From the Department of Medical Biochemistry & Biophysics
Division of Biochemistry
Karolinska Institutet, Stockholm, Sweden

SHEDDING LIGHT ON THE STAT3 SMALL MOLECULE INHIBITOR FIELD

Sander Busker

Karolinska Institutet

Stockholm 2019

Published by Karolinska Institutet.
Printed by E-Print AB, 2019

ISBN 978-91-7831-478-2

Shedding light on the STAT3 small molecule inhibitor field
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Sander Busker

Principal Supervisor:
Professor Elias S. J. Arnér
Karolinska Institutet
Department of Medical Biochemistry & Biophysics
Division of Biochemistry

Co-supervisor(s):
Assistant Professor Brent D. G. Page
University of British Columbia
Department of Pharmaceutical Sciences

Docent Katja Pokrovskaja-Tamm
Karolinska Institutet
Department of Oncology-Pathology

Assistant Professor Daniel Hägerstrand
Karolinska Institutet
Department of Oncology-Pathology

Opponent:
Professor Masayuki Yamamoto
Tohoku University
Department of Basic Medicine
Division of Medical Biochemsitry

Examination Board:
Professor Anna Karlsson
Karolinska Institutet
Department of Laboratory Medicine
Division of Clinical Microbiology

Assistant Professor Giovanna Zinzalla
Karolinska Institutet
Department of Microbiology, Tumor & Cell Biology

Professor Helena Danielson
Uppsala University
Department of Chemistry
Division of Biochemistry

To Christina and our family

We are the music makers,
And we are the dreamers of dreams,
Wandering by lone sea-breakers,
And sitting by desolate streams; —
World-losers and world-forsakers,
On whom the pale moon gleams:
Yet we are the movers and shakers
Of the world for ever, it seems.

With wonderful deathless ditties
We build up the world's great cities,
And out of a fabulous story
We fashion an empire's glory:
One man with a dream, at pleasure,
Shall go forth and conquer a crown;
And three with a new song's measure
Can trample a kingdom down.

We, in the ages lying,
In the buried past of the earth,
Built Nineveh with our sighing,
And Babel itself in our mirth;
And o'erthrew them with prophesying
To the old of the new world's worth;
For each age is a dream that is dying,
Or one that is coming to birth.

Ode by Arthur O'Shaughnessy

ABSTRACT

STAT3 is one of the seven family members of the STAT transcription factor family. STAT3 has become a very attractive target for cancer therapy and other diseases. It has a prominent role in cancer development as well as progression. However, there are still no STAT3 inhibitors applied in the clinic. One of the underlying reasons might be the complicated regulation of STAT3 function. Not only do many signaling pathways converge on STAT3, it is also a redox sensitive transcription factor. Therefore, STAT3 activity is determined by a plethora of different intracellular and extracellular signals. STAT3 also affects many different cellular functions and has a role in each hallmark of cancer, either through its role as a transcription factor or through one of its diverse non-transcriptional functions.

This thesis focuses on the STAT3 small molecule inhibitor field, by trying to further explore the mechanism of action of novel and widely used STAT3 inhibitors. In addition, we developed a new method to discover specific STAT3 inhibitors. Interestingly, we found that STAT3 was not targeted directly, but rather STAT3 is inactivated due to oxidation of cysteine residues on the protein.

In **Paper I**, we developed a cell-based high-throughput screening system to evaluate STAT3 transcriptional activity. This was used to screen 28.000 compounds, where after several additional screening steps four lead compounds were selected for further evaluation. All four compounds inhibited STAT3 transcriptional activity. Their mechanisms of action however appeared to be diverse. One compound, KI16, was found to preferentially inhibit STAT3 function compared to STAT1, and inhibited STAT3-driven phenotypes.

In **Paper II**, we used differential scanning fluorimetry (DSF) to highlight some of the shortcomings of other *in vitro* STAT3 inhibitor methods. Two STAT3 protein truncations were used to evaluate SH2 domain binders of STAT3 as well as binding specificity of well-known STAT3 small molecule inhibitors. Phosphopeptides were able to specifically bind to the STAT3 SH2 domain and increase protein thermal stability. While two small molecule inhibitors did not affect thermal stability of either truncation, two other small molecules, Stattic and BP1-102 decreased stability of both truncations. Which was an indication that both small molecules are not specific SH2 domain binders, but rather target STAT3 in multiple sites leading to its destabilization.

In **Paper III**, we performed structure-activity relationship (SAR) on a selected series of compounds that were identified in the high-throughput screening in **Paper I**. Sub 1 μM compounds were generated that potently inhibited STAT3 transcriptional activity. These compounds were found to have electrophilic properties, which were essential for STAT3 inhibition. With the use of a fluorescently-tagged compound we were able to pinpoint the cellular protein target, thioredoxin reductase 1 (TrxR1). The top compounds as well as Stattic were potent inhibitors of TrxR1 function, and were found to induce oxidative stress. Vice versa, TrxR1 inhibitors were also able to inhibit STAT3 transcriptional activity. Oxidative stress induction leads to the oxidation of Prx2, which can relay its oxidative equivalents to

STAT3 that forms oxidized dimers and is transcriptionally inactivated. TrxR1 is the main reductase that reduces Prx2 and STAT3 through consumption of NADPH. However due to the compounds inhibiting its function, the proteins remain oxidized, and eventually leads to cell death.

LIST OF SCIENTIFIC PAPERS

I. Iryna Kolosenko, Yasmin Yu, **Sander Busker**, Matheus Dyczynski, Jianping Liu, Martin Haraldsson, Caroline Palm Apergi, Thomas Helleday, Katja Pokrovskaja-Tamm, Brent D. G. Page. Identification of novel small molecules that inhibit STAT3-dependent transcription and function. *PLoS One* 2017 June 21; 12(6): e0178844

II. Matthieu Desroses, **Sander Busker**, Juan Astorga-Wells, Sanaz Attarha, Iryna Kolosenko, Roman A. Zubarev, Thomas Helleday, Dan Grandér, Brent D. G. Page. STAT3 differential scanning fluorimetry and differential scanning light scattering assays: Addressing a missing link in the characterization of STAT3 inhibitor interactions. *JPBA* 2018 July 14; 160: 80-88

III. **Sander Busker**, Weixing Qian, Martin Haraldsson, Lars Johansson, Belen Espinosa, Sanaz Attarha, Iryna Kolosenko, Jianping Liu, Markus Dagnell, Dan Grandér, Elias S. J. Arnér, Katja Pokrovskaja-Tamm, Brent D. G. Page. Disrupting oncogenic STAT3 activity by targeting TrxR1 with irreversible covalent inhibitors. *Submitted manuscript*.

CONTENTS

LIST OF ABBREVIATIONS

ABL	Abelson murine leukemia viral oncogene homolog 1
ADME	Absorption, distribution, metabolism and excretion
ALK	Anaplastic lymphoma kinase
AML	Acute myeloid leukemia
ANXA2	Annexin A2
APML	Acute promyelocytic leukemia
ARE	Antioxidant response elements
ASK1	Apoptosis signal-regulating kinase
ATP	Adenosine triphosphate
Bcl-2	B-cell lymphoma 2
BCR	Breakpoint cluster region protein
BSF-2	B-cell stimulatory factor 2
BSO	Buthionine sulfoximine
CBS	Cystathione β-synthase
CD	Cluster of differentiation
CDK	Cyclin-dependent kinase
CETSA	Cellular thermal shift assay
CLL	Chronic lymphocytic leukemia
CML	Chronic myeloid leukemia
COX-2	Cyclooxygenase 2
CRP	C-reactive protein
CXCL	C-X-C motif chemokine
DNA	Deoxyribonucleic acid
DSF	Differential scanning fluorimetry
DSLS	Differential scanning light scattering
DTNB	5,5′-dithiobis-(2-nitrobenzoic acid)
EGFR	Epidermal growth factor receptor
EMSA	Electrophoretic mobility shift assay
EMT	Epithelial-mesenchymal transition
EpRE	Electrophilic response elements
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
ErbB	Erythroblastic leukemia viral oncogene
FAD	Flavin adenine dinucleotide
FGFR	Fibroblast growth factor receptor

