, expression of the IRDS genes were significantly reduced (**Paper II**, Figure 2a-c). JAK-STAT3 is activated by cytokines that belong to the IL6-family who all signal through the receptor subunit gp130 (Silver and Hunter 2010). Blocking gp130 signaling, using two different inhibitors, had the same effect as the JAK inhibitors in HCT116 MCS (**Paper II**, Figure 2d-e), suggesting that the activation of STAT3 and the induction of IRF9 and the IRDS genes are downstream of the gp130-JAK signaling pathway.

The involvement of gp130 indicated that a cytokine could be responsible for activating this signaling pathway. Thus, we transferred conditioned medium (CM) from confluent monolayer culture to freshly seeded non-confluent cells, and observed phosphorylation of STAT3, increased protein levels of IRF9 and a significant upregulation of the IRDS genes (**Paper II**, Supplementary Figure 2a-b). In accordance with what we observed in MCS, this effect was blocked by the JAK or gp130 inhibitors, suggesting a common mechanism (**Paper II**, Figure 2f, Supplementary Figure 2d-e). IFNs can be secreted by the tumor cells or cells in the tumor microenvironment (Cheon, Borden, and Stark 2014), hence, we investigated if IFNs could be involved in the induction of IRF9 and the IRDS genes in our model. No induction of IFN mRNA was observed in our microarray (**Paper I**, Supporting Information Table 1), nor could we detect secreted IFNα, IFN β or IFNγ in CM by ELISA. Furthermore, IRF9 and the IRDS genes were readily induced in confluent U5A cells, a sub-line of the 2fTHG cell line that lack a functional IFNAR2, thus impairing the response to type I IFNs (**Paper I**, Supporting Information Figure S3g). The fact that gp130 inhibitors effectively blocked the induction of STAT3, IRF9 and the IRDS genes suggested the involvement of a member of the IL6 family of cytokines. IL6 is a known inducer of IRF9 expression, however, not directly through STAT3 (Weihua et al. 2000). We did not observe any induction of IL6 in MCS (**Paper I**, Supporting Information Table 1) or in CM from confluent monolayer culture. In addition, the use of an IL6-neutralizing antibody did not affect STAT3 phosphorylation induced by CM (**Paper II**, Supplementary Figure S3a). Among the other members of the IL6 family of cytokines, we found that mRNA levels of both oncostatin M (OSM) and the OSM-receptor (OSMR) were upregulated in MCS compared to 2D culture (**Paper I**, Supporting

Information Table I, **Paper II**, Supplementary Figure S3b). However, addition of an OSM-neutralizing antibody did not block the phosphorylation of STAT3 or the induced expression of IRF9 by CM in non-confluent monoculture (**Paper II**, Supplementary Figure S3c). Thus, the factor responsible for activating the signaling pathway that leads to STAT3 activation, IRF9 expression, and the subsequent upregulation of the IRDS genes still remains unknown.

To summarize, our findings demonstrate that a set of ISGs is induced in cancer cell lines of different origin cultured at high cellular density, such as multicellular spheroids. STAT1 augments this effect but is not required for this upregulation in HCT116 cells. We found that overexpression of IRF9 alone was sufficient to induce the expression of these ISGs and decrease the sensitivity to chemotherapeutic drugs. Furthermore, we show that STAT3 is activated, through gp130-JAK signaling, in conditions of high cellular density and directly drives the expression of IRF9. We propose a novel mechanism where STAT3 activation, in conditions of high cellular density, induces the expression of IRF9 and subsequently IRDS genes, underlining a mechanism by which drug resistance may be regulated in tumors.