FLT3	FMS-like tyrosine kinase 3
GAS	Gamma-activated sequence
Gp130	Glycoprotein 130
GPx	Glutathione peroxidase
GR	Glutathione reductase
Grx	Glutaredoxin
GSH	Glutathione (reduced)
GSSG	Glutathione (oxidized)
H_2O	Water
H_2O_2	Hydrogen peroxide
HDAC	Histone deacetylase
HDX	Hydrogen-deuterium exchange
HER2	Human epidermal growth factor receptor 2
HIF	Hypoxia-inducible factor
IC_{50}	Half maximal inhibitory concentration
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL6R	Interleukin 6 receptor
iNOS	Inducible nitric oxide synthase
IRDS	IFN-related DNA damage resistance signature
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
Juglone	5-hydroxy-1,4-naphthalenedione
kDa	Kilodalton
Keap1	Kelch-like ECH associated protein 1
KO	Knockout
LC8	Dynein light chain 8
LIF	Leukemia inhibitory factor
LIFR	Leukemia inhibitory factor receptor
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MDR	Multi-drug resistance gene
MEK	Mitogen-activated protein kinase kinase
MitoSTAT3	Mitochondrial STAT3
MMP	Metalloproteinase

MPN	Myeloproliferative neoplasm
mTOR	Mammalian target of rapamycin
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NF-κB	Nuclear factor-kappaB
NK cell	Natural killer cell
Nox	NADPH oxidase
Nrf2	NF-E2 related factor 2
O_2	Oxygen
$O_2^{\cdot -}$	Superoxide anion
ODN	Decoy oligonucleotide
$OH^{\cdot}$	Hydroxyl radical
OH^-	Hydroxyl anion
OSM	Oncostatin M
OSMR	Oncostatin M receptor
OXPHOS	Oxidative phosphorylation
PARP	Poly (ADP-ribose) polymerase
PD-1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1
PDGFR	Platelet-derived growth factor receptor
PDI	Protein disulfide-isomerase
PI3K	Phosphonositide 3-kinase
PK/PD	Pharmacokinetics/pharmacodynamics
pKa	Acid dissociation constant
Prx	Peroxiredoxin
PTEN	Phosphatase and tensin homolog
PTP	Protein tyrosine phosphatase
RA	Rheumatoid arthritis
RNR	Ribonucleotide reductase
ROS	Reactive oxygen species
Sec	Selenocysteine
SecTRAP	Selenium compromised thioredoxin reductase-derived apoptotic proteins
SH2	Src homology 2
SIE	STAT inducible elements
STAT	Signal transducer and activator of transcription
TGF	Transforming growth factor
TGR	Thioredoxin glutathione reductase

T_m	Melting temperature
TNF	Tumor necrosis factor
Trp	Tryptophan
Trp14	Thioredoxin related protein 14kDa
Trx	Thioredoxin
TrxR	Thioredoxin reductase
TYK2	Tyrosine kinase 2
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

1 INTRODUCTION

1.1 CANCER THERAPY DEVELOPMENT

Cells are the building blocks of tissues, constituting organs, which form functional systems, allowing a multicellular organism to become a complex functioning being. In order to keep this complexity organized, on a cellular level processes like cell division, cell death and metabolism need to be tightly controlled [1]. Cancer is defined by the loss of this control thereby allowing cells to divide unregulated. The disease itself is easily defined, but because the underlying cause is different per patient, the development, therapeutic response and clinical outcome are as well [2]. Consequently, it is very difficult to find the appropriate treatment for each patient.

Long after surgery and radiotherapy dominated cancer therapy, the field of chemotherapeutics was initiated. After the development of tumor model systems, transplantable tumors in rodents, the field started to advance. There were initial failures with drug screenings, however it was Charles Huggins who introduced hormonal therapy when he treated prostate cancer patients with hormones, earning him a Nobel Prize. Of course as many other big discoveries were found by accident, the first observation that kick started the testing of chemotherapeutic small molecule drugs came during the Second World War. An accidental spill of sulfur mustards on troops in Bari Harbor, Italy led to the discovery that the soldiers exposed had their bone marrow and lymph nodes significantly depleted. This led to the administration of nitrogen mustard to patients with non-Hodgkin's and other lymphomas, resulting in tumor regression [3]. This paved the way for synthesis and testing of related alkylating agents, such as Chlorambucil and Cyclophosphamide [4]. Around the same time the first antimetabolite was developed, the folic acid antagonist, methotrexate, and antitumor antibiotics as well, e.g. Actinomycin D. In the wake of these discoveries many different types of chemotherapeutics have been developed, the major groups being: alkylating agents, plant alkaloids, antitumor antibiotics, antimetabolites, topoisomerase inhibitors and others [4].

Early observations led to other sophisticated ways to treat specific tumors. Instead of systemic chemotherapeutics, targeted therapy is based on specific traits of tumors. Radioactive Iodine-131 for thyroid cancer patients was one of the first targeted therapies [5]. A more famous example is Tamoxifen targeting ER in breast cancer [6]. However, the first breakthrough discovery for molecular targeted therapy was Imatinib [7]. It was designed to target BCR-ABL fusion kinase in chronic myeloid leukemia (CML). BCR-ABL is the result of a specific chromosomal translocation of chromosome 9 and 22. It significantly improved survival and clinical outcome for CML patients since its FDA approval in 2001 [8]. This exploded the targeted therapy research and many small molecules and monoclonal antibodies have been approved for clinical use since, targeting specific oncogenes and kinases, e.g. EGFR, HER2, ALK, PARP, PI3K, VEGFR, PDGFR, Braf, MEK, CDK etc.

The importance of reactive oxygen species (ROS) and if their levels are higher in cancer cells has been a research topic under debate for many years. ROS are free radical and non-radical

oxygen species through derivation of oxygen [9]. In tolerable levels they have been shown to affect essential biological processes, e.g. proliferation, differentiation, migration and cellular signaling [10]. If there aren't enough cellular antioxidants to counteract the ROS, oxidative stress is induced, leading to DNA and other cellular damages, promoting oncogenesis [11]. Therefore, high basal levels of ROS are associated with oncogenesis. More important, the high cellular energy upkeep cancer cells demand in order to keep dividing also increases cellular oxidative stress [12]. In order to cope, oncogenes drive the expression of more antioxidants and subsequent elimination. This upward shift in the redox balance has been suggested to make cancer cells more sensitive to sudden increases in oxidative stress leading to selective cell death [12]. Interestingly, one of the main mechanisms of many chemotherapies and radiotherapy is their associated induction of oxidative stress [13,14].

1.2 IL6 SIGNALING IN CANCER

The cytokine Interleukin 6 (IL6) has a pleiotropic effect on immune response, inflammation and hematopoiesis. It was named BSF-2 for its ability to induce activated B-cells into antibody-producing cells, IFN-β2 for its antiviral activity, HPGF owing to its enhancement of fusion cell growth of plasma and myeloma cells, and HSF for its effect on acute phase protein synthesis in hepatocytes [15–19]. When BSF-2 cDNA was cloned in the lab of Kishimoto however, it was discovered that all these molecules were all the same gene. Henceforth the molecule has been named IL6 [20]. It is synthesized and can be secreted by many different cell types, including fibroblasts, T-cells and monocytes [21]. The classical pathway of IL6 signaling commences with IL6 binding to its receptor IL6R [22]. This receptor is mainly expressed on the membrane of specific immune cells and hepatocytes, but the receptor can be proteolytically cleaved and become a soluble factor as well [23]. The complex of cytokine and receptor has the capacity to bind to gp130 receptor, which is ubiquitously expressed on cells. These receptors form a heterotetramer, which can activate several downstream signaling pathways: Ras/MAPK. PI3K/Akt and JAK/STAT are all activated through gp130 tyrosine phosphorylation and drive cell proliferation and survival [24].

IL6 plays a role in many autoimmune diseases. Persistent IL6 signaling has been related to rheumatoid arthritis, arterosclerosis, asthma and lupus [25]. And persistent signaling has been shown to facilitate different facets of oncogenesis, progression and drug resistance [26]. IL6 and IL6R targeting drugs only have shown their clinical efficacy in rheumatoid arthritis (RA) and Castleman's disease, where Tocilizumab and Siltuximab were shown to have a beneficial effect over placebo treatment [27]. However, in various cancer types they have no demonstrated clinical efficacy [24]. This is not due to ineffectiveness of the drug, since it efficiently inhibits IL6 driven production of C-reactive protein (CRP) *in vivo* by hepatocytes of cancer patients [24,28]. Lack of efficacy is more likely due to cancer cell plasticity, and the multiple downstream signaling effects that IL6 inhibition has in cancer cells.