3.2 PAPER III

STAT3 interacting proteins identified in a complex with DNA affect expression of STAT3-dependent genes

Constitutive activation of STAT3 has been observed in a large variety of cancers, both solid tumors and hematological malignancies. Activated STAT3 induces the expression of target genes that regulate a number of oncogenic processes, such as proliferation, inhibition of apoptosis, migration, angiogenesis and tumor-associated inflammation (Yu and Jove 2004). The specificity of the STAT3-induced genes is most likely dependent on the nature of the stimuli and the cell type, as well as additional factors that cooperate with STAT3 in gene regulation. However, the exact mechanisms behind the cell- and tissue specific STAT3-dependent response remains largely unknown. IL6 is a well-described activator of STAT3 and has been linked to the pathogenesis of several cancers, including colorectal carcinoma (Becker et al. 2005; Schafer and Brugge 2007). A number of studies have previously attempted to identify interacting partners of STAT3, using different proteomics-based approaches (Zheng et al. 2012; Yeh et al. 2015; Blumert et al. 2013). In this study we aimed to identify proteins that interact with STAT3 on DNA upon IL6 stimulation, potentially affecting transcription of STAT3-target genes.

To this end, we performed immunoprecipitation (IP) with anti-STAT3 antibodies, followed by DNA biotinylation, pull down of DNA and finally mass spectrometry (MS) to identify interacting proteins (**Paper III**, Figure 1a). In order to be able to distinguish the hits from the background, we used two cell lines derived from colorectal carcinoma cell line DLD1; A4 where STAT3 was homozygously deleted, and A4wt, where A4 were reconstituted with wild type STAT3. The identified proteins were sorted according to protein area, A4 cells from lowest to highest and A4wt from highest to lowest (**Paper III**, Figure 1f). Proteins identified in A4wt cells treated with IL6, which were not present in A4 cells, were considered hits. STAT1, which is known to form heterodimers with STAT3, was also identified in our system (Delgoffe and Vignali 2013). Among the hits, we chose three proteins to investigate further;

histone deacetylase 2 (HDAC2), thyroid hormone receptor 3 (THRAP3) and chromobox protein 3 (CBX3). HDAC2 has previously been identified as an interacting protein of STAT3, although, whether or not they interact on DNA has not been investigated before (Z.-L. Yuan et al. 2005). CBX3 is a component of heterochromatin and has been linked to tumor cell proliferation and cancer stem cells (Fan et al. 2017; Saini et al. 2012). THRAP3 is involved in pre-mRNA splicing and has been demonstrated to regulate the DNA damage response (Vohhodina et al. 2017). Neither CBX3 nor THRAP3 have previously been identified as STAT3-interacting proteins.

In order to investigate if these potential interacting partners affect STAT3-dependent transcription, we utilized the A4wt cells carrying a luciferase reporter containing the STAT-inducible element (SIE), derived from the promoter of the STAT3-inducible gene CFOS (Kolosenko et al. 2017). We used RNAi to knock down STAT1, HDAC2, CBX3 and, THRAP3 and monitored the activity of the reporter in response to IL6. Cells were set up in parallel and harvested for RT-qPCR analysis in order to be able to assess the knock down efficacy. In addition, in these samples we also analyzed the mRNA expression of three STAT3 target genes; JUNB, MUC1, and BCL3 (Kolosenko et al. 2017). Knockdown of STAT3 and each of the candidate interacting proteins resulted in a decreased activity of the luciferase reporter (**Paper III**, Figure 2b). Efficient knockdown was observed for all targets except STAT3, possibly due to the constitutive expression of STAT3 from the cytomegalovirus (CMV) promoter in these cells (**Paper III**, Figure 2c, Supplementary Figure 3a). JUNB, MUC1 and BCL3 mRNA were all induced by IL6. However, since we failed to knock down STAT3 in these cells, we cannot conclude that the induction of JUNB, MUC1, and BCL3 is STAT3 dependent. Out of the three potential interacting partners, only HDAC2 knock down had a consistent inhibitory effect on the expression of these genes, suggesting that HDAC2 positively affects STAT3 transcriptional activity in response to IL6 stimulation (**Paper III**, Figure 2d, Supplementary Figure 3b-d). In order to confirm our findings, we knocked down STAT3 and HDAC2 in HCT116, another colorectal cancer cell line. Out of the three STAT3-target genes, only MUC1 was readily induced by IL6, demonstrating the cell line specificity of STAT3 signaling. Efficient knockdown of HDAC2 resulted in a significant reduction of MUC1 mRNA expression compared to IL6-treated siRNA control (**Paper III**, Figure 3c).

In terms of general transcription, deacetylation of histones by HDACs result in a more condensed chromatin and thereby, transcriptional inactivation (Gallinari et al. 2007). Type I HDACs (HDAC1-3) have also been shown to directly repress STAT3 transcriptional activity through deacetylation of STAT3 on Lys685 (Z.-L. Yuan et al. 2005). However, recent studies suggest that the function of lysine acetylation of STATs is multifaceted, and that HDAC activity, under specific conditions, may be required for STAT-dependent transcription (Icardi, De Bosscher, and Tavernier 2012). Furthermore, HDAC inhibitors have been shown to block transcription elongation[2], suggesting that HDAC activity could induce transcription (Greer et

[2] The process of copying DNA into RNA by RNA polymerase.

al. 2015). Our data show that IL6-induced STAT3-dependent transcription is repressed upon HDAC2 knockdown, suggesting that HDAC2 positively influence transcription of STAT3-dependent genes in this system. It has been suggested that down-regulation of HDAC2 could result in a compensating increase in expression of the other type I HDACs, which could be responsible for this effect (Senese et al. 2007). However, we did not detect any changes in mRNA expression of HDAC1 or 3 upon HDAC2 knockdown (**Paper III**, Figure 3d). The effect of HDAC2 knockdown on the protein levels of HDAC1 and 3 and the acetylation of STAT3 in our system remains to be investigated.

To summarize, we attempted to identify novel STAT3-interacting proteins that associate with STAT3 when bound to DNA. To do this we used a modified ChIP-protocol with antibodies targeting STAT3, pulled down DNA and identified proteins using mass spectrometry. The hits were known STAT3 interacting proteins as well as new potential interactors. Among the candidates, only HDAC2 knockdown displayed a consistent negative effect on STAT3 transcriptional activity. Thus, our data suggests that HDAC2 positively influences STAT3 transcription. However, HDACs are known to regulate transcription by several different mechanisms and further studies are required to determine the exact role of HDAC2 in STAT3-driven transcription. Additional experiments are also warranted to verify that the identified proteins do interact with STAT3 upon IL6 stimulation.

3.3 PAPER IV

RNAi prodrugs targeting Plk1 induce specific gene silencing in primary cells from pediatric T-acute lymphoblastic leukemia patients

While the cure rate for pediatric ALL has greatly improved over the past decades, long term side effects from the treatment, such as secondary cancers or cardiac toxicity are common (Robison et al. 2009). Thus, novel targeted therapies are warranted in order to reduce acute and long-term side effects and further improve overall survival. Cell cycle kinase Plk1 is upregulated in multiple different types of cancer and several small molecular inhibitors targeting this kinase have been developed (Juping Yuan et al. 2011; Rudolph et al. 2009). However, small molecules are often unspecific and may inhibit other members of the Plk family or other kinases. The high specificity of RNAi makes it an appealing strategy for treatment of human disease. RNAi prodrugs, called short interfering ribonucleic neutrals (siRNNs), are modified siRNA molecules whose phosphate backbone contain neutral phosphotriester groups, which allows for delivery into cells (Meade et al. 2014). Once inside, the phosphotriester groups are cleaved by cytoplasmic thioesterase and the remaining native phosphodiester siRNA is loaded into RISC and complementary mRNA is cleaved. Previous studies have suggested that depletion of Plk1 using RNAi results in cancer cell-specific toxicity (X. Liu, Lei, and Erikson 2006). Thus, in this study we investigate the effects of Plk1 knockdown in samples from pediatric ALL patients and healthy donors using self-delivering siRNNs. The T-ALL patient samples were collected at the time of primary diagnosis. Mononuclear cells were isolated from peripheral blood and/or bone marrow and cryo-preserved in liquid nitrogen.