1.3 SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION (STATS)

The protein family of STATs was discovered during the exploration of IFN-α and IFN-γ signaling specificity [22,29]. The family is comprised of STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6 [30]. These predominantly cytoplasmic transcription factors were found to be phosphorylated on a tyrosine residue located around amino acid 700. It was later found that other receptors are also able to catalyze this tyrosine phosphorylation, e.g. growth factor receptors (EGFR, FGFR, PDGFR) and gp130 receptor complexes (IL6R, OSMR, LIFR), other cytokine receptors and non-receptor Tyrosine kinases [31]. Several STATs also contain a Serine residue further down the transactivation domain, which also can be phosphorylated [30]. Phosphorylation of this residue contributes to the transcriptional activity of STATs and was shown to influence non-transcriptional functions of STAT3 [32–34].

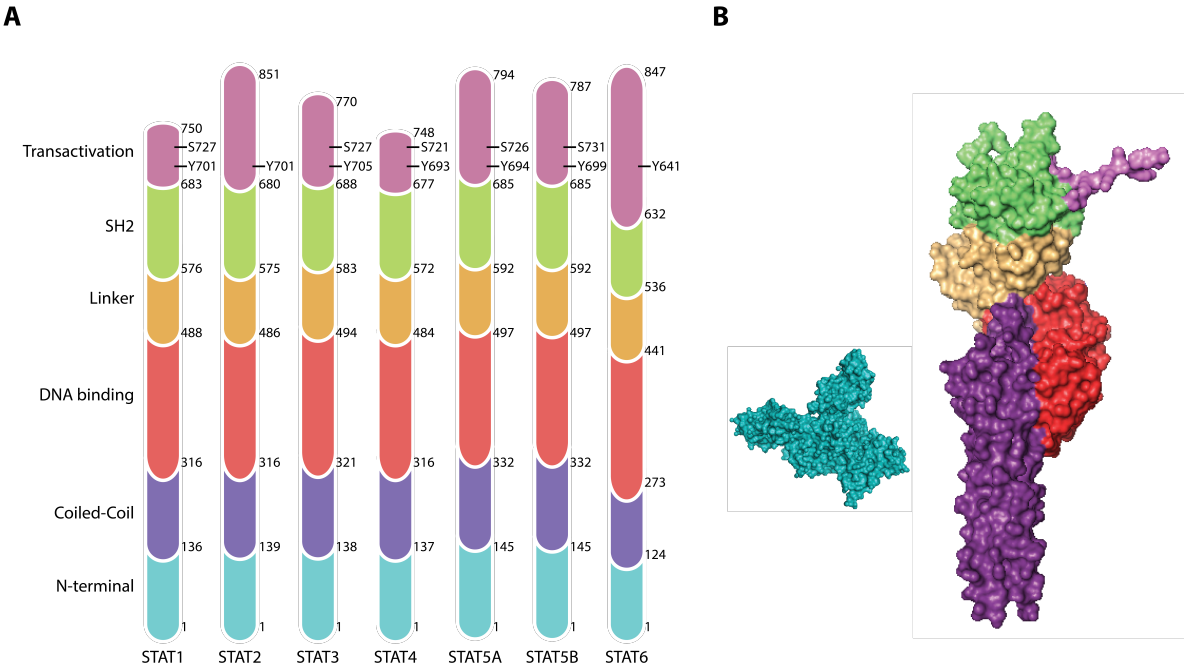

Figure 1. STAT family member structural domains. A. The general structural domains of all the STAT family members. The transactivation domain interlinks two monomers to form an activated dimer upon tyrosine phosphorylation. Serine phosphorylation enhances transcriptional activity. **B.** Surfaces of resolved crystal structures of STAT3 domains, colors correspond to the domains in A. Full length STAT3 protein was never resolved due to the inherent flexibility of transactivation domain, as well as the connection between N-terminal and Coiled-coil domain. The N-terminal domain alone and unphosphorylated STAT3β core protein (bound to DNA target sequence oligo (omitted)) crystal structures were resolved separately [35–37].

There is a lot of homology between the STAT family members. Each member is comprised of five structural domains: an N-terminal, coiled-coil, DNA-binding, Linker, Src homology 2 (SH2) domain and transactivation domain (Figure 1) [30]. The canonical pathway of activation is also similar for all members [22,30]. An extracellular signaling molecule, like IL6 described

above, binds its membrane receptor, which leads to receptor homodimerization and autophosphorylation of Janus Kinases (JAKs). JAKs in-turn phosphorylate tyrosine residues on the intracellular domain of the receptor. This enables STATs to bind to the receptor and become phosphorylated. When activated, STATs form homo- or heterodimers with other family members and translocate to the nucleus, where they bind directly to DNA consensus sequences or cooperate with other transcription factors to regulate gene transcription (Figure 2). The functions of different STATs are rather particular and quite diverse [22,30]. The specific functions of STAT3 and its close family members, STAT1, STAT5a and b play an important role in cancer [38,39].

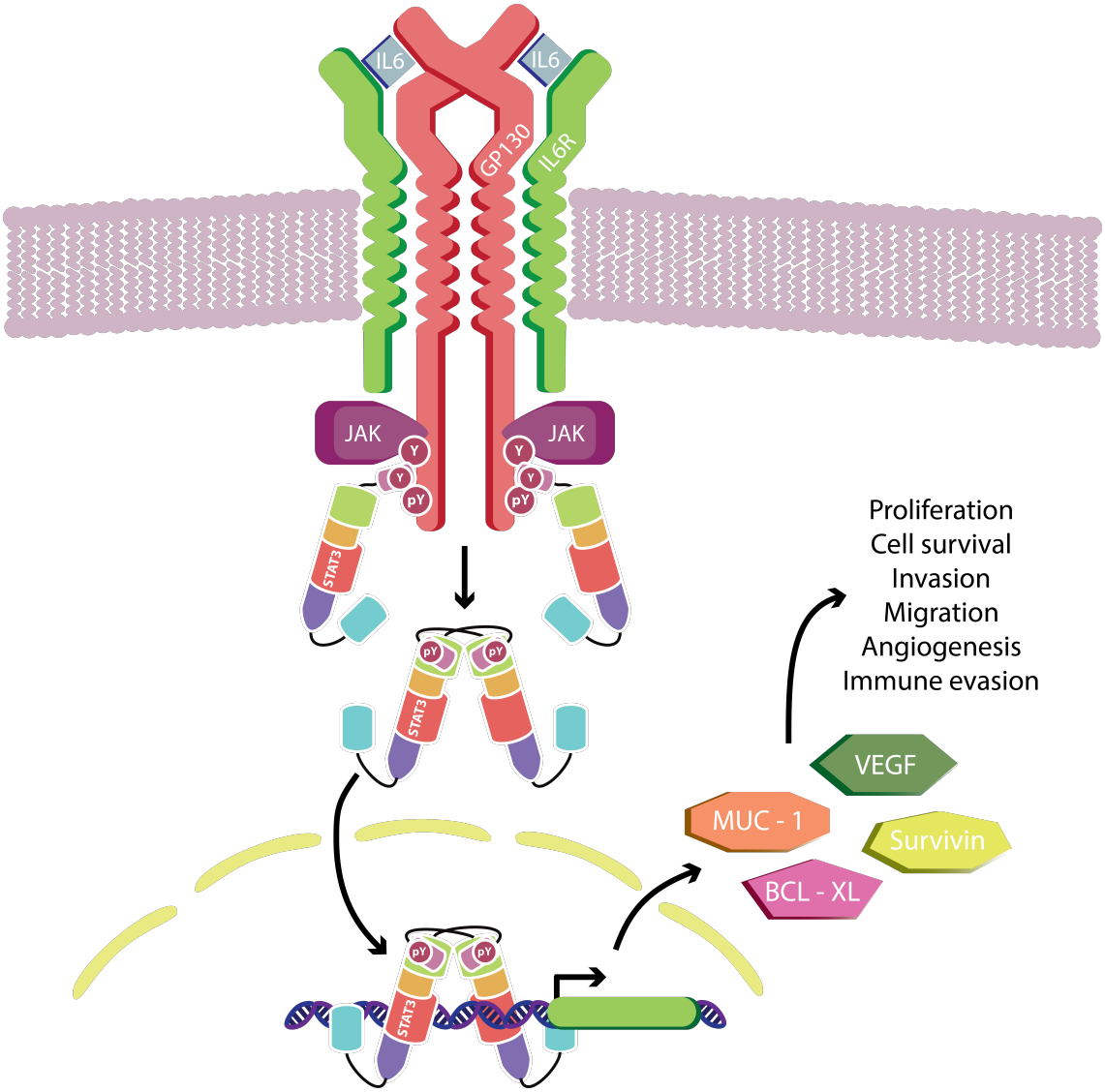

Figure 2. Depiction of the cellular IL6/JAK/STAT3 activation pathway. Two IL6 molecules bind to IL6R, forming a heterotetrameric structure with GP130 in the plasma membrane. This stimulates the autophosphorylation of JAKs, localized on the intracellular domain of gp130. JAKs phosphorylate tyrosine residues on the intracellular domain of gp130, which in turn phosphorylate STAT3. This drives STAT3 dimerization and consecutive transport to the nucleus, where transcription of target genes is initiated.

1.3.1 STAT1

Cellular antiviral, immunological and antiproliferative responses are mainly regulated by IFNs [40–42]. IFNs stimulate the activation of STAT1, driving the cellular immune response towards viral infection clearance [43]. STAT1 knockout mice lack selective signaling in their response to IFN stimulation, causing high sensitivity towards infection by viral and microbial infections [44,45]. Context and cell-type are key elements when exploring the functions of all STATs. In this respect, STAT1 has been found to have tumor suppressive and tumor supporting functions [46]. A precautionary note, many studies have performed correlation studies of protein levels of total STAT1, unphosphorylated STAT1, pSTAT1, and STAT1 mRNA levels. While STAT1 localization and expression can both determine its transcriptional function in cancer. Both parameters should be taken into consideration when exploring contradictory reports on STAT1.

Its suppressive function was described in STAT1 knockout mouse models. In the absence of tumor suppressor p53, STAT1 depletion curtailed spontaneous tumor development time [47]. Deletion of STAT1 led to more rapid mammary tumor development with concomitant expression of ErbB2/neu oncogene [48]. STAT1 deletion also increased spontaneous mammary adenocarcinoma formation in female mice [49]. These studies have established STAT1 as a tumor suppressor in cancer cells and the tumor microenvironment. The established role of STAT1 in signaling of all IFNs has elucidated its role in cell cycle inhibition, protein expression stimulating apoptosis, as well as other forms of cell death induction [50]. STAT1 represses the transcription factor c-myc, which stimulates cell cycle progression [51]. Additionally, STAT1 drives the expression of CDK inhibitors p21 and p27, or induces cell cycle arrest through direct interaction with Cyclin D1-3 or CDK4 in fibrosarcoma [46,52,53].

It is rather conflicting however that STAT1 also has multiple tumor supporting functions. STAT1 expression levels as well as activation levels correlated with poor patient prognosis in breast cancer and sarcoma patients [54,55]. STAT1 mRNA and pSTAT1 levels however not always correlated in those studies, and therefore STAT1 activity was also correlated with a favorable prognosis [54,56–60]. Gene expression profiling of radioresistant squamous cell carcinoma cells that activated STAT1 signaling revealed that mainly IFN-stimulated genes were highly expressed. The profile was termed IFN-related DNA damage resistance signature (IRDS) [61]. IRDS induction was detected in different xenograft models of breast, as well as head and neck cancer [62,63]. High IRDS also correlated with poor prognosis in glioblastoma patients [64]. Different mechanisms have been described to confer STAT1-induced therapy resistance. STAT1 knockdown cells were shown to alter the expression of genes involved in cellular metabolism, while another study reported a STAT1-dependent upregulation of HDAC4 and the P-glycoprotein 1 (MDR1) gene, regulating multidrug resistance [65,66]. STAT1 was also shown to mediate immune evasion of tumor cells. STAT1, in combination with STAT3, drives the expression of programmed death-ligand 1 (PD-L1) expression on tumor cells, whereby cytotoxic T-cell and NK cell functions are impeded [67]. Inhibition of either STAT1, or STAT3 leads to partial downregulation of PD-L1 expression, while inhibition of

both abolished PD-L1 expression completely. STAT1 and STAT3 moreover form heterodimers that induce gene transcription [68].

1.3.2 STAT3

Mouse studies have revealed that knockouts of STAT genes generate rather specific phenotypes, where each STAT could be linked to a relatively discrete pathway [69]. Unlike its family members, STAT3 knockout causes embryonic lethality [70]. STAT3 is activated in response to a platitude of cytokines and growth factors, e.g. IL1, IL5, IL6, LIF, OSM, IFNs, EGF, FGF, and PDGF. Moreover, it plays an important role in many different tissues and cellular functions. Many important and striking functions will be described in this section.

1.3.2.1 Immune system

The functions that STAT3 has in the immune system are very two-faced, where it can facilitate both pro- and anti-inflammatory functions. It pro-inflammatory functions are primarily depicted by the maturation of different immune cells. For example, STAT3 is essential for pro-B-cell development and IgG production in maturated B-cells [70]. STAT3 gain of function mutations can cause autoimmunity conditions, with broad infectious phenotypes encompassing hepatitis, arthritis, enteropathy, cytopenias and lymphocytic interstitial pneumonia [43]. On the other hand, genetic missense and in-frame deletions in the DNA binding domain or SH2 domain causing dominant negative forms of STAT3 can cause Hyper-IgE syndrome [71,72]. The observed immunodeficiencies of patients include different abscesses, eczema, chronic mucocutaneous candidiasis, fungal infections, mucocutaneous and gastrointestinal infecions caused by different pathogens, as well as meningitis among others [43].

Several studies have suggested that STAT3 mediates immunosuppression in the tumor microenvironment. STAT3 drives immunosuppression by upregulation of several anti-inflammatory genes, e.g. IL6, IL10, VEGF and TGFβ, but also the downregulation of pro-inflammatory genes, e.g. IFN-β, IFN-γ, IL12, TNF, CD80, CD86, and CXCL10 among others [24,28,70,73]. Constitutive activation of STAT3 in different immune cells affects their ability to maturate. In dendritic cells this affects their antigen presentation function, whereby cytotoxic T-cell responses are affected [70]. Thereby, more T regulatory cells are present in the tumor microenvironment promoting tumor growth. In addition, studies in mice have shown that STAT3 KO in NK cells and neutrophils leads to increased anti-tumor cytotoxic responses, in particular when cells detect specific tumor-derived factors [74]. This indicates that STAT3 can act as a brake in different immune cells, regulating their anti-tumor response. STAT3 is also a main player in immune response evasion by tumor cells by effectively regulating PD-L1 and PD-1 expression on tumor and different immune cells [75–79]. Moreover, it has been suggested that STAT3 is also activated in melanoma patients that do not respond to nivolumab, PD-1 antibody treatment, where the induced immune response is suppressed by increased STAT3 phosphorylation that drives IL10 expression, which increases regulatory T-cell percentages [80]. Thus, STAT3 is involved in the formation of an immunosuppressed

tumor microenvironment, it can affect the cytotoxicity of immune cells, and contributes greatly to tumor cell immune evasion.

1.3.2.2 Embryonic development

The importance of STAT3 in embryonic development became apparent very early on. Gene targeting in mice revealed that the genetic knockout of STAT3 is embryonically lethal, while genetic knockout of any other STAT gene leads to either impaired responsiveness to interferons, specific interleukins or growth hormones [30,69]. STAT3 is essential during embryogenesis because of its regulation of stem cells [81]. The propagation of undifferentiated pluripotent embryonic stem cells is dependent on LIF [82]. Expression of dominant negative STAT3 forms in embryonic stem cells promotes differentiation [83]. Moreover, regulated STAT3 gene copy reduction results in the loss of STAT3 activity and potency of stem cells to self-renew [83].

1.3.2.3 Cardiovascular system

Physiological levels of ROS production together with cytokine signaling activates the JAK/STAT3 pathway, while elevated ROS production due to cellular stresses inhibits the STAT3 pathway [39,84–86]. This is very important during myocardium remodeling upon exposure to different stressors, like exercise, infections and infarctions. STAT3 is a key regulator of angiogenic factors. Target gene VEGF-A promotes vascularization and vascular permeability, while Bcl-XL and MnSOD promotes survival of cardiomyocytes [87,88]. Upon myocardial ischemia, STAT3 is activated and plays a cytoprotective role [89]. During ischemia several different cytokines are released from myocardial cells, the cytokines activate the STAT3 pathway in cardiac progenitor cells inducing their differentiation into endothelial cells. Furthermore, progenitor cell differentiation is reduced in STAT3 knockout mice [90].