Aberrant Plk1 expression has been reported in adult and pediatric ALL patient samples and cell lines (Ikezoe et al. 2009; Hartsink-Segers et al. 2013). In line with this, we found that Plk1 mRNA expression was higher in pediatric T-ALL cell lines and patient samples compared to peripheral blood mononuclear cells (PBMCs) from healthy donors (**Paper IV**, Figure 1a). Plk1 protein was observed in three T-ALL cell lines while undetectable in PBMCs by Western blot (**Paper IV**, Figure 1b). It is important to note that Plk1 is a cell cycle kinase and its expression correlates to the mitotic index. PBMCs are quiescent and, therefore, express very low levels of this kinase. Stimulation of PBMCs with anti-CD3 and IL2 did induce mRNA and protein expression of Plk1 (**Paper IV**, Figure 5c-d), however, we did not compare the levels in cycling PBMCs to that of the ALL cell lines or patient samples.

Next, we analyzed the ability of the siRNNs to enter cells and deplete the target mRNA. Reduced protein levels of Plk1 was observed in two T-ALL cell lines, Jurkat and CCRF-CEM, 48h after treatment (**Paper IV**, Figure 2a). Knockdown or inhibition of Plk1 leads to cell cycle arrest and apoptosis. Phosphorylation of histone H3 (pH3) is a marker for G2 arrest and was induced by Plk1 siRNN as well as by BI6727, an ATP-competitive small molecular inhibitor of Plk1. Apoptosis was induced by both Plk1 siRNN and BI6727 as detected by Western blot analysis of cleaved Poly (ADP-ribose) polymerase (PARP) and Annexin V (AxV)/Propidium Iodide (PI) staining by flow cytometry. The negative control siRNN targeting luciferase (Luc), did not induce cell cycle arrest or apoptosis compared to the untreated control (**Paper IV**, Figure 2a-c). Thus, the siRNN prodrugs are able to enter the cells and reduce the levels of Plk1, which leads to cell cycle arrest and apoptosis.

The majority of the cells in the pediatric T-ALL patient samples were not cycling upon thawing (**Paper IV**, Figure 3a). Our results in cell lines showed that the full effect of siRNN treatment is reached after 48h, hence, the patient cells had to be viable in culture for at least 3 days. In order to achieve this, we used a kit for expansion/activation of T-cells and IL-2 treatment to induce proliferation in two peripheral blood-derived patient samples. Plk1 protein expression was readily induced at day 4 and 10 after initiation of stimulation. Expression of Plk2 showed the inverse pattern, the protein was detected at day 0 in both patient samples and were undetectable by Western blot at day 4 and 10 (**Paper IV**, Figure 3c). Treatment of these patient samples with BI6727 did not induce apoptosis as assessed by flow cytometry of AxV-stained cells (**Paper IV**, Figure 3e-f). Plk1 siRNN induced an 8% increase in apoptotic cells in patient sample A3834 compared to Luc siRNN control (**Paper IV**, Figure 3e). In patient sample A211 Plk1 siRNN and Luc siRNN control increased the number of apoptotic cells with 19% and 15% respectively, compared to untreated control (**Paper IV**, Figure 3f). In order to further investigate the effects of Plk1 siRNN treatment we stimulated two additional patient samples, one isolated from peripheral blood and one from bone marrow. A decrease in Plk1 mRNA and protein was observed in both samples 48h after Plk1 siRNN treatment. Importantly, the mRNA levels of Plk2 and Plk3 were not affected by Plk1 knockdown (**Paper IV**, Figure 4a-h). Plk1 siRNN induced cell cycle arrest in the bone marrow sample alone, while BI6727 induced arrest in both samples as assessed by pH3 detected by Western blot (**Paper IV**, Figure 4a-b). An increase of apoptotic cells was observed by both BI6727 (9%) and Plk1 siRNN (15%) treatment in the peripheral blood