The cellular communication between myocytes and fibroblasts is tightly regulated by STAT3. STAT3 knockout mice additionally develop severe cardiac fibrosis, which affects cardiac function, heart failure symptoms and increases mortality [91]. Loss of STAT3 in cardiac fibroblasts enhances their proliferation, whereby cardiomyocytes can induce fibroblast proliferation [91]. This indicates that STAT3 is essential for impeding cardiac fibrosis, which was further confirmed in STAT3 knockout mice showing a reduction in myocardial capillary density, while interstitial fibrosis was increased causing cardiomyopathy, impaired cardiac function and increased mortality [90]. Thereby, STAT3 appears to play a complex part in the regulation and function of different cardiac cells upon cardiac stress.

1.3.2.4 Mammary gland involution

The role STAT3 has in mammary gland involution is one of the most striking, because it is the only biological process where STAT3 activation promotes cell death [92]. Upon the loss of a suckling stimulus and consequential milk stasis, STAT3 becomes phosphorylated due to high levels of LIF expression [93]. This induces mammary gland involution, which is the process of regression of mammary glands after lactation ceases. Conditional deletion of

STAT3 in mammary epithelial cells significantly delays the initiation and progression of involution [94]. The cell death of the epithelial cells is caspase-independent and therefore not apoptotic, but is rather induced through the activation of a lysosomal-mediated cell death program [93,95]. STAT3 drives the formation of cellular lysosomal vacuoles. Also, STAT3 activation stimulates the uptake milk fat globules by the epithelial cells, switching their function from secretion to uptake of the globules. The globules contain the hydrolysate oleic acid that induces lysosomal leakage eventually leading to cell death [93].

1.3.2.5 Mitochondrial STAT3

The most extensively explored non-transcriptional function of STAT3 is its role in mitochondria. Mitochondria regulate cellular metabolism, cellular calcium gradients, and also produce reactive oxygen species through energy metabolism [96]. The initial study that identified that STAT3 also resides in mitochondria, demonstrated mitoSTAT3 is serine 727 phosphorylated and STAT3 KO affects function of electron transport chain complex I and II as well as decreases mitochondrial O_2 consumption rates [34]. Interestingly, the MEK-ERK pathway is essential for serine 727 phosphorylation of mitoSTAT3 [97]. MitoSTAT3 is oncogenic without assistance from STAT3 transcriptional activity, since Ras-dependent transformation requires serine 727 but not tyrosine 705 phosphorylation [33]. Moreover, Ras induces serine 727 phosphorylation on STAT3, which increases mitochondrial respiration and facilitates Ras-dependent oncogenic transformation. MitoSTAT3 thus plays an important role in sustaining the metabolism needs during oncogenic transformation, which may sustain oxidative phosphorylation and decreased ROS accumulation in cancer cells. Thereby, it potentially could provide enough energy to sustain cell proliferation and tumor growth.

1.3.2.6 STAT3 in cancer

Constitutively activated STAT3 has been found in prostate, pancreatic, ovarian and brain tumors, as well as in breast carcinoma, head and neck carcinoma, melanoma, leukemia and lymphoma [38,98]. Experiments performed with STAT3C, a STAT3 construct that is constitutively in an active dimer formation through intermolecular cysteine-cysteine bonds, led to the description of STAT3 as an oncogene [21]. Implantation of normal mouse fibroblasts expressing STAT3C into nude mice leads to the formation of tumors. In addition, STAT3C expression in different cell types leads to the upregulation of oncogenic and angiogenic factors, driving oncogenesis and progression [26]. STAT3 drives the transcription of multiple genes that are critically important to oncogenic transformation and the maintenance of the hallmarks of cancer (Figure 3; [2,28,99]).

STAT3 affects cell cycle progression through its regulation of genes like Cyclin-D1 and c-Myc [28]. It also contributes to cell survival through the upregulation of anti-apoptotic proteins of the Bcl-2 family and Survivin. Furthermore, it can also act as a repressor of the P53 gene by binding to its promoter, effectively preventing apoptosis [28]. STAT3 in addition induces angiogenesis by facilitation of the expression of VEGF-A and b-FGF. Furthermore, STAT3 drives the expression of metalloproteinases (MMPs), which promote tumor invasion by the

degradation of the extracellular matrix [28]. It also drives tumor metastasis by upregulating several proteins, e.g. Vimentin, Snail, Twist and N-cadherin [99]. The constitutive activation of STAT3 has also been described as a mechanism of chemotherapy and radiation therapy resistance in different preclinical tumor models, as well as patients. A positive feedback loop driving STAT3 activation is found in multiple drug-treated, oncogene-driven cancer cells. It is activated in melanomas that have become resistant to Vemurafenib, EGFR-driven lung cancer cells that are resistant to Erlotinib, Her2-driven cells resistant to Lapatinib and Trastuzumab, ALK-driven cells resistant to the ALK inhibitor TAE684 and Met-driven cancer cells resistant to Crizotinib [100–102]. STAT3 activation also occurs upon resistance to different TKI inhibitors in myeloproliferative neoplasms (MPNs), e.g. different JAK 2 inhibitors, BEZ235 a dual inhibitor of PI3K/AKT and mTOR signaling [103,104]. Lastly, inhibition of STAT3 function leads to synthetic lethality in Imatinib-resistant BCR-ABL1-positive CML cells [105].

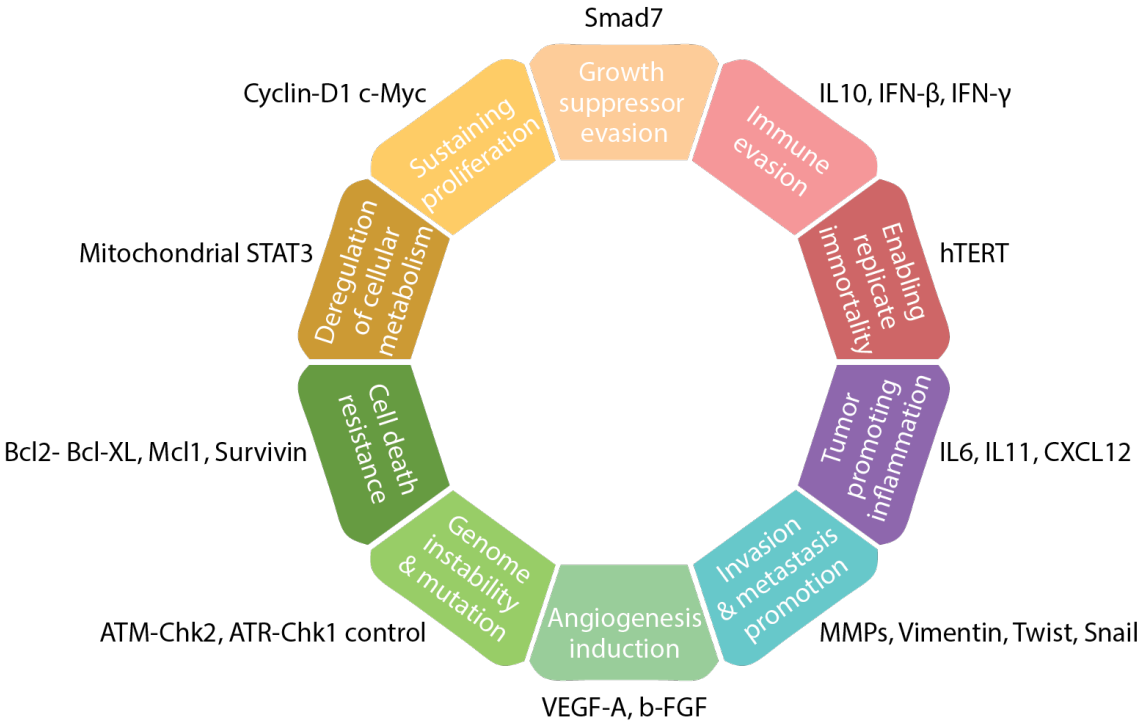

Figure 3. STAT3 functions and target genes delineated by cancer hallmark. The wide range of STAT3 functions and target genes can affect all hallmarks of cancer [2,28,99].