patient sample, compared to their individual controls (**Paper IV**, Figure 4i). In the bone marrow patient sample both Luc siRNN control and Plk1 siRNN treatment resulted in an increase in AxV-positive cells (26% and 33% respectively) compared to the untreated control (**Paper IV**, Figure 4j). Lastly, one additional patient sample was stimulated to proliferate and treated with BI6727 or siRNNs. A reduction of Plk1 protein was observed in the Plk1 siRNN treated sample. Cell cycle arrest and DNA damage was induced by both BI6727 and Plk1 siRNN, as assessed by pH3 and phosphorylated histone H2AX (pH2AX) by Western blot (**Paper** IV, Figure 5e). These data show that the siRNN prodrugs induce specific RNAi-mediated reduction of Plk1 mRNA in cycling cells from ALL patients, which resulted in induction of apoptosis and, in two samples, cell cycle arrest. The toxicity induced by Luc siRNN control needs to be investigated and more patient samples analyzed before any general conclusions can be drawn regarding the effect of the siRNN prodrugs.

Plk1 plays an important role during several stages of the cell cycle. Injecting Plk1 specific antibodies into HeLa cells resulted in failure to correctly assemble the mitotic spindle and subsequent cell death (Lane and Nigg 1996). By contrast, non-immortalized Hs68 fibroblasts arrested in G2, but did not display this aberrant spindle, possible due to intact cell cycle checkpoints that require a functional Plk1 (Suqing Xie et al. 2005). Furthermore, expression of dominant negative Plk1 induced apoptosis in two cancer cell lines, while normal epithelial cells displayed decreased proliferation but very low apoptosis (Cogswell et al. 2000). These studies suggest that non-transformed cells are less sensitive to Plk1 inhibition, possibly due to intact mitotic checkpoints that have been disrupted in cancer cells. In order to investigate the effect of Plk1 inhibition or depletion in healthy cells we used the same CD3/IL2-stimulation protocol, previously applied to the T-ALL patient cells, to induce proliferation as well as Plk1 mRNA and protein expression in PBMCs from healthy donors (**Paper IV**, Figure 5a-d). Cycling PBMCs from one donor were treated with BI6727 and Plk1 siRNN prodrugs for 48h. BI6727 induced G2/M arrest and DNA damage as assessed by pH3 and pH2AX by Western blot. Plk1 siRNN treatment did not induce cell cycle arrest or DNA damage to the same extent as BI6727. However, no obvious reduction in Plk1 protein expression was observed upon siRNN treatment (**Paper IV**, Figure 5d). Hence, further experiments are required in order to determine the toxicity of Plk1 siRNN treatment in healthy proliferating cells.

The major hurdle for successful RNAi therapeutics is to effectively deliver the siRNA into cells. The neutral phosphotriester backbone of siRNNs provides a possible solution to this problem. In order to further facilitate the delivery, a cell-penetrating TAT-peptide has been conjugated to the phosphotriesters. Furthermore, tissue-targeting domains can be conjugated to the siRNNs in order to improve the specificity. Systemic delivery of siRNNs conjugated to a hepatocyte-targeting moiety resulted in a decrease of the target mRNA in the liver of mice. Importantly, siRNNs enter cells without eliciting an innate immune response and, in contrast to siRNAs, bind to serum and thus improving the pharmacokinetic properties (Meade et al. 2014). However, we observed a drastic reduction in transfection efficacy of the siRNNs in the presence of physiologically relevant levels of serum. All transfections using siRNNs in this study were therefore performed in serum-free conditions. Studies in pediatric ALL patient-

derived xenografts in mice could be the way forward to determine the delivery and action of the siRNN prodrugs.

To summarize, we have attempted, for the first time, to assess the effect of siRNN prodrugs in T-ALL cell lines and patient samples. Our data shows that the siRNN prodrugs can enter cells and induce RNAi-mediated degradation of Plk1 mRNA, which leads to cell cycle arrest and apoptosis. The observed toxicity of the control siRNN needs to be investigated and the effect of Plk1 siRNN treatment in healthy cells also requires further studies.