1.3.3 STAT5a & STAT5b

Even though STAT5a and b are encoded by different genes, their sequences minutely differ in their C-terminus, where STAT5a has 20 and STAT5b has 8 unique amino acids [106]. Both drive gene transcription through binding to gamma-activated sequence (GAS) elements [106]. While many studies have shown that different STATs can form dimers without necessity of phosphorylation [82,101,107,108]. It remains unclear how dimerization of unphosphorylated STATs is regulated. The only unphosphorylated dimerized STAT crystal structure that was resolved

was of STAT5a This revealed that both monomers do not interact by SH2 domains, but rather interact by their coiled-coil and DNA-binding domains in an antiparallel conformation [109].

STAT5 plays an important role in mammary gland and hematopoietic lineage differentiation [38,50]. Therefore, aberrant STAT5 activity is mostly found in breast and hematopoietic malignancies [110–113]. Often, constitutive activation of STAT5 is regulated through upstream kinases mutations. In chronic CML STAT5 is activated through the BCR-ABL kinase, a specific mutation that drives oncogenic transformation of cells in many CML patients [114,115]. In acute myeloid leukemia (AML) FMS-like tyrosine kinase 3 (FLT3) drives STAT5 activation [116]. And in MPNs the JAK2 point mutation V617F activates STAT5 [117]. In cancer STAT5 regulates expression of genes stimulating tumor cell survival, proliferation and the apoptosis resistance.

1.4 REACTIVE OXYGEN SPECIES

The utilization of the aerobic environment by living organisms permitted the evolution of many different complex species. The only more complex organisms that are anaerobic are several Loricifera, found at the bottom of the Mediterranean Sea [118]. The oxygen metabolism by organisms therefore facilitated the development of complex life, however it has some inherent drawbacks. The metabolism of oxygen by cells can lead to the production of free radicals and ROS [96,119,120]. Free radicals are molecules that contain one or more unpaired electrons [119].

Endogenous ROS are mainly created in the mitochondria. They are an abundant side product from ATP production through oxidative phosphorylation (OXPHOS) by the electron transport chain [120,121]. Inefficiency of the final step of OXPHOS where O_2 is usually directly reduced to H_2O, a small portion gets successively reduced, forming superoxide anions ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\cdot}$) and hydroxyl anions (OH^-), eventually forming H_2O (Figure 4). Other cellular organelles that utilize and consume oxygen are the endoplasmic reticulum (ER) and Peroxisomes. In order to facilitate proper protein folding the ER is kept in a constant state of oxidation [122]. Through the activity of specific enzymes oxygen is used to oxidize cysteine residues on proteins to induce cysteine-cysteine bonding, in due process H_2O_2 is produced. Peroxisomes induce the β-oxidation of fatty acids, the most prevalent form of ROS is H_2O_2 [123].

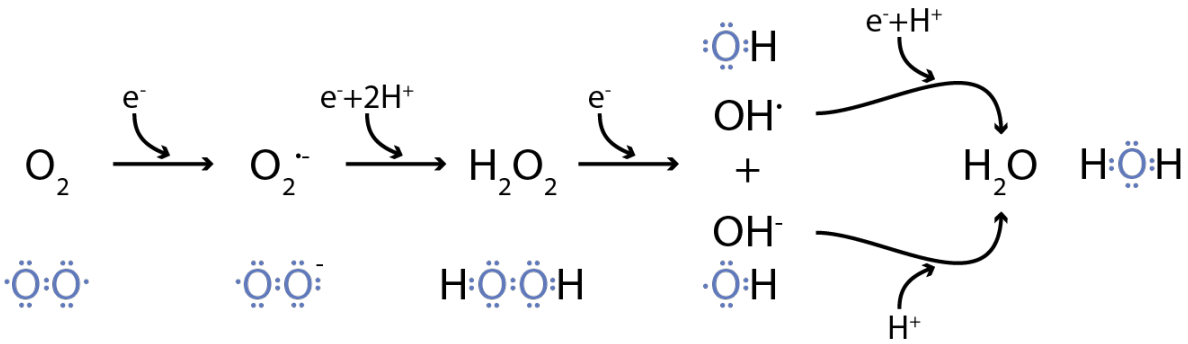

Figure 4. A simplified scheme of oxygen reduction into water. In the final step of OXPHOS a small portion of O_2 gets successively reduced, forming superoxide anions ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\bullet}$) and hydroxyl anions (OH^-), eventually forming H_2O

Balancing ROS is important for cell survival and cellular signaling. In normal cells ROS production is counterbalanced by antioxidant scavenging systems, creating redox homeostasis. There are different types of ROS species, in the form of ionic, free radical and non-radical species, which are synthesized as by-products of oxygen metabolism. In redox homeostasis these species play an important role in cellular signaling. It is well described that different cytokines and growth factors induce cellular ROS, which is essential to induce downstream target phosphorylation [124–127]. This mechanism involves NADPH oxidases (Nox) and negative regulators of phosphorylation, the protein tyrosine phosphatases (PTPs) [124,128,129]. Many PTPs, like PTP1B, are sensitive to increases to ROS, and become inactivated due to protein oxidation, which facilitates subsequent phosphorylation of cytokine and growth factor receptors and downstream kinases [130].

Different oncogenes can induce ROS in transformed cells, which is even required for the tumorigenicity of the oncogene in specific instances [12]. In combination with the ability of proto-oncogenes, including RAS, to induce antioxidant expression to counteract the higher ROS levels suggests that cancer cells have increased rates of ROS generation and elimination, as well as aberrant regulation of redox homeostasis [131]. Moreover, this may make cancer cells more susceptible to abrupt alterations in redox balance and lead to selective cell death [12,132]. Therefore therapeutics inducing ROS production, as well as its metabolism is widely and intensively explored in research (Figure 5) [12,132].

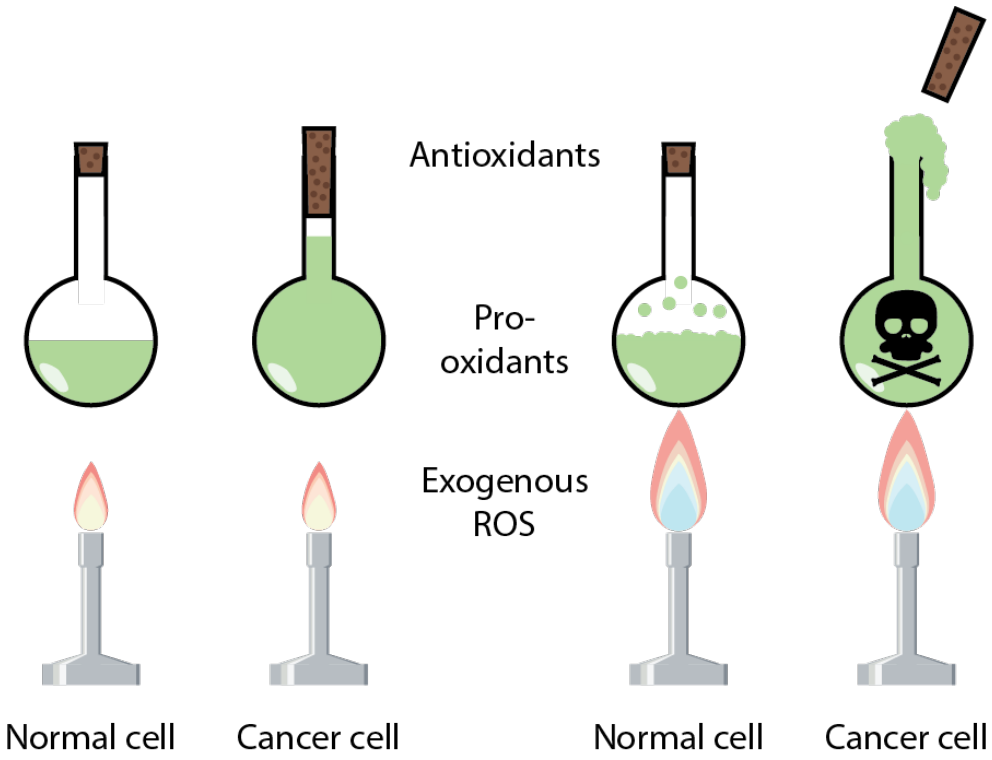

Figure 5. Targeting ROS generation and elimination as anticancer strategy. Normal cells have low basal levels of ROS and therefore also their antioxidant systems are utilized at low levels. Due to increased cellular metabolism and the tumor microenvironment, cancer cells have higher levels cellular ROS and their antioxidant systems are more engaged in scavenging to prevent cell death. Cancer therapeutics can induce higher ROS levels affect normal cells, however the cellular antioxidant systems are capable of scavenging the additional ROS. If ROS levels become too high cancer cells are unable to further engage their antioxidant systems, which causes increased cellular stress and inevitable cell death [12].