4 CONCLUDING REMARKS

In **Paper I** and **Paper II** we show that STAT3 is activated in conditions of high cellular density, downstream of gp130-JAK signaling, and drives the transcription of IRF9. IRF9 subsequently induces the expression of a subset of ISGs that confer resistance to chemotherapeutic drugs. This finding further implicates STAT3 as a mediator of drug resistance and identifies IRF9 as the main factor driving the expression of the ISGs. Furthermore, we demonstrate that IRDS genes are induced in conditions of high cell density, suggesting that this could be one of the mechanisms contributing to multicellular drug resistance. Despite our efforts, we did not succeed in identifying the factor responsible for inducing this signaling pathway. However, our results suggest that it is member of the IL6 family of cytokines and further experiments, for example using neutralizing antibodies, could identify the responsible factor.

In order to find novel STAT3-interacting proteins that affect transcription of STAT3-target genes we used a modified ChIP protocol, combined with mass spectrometry to identify proteins that interact with STAT3 on DNA (**Paper III**). Among the potential interacting proteins, only HDAC2 knockdown had a consistent negative effect on the expression of STAT3 target genes induced by IL6 stimulation. Thus, our data suggests that HDAC2 positively regulates STAT3 transcriptional activity. However, further studies are required in order to verify that the identified proteins do interact with STAT3 and to elucidate the role of HDAC2 in regulating STAT3 transcriptional activity.

In **Paper IV** we showed that Plk1 siRNN prodrugs successfully enter T-ALL cells and induce specific RNAi mediated degradation of Plk1 mRNA. The reduction of Plk1 levels led to cell cycle arrest and apoptosis in pediatric T-ALL patient samples, which were cultured ex vivo and stimulated to proliferate. The observed toxicity induced by the Luc siRNN control needs to be investigated and more patient samples analyzed before any general conclusions can be drawn regarding the effect of the siRNN prodrugs.

5 ACKNOWLEDGEMENTS

I would like to express my gratitude to everyone who in various ways have supported me during my PhD!

Katja, supervisor, extra-mom, and friend. Your never-ending curiosity and love for science is inspiring. Thank you for always looking out for me, for taking the time to talk (about science or life), and all of your support during these years.

Danne, thank you for taking me in, even though you already had too many students. Rest in peace.

To **Caroline**, for giving me the opportunity to do my PhD and for helping me prepare this thesis.

To my mentor, **Maggan,** for moral support and guidance during these years.

To **Nathan**, the man with all the best dance moves. Work is never boring when you are around.

Linda, the number of NERF-gun fights decreased significantly since you left, and I don't like it. Please come back and gossip with me in the cell lab.

Jason, thank you for all the support, personal and scientific.

Matheus, the founder of the Moist group, you are one of a kind. Thank you for all the laughs.

Yasmin, so talented and enthusiastic. It is a joy to have you around.

To **Martin** and **Vasilios**, thank you for making lunchtime a lot of fun!

To **Sander**, my STAT3 buddy, CCK was not the same without you.

To the past and present members of the Grandér/Pokrovskaja/Enge lab: **Angelo**, **Huaitao, Iryna, Lotte, Santi and Tiago.**

My, my best friend, postdoc on-call, and statistics guru. Thank you for always being there. I truly could not have done this without you.

To **Ana** and **Robin**, I could not ask for better friends. Thank you for being there and always making me laugh. ¡Manzanas!

To all the KI people who have made these years so enjoyable: **Alex, Axel, Carolina, Chiara, David, Francesca, Joanna, Marc, Mimmi, Muyi, Nicole, Nikos, Ran, Sofie, Sophia, William, Xinsong,** and everyone else from the third floor of CCK!

Thank you to **Anna**, **Janne**, and all the four-legged members of Cirkus Björklund <3

Lastly, to my amazing family, for endless support.

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