1.5 REDOX & ANTIOXIDANT PATHWAYS

There are two major pathways that control cellular redox signaling and antioxidants. Both pathways are intertwined in a wide range of cellular processes, e.g. proliferation, antioxidant activity, kinase pathway signaling and redox homeostasis [133–135]. The pathways are named after predominant components, namely the glutathione (GSH) and the thioredoxin (Trx) pathways [133–135]. Both pathways have mechanisms and functions that are distinct as well as complementary. Notably, functions of both pathways have been intricately linked to oncogenesis, cell proliferation and tumor progression [12]. Both systems and their function in cancer will be described below.

1.5.1 Glutathione pathway

The GSH pathway is composed of glutathione, glutathione peroxidase (GPx), glutaredoxin (Grx) and glutathione reductase (GR) [134]. GSH is a small tripeptide, consisting of glutamate, cysteine and glycine. It is synthesized by two different synthetases, first glutamate and

cysteine form γ-glutamylcysteine via glutamate cysteine ligase, and second glycine is added forming GSH via GSH synthetase [136,137]. It is a highly abundant intracellular peptide, with a range between 1 and 10 mM in mammalian cells [137]. In its reduced state GSH is a single tripeptide, while oxidized (GSSG) it forms a pair through a disulfide bond of two cysteine thiols. GSSG can be reduced again by GR through consumption of Nicotinamide adenine dinucleotide phosphate (NADPH) [134]. The process of S-glutathionylation is important for protecting protein thiols from irreversible oxidation when oxidative stress levels are increased. When the GSH/GSSG ratio is disrupted GSH forms disulfide bonds with reactive thiols, in order to prevent irreversible thiol oxidation of proteins [138]. Apart from the importance of GSH for cellular protection against different stressors, GSH is also an important signaling peptide [139].

1.5.1.1 Glutathione Peroxidase

GPxs are peroxidases that catalyze the removal of hydrogenperoxide and other hydroperoxides from cells by utilization of GSH [139]. Dependent on the peroxide this catalysis leads to the generation of H_2O or alcohols [140]. The human genome encodes eight different GPx genes, of which five (GPx1-4 and GPx6) contain a rare selenocysteine (Sec) in their active site, while other isoforms contain a cysteine [141]. Substitution of Sec to cysteine leads to drop in activity in a magnitude of two to three orders [142]. Sec-containing GPxs have a very high rate, 10^5-10^8 $M^{-1}s^{-1}$, of reaction with H_2O_2 [143]. GPx4 is unique in that it is the only isoform that also directly reduces lipid peroxides, e.g. phospholipids, cholesterol and fatty acids [144–146]. When GPx4 is inhibited the peroxidation of polyunsaturated fatty acids can drive ferroptosis, a regulated nonapoptotic form of cell death [146].

1.5.1.2 Glutaredoxin

Humans express four different Grx family members [134]. They can be subdivided by their active site motif, where Grx1 and Grx2 contain a dithiol CXXC active site motif, while Grx3 and Grx5 contain a monothiol CXXS active site motif [134]. Grx1 and Grx3 are mostly found in the cytosol, while Grx2 and Grx5 mainly reside in the mitochondria where they exert different redox-related functions. Grxs mainly are oxidized by different cellular substrates, e.g. Ribonucleotide reductase (RNR), apoptosis signal-regulating kinase (ASK1), and are reduced by GSH in a two-step reaction [134]. In addition to their main functions, Grxs were also found reduce Trx1 and peroxiredoxins (Prxs), which is a good illustration of mechanical complementation between both cellular antioxidant pathways [147,148].

1.5.2 Thioredoxin pathway

The Trx pathway is composed of Prx, Trx and Thioredoxin Reductase 1 (TrxR1) [133]. Because of the focus of the thesis, TrxR1 will be discussed separately after this section.

1.5.2.1 Peroxiredoxin

Analogous to the GPxs in the GSH pathway, Prxs are the main thiol peroxidases for the Trx pathway [133]. They have a similar reactivity rate with H_2O_2 compared to Gpxs [143,149]. The Prx

family contains six different members Prx1-6 [135,150]. Also here some members, Prx1-4, have a dithiol active site motif, which upon thiol oxidation form a dimer through intermolecular disulfide bonding [135]. Prx5 also contains a dithiol active site, however in contrast with Prx1-4 it forms an intramolecular disfulde bond, thereby remaining monomeric when oxidized. Prx6 is the only monothiol Prx, and is another crossover between both antioxidant pathways because it utilizes GSH instead of Trx as its reductant [151]. Prx1 and Prx2 are mainly located in the cytosol and appear to have some redundant functions [151]. Prx3 resides in the mitochondria, while Prx4 is found in the ER [151]. Prx5 has been detected in the cytosol, mitochondria and peroxisomes [151]. The function of Prxs in cells that are exposed to H_2O_2 is under debate. Different studies from primarily the lab of Tobias Dick claim that different Prxs can form redox relays together with the other players of the Trx pathway [149,150]. Where Prx can relay oxidative equivalents directly to Trx or other substrate proteins, amongst which is STAT3 (Figure 6) [149]. Knockdown or knockout of Prx2 or both Prx1 and Prx2 prevents the oxidation of these redox-regulated proteins upon H_2O_2 exposure [150]. A shortlist of Prx1 and Prx2 substrate proteins are mentioned in the Thioredoxin subsection below.

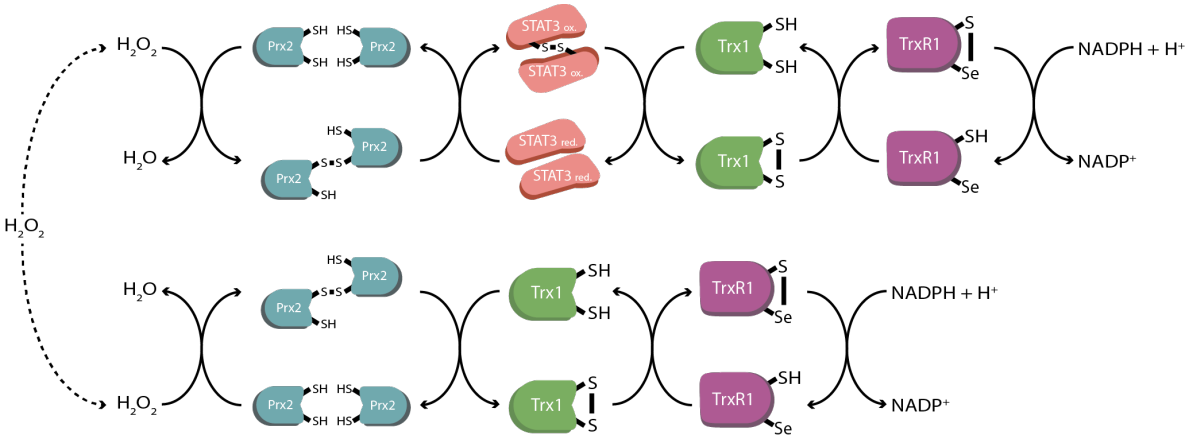

Figure 6. Kinetic model of Prx-mediated redox relay involving STAT3. H_2O_2 oxidizes a cysteine in the Prx2 active site, causing Prx2 dimerization. Oxidized equivalents are relayed to either reduced STAT3 and subsequently passed to Trx1, or directly to Trx1, which is kept in reduced form by TrxR1 enzymatic activity driven by NADPH consumption [152].

1.5.2.2 Thioredoxins

Trx is the pivotal enzyme of the Trx pathway. The three different Trx proteins all have the same disulfide motif in their active site, Cys-Gly-Pro-Cys. Trx1 resides in the cytosol, nucleus and is even found extracellularly, Trx2 is located in the mitochondria and SpTrx is only found in spermatozoa [135,153]. Trxs mainly keep Prxs and other substrate proteins reduced, and Trx is consequently reduced mainly by TrxR [133,154]. However, another functional crossover between the two pathways is that Grx2 can also reduce Trx1 [155]. A group of different cellular proteins that are substrates of Trx, besides Prxs, are amongst others: STAT3, p53, RNR, ASK1, phosphatase and tensin homolog (PTEN), annexin A2 (ANXA2),

cystathione β-synthase (CBS) and PTPs [149,150,156–158]. The reduction of these substrates determines the activity and function of these substrates. Trx is therefore a very important enzyme for a wide scale of cellular processes.

1.6 THIOREDOXIN REDUCTASE 1

TrxRs consist of only three family members, cytosolic TrxR1, mitochondrial TrxR2 and testis specific thioredoxin glutathione reductase (TGR) [154]. They are a family of pyridine nucleotide-disulphide oxireductases with a redox catalytic site similar to that of GRs [154]. However, TrxRs also contain a unique amino acid, a Sec residue is situated at the penultimate position of the protein amino acid chain [159]. In its oxidized state, the selenolate of Sec is three orders in magnitude more reactive with electrophilic moieties than the thiolate of a cysteine [160]. TrxR consumes NADPH to reduce Trx, and therefore is the catalyst of the Trx pathway [154]. Knockout of either TrxR1 or TrxR2 is embryonically lethal in mice. As catalyst, cytosolic TrxR1 therefore regulates different cellular processes, e.g. activation of signaling pathways and antioxidant activities. In addition, TrxR1 is targeted by a plethora of anticancer drugs and TrxR1 inhibition was shown to contribute to their therapeutic effects [159,161]. Thus, TrxR1 has been proposed as a good therapeutic target for cancer treatment. The function, selenoprotein synthesis machinery, substrates, small molecule inhibitors, as well as its function in cancer will be discussed in this section.

1.6.1 Structure and activity

TrxR1 has a size of approximately 55 kilodalton (kDa) and usually resides as a homodimer through a covalent bond of two oxidized tryptophan residues (Trp114) that are solvent exposed on the protein surface [162]. Upon oxidation it can relay electrons with the flavin adenine dinucleotide (FAD) moiety of TrxR1. The oxidation state of Trp114 determines the ability of TrxR1 to form dimers, and even facilitates tetramer and higher oligomer formation [162]. In order to reduce Trx, TrxR1 reduces itself by transferring electrons from the FAD-containing cysteine dithiol active site of one subunit to the Sec-containing active site of the other subunit [163]. The FAD moiety trasnfers electrons from NADPH upon its binding. The electrons are then transferred from the FAD to the cysteine dithiol of the same active site and subunit [163]. This reduced dithiol motif is then able to reduce the selenothiol motif on the other subunit of the dimer, which then is able to reduce oxidized Trx or other substrates [162,163].

In its function the Sec amino acid is the same as that of cysteine, the only difference is the substitution of a selenium atom for that of a sulfur atom, which even posses an equal amount of valence electrons [164]. Sec is highly reactive however, significantly more than cysteine, their respective dissociation constants are pKa = 5.5 and pKa = 8.5 [159]. The natural presence of selenium is however much lower than sulfur, and only 25 human proteins are encoded to contain a Sec residue [165].

1.6.2 Substrates

TrxR1 has a wide range of different substrates, of which the most prominent is Trx1. Other proteins that are reduced by TrxR1 include thioredoxin related protein 14kDa (Trp14), Grx2, protein disulfide-isomerase (PDI) [124,135]. It is interesting that the list of TrxR1 substrates not only encompasses cellular substrates. There are multiple cellular molecule substrates, e.g. cytochrome C, selenite, selenocysteine, menadione and H_2O_2, but also substrates not found in cells, e.g. 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) and 5-hydroxy-1,4-naphthalenedione (Juglone) [154,166]. The different cellular functions of the known substrates of TrxR1 effectively link its function to different processes including proliferation, apoptosis, regulating redox homeostasis and protein folding [161].

1.6.3 TrxR1 in cancer

Due to the variety of different substrates described above TrxR1 facilitates oncogenesis, proliferation, angiogenesis, apoptosis resistance, metastasis, and supports tumor cells further by driving the antioxidant response needed for cancer cell survival due to higher levels of ROS created by the tumor microenvironment and increased cellular metabolism [12,167]. Metabolism in cancer cells is moreover often erroneous leading to additional ROS synthesis [12]. In addition to its direct involvement as an antioxidant enzyme, TrxR1 has been linked to many different oncogenic processes indirectly. TrxR1 protein expression is upregulated in different tumors, including breast cancer where tumor tissue expresses significantly higher levels of Trx1 and TrxR1 compared to healthy breast tissue of the same patients and Trx1 and TrxR1 were both correlated with poor overall survival, disease free survival and distant metastasis free survival [168]. It is further upregulated in malignant melanoma, colon cancer, non-small cell lung cancer, thyroid cancer and oral squamous cell carcinoma [169–173]. Moreover, TrxR1 expression levels between cancer patients have been positively correlated to a worse overall prognosis and overall survival [174,175].

TrxR1 facilitates cellular proliferation through the reduction of RNR by the Trx pathway. RNR reduces ribonucleotides into deoxyribonucleotides, the building blocks of DNA [124]. It was shown that RNR activity is decreased upon inhibition of TrxR1 [176]. ASK1 is a well-described substrate of Trx1 and is kept in a reduced state to prevent its autophosphorylation [177]. It is a MAPK family member that when activated it induces apoptosis through activation of p38 and JNK [177]. Overexpression of Trx1 under hypoxic conditions increases HIF-1 levels, an important oxygen sensing transcription factor induced during hypoxia, and leads to higher levels of VEGF stimulating angiogenesis [178]. Furthermore, Trx1 in breast cancer cells leads to a more invasive phenotype. While Trx1 knockdown decreased invasiveness and cellular migration, the mechanism of invasion and migration might involve the upregulation of MMP-9 [168,179].

1.6.4 Targeting TrxR1

Targeting of either the Trx or GSH pathway is considered an interesting therapeutic avenue for anticancer treatment, since normal cells only require one of the two pathways to be

functional for survival [180,181]. Therefore, simultaneous disruption of both pathways could lead to adverse side effects. Many pan-TrxR inhibitors have been described and two are approved for clinical use, Auranofin for rheumatoid arthritis and arsenic trioxide for treatment of acute promyelocytic leukemia (APML) [182–184]. Auranofin is a gold-containing compound, which causes mild adverse toxicities in humans. Different clinical trials are being conducted to assess the efficacy of Auranofin as monotherapy for ovarian cancer, fallopian tube cancer, and chronic lymphocytic leukemia (CLL) [185,186]. Gold is a transition metal, due to their electrophilic nature many transition metal containing compounds have been shown to be potent inhibitors of TrxR1 [187]. One platinum-containing compound, Cisplatin, is a widely used chemotherapeutic that alkylates purine bases on DNA, thereby preventing DNA repair mechanisms and causing replicative stress [188]. Interestingly, Cisplatin was also shown to target TrxR1, and Cisplatin-resistant cells were shown to upregulate Trx1 and TrxR1 expression [189,190]. Furthermore, Curcumin, a plant-derived polyphenol, is another inhibitor of TrxR1 that irreversibly binds to the Sec and cysteine residues in the TrxR1 active site [191]. It and its many derivatives are well known for its anticancer and antiangiogenic effects [192,193]. In addition, it was shown in multiple studies that both the STAT3 as well as nuclear factor-kappaB (NF-κB) pathways are inhibited by Curcumin [192,194,195].

There are multiple efficacious inhibitors of TrxR1, however none do not affect mitochondrial function indicating simultaneous targeting of TrxR2 [196]. Consequently, one recent study published by the Arnér lab performed a high throughput screening campaign to identify specific small molecule inhibitors of TrxR1 that did not affect TrxR2 function [197]. The study focused on the *in vitro*, cellular and *in vivo* effects of patented compounds TRi-1 and TRi-2, while a third patented compound was also identified TRi-3, it was omitted from the final published work. TRi-3 is mentioned here because of its structural similarity to the compounds identified in **Paper III** of this thesis. TRi-1 and TRi-2 were shown potently inhibit TrxR1 function, have minimal effect on mitochondrial function [197]. Both were cytotoxic to cancer cells, in comparison normal cells were relatively unaffected. Both compounds showed minimal toxicity in mice and showed good efficacy in several *in vivo* mouse tumor models.

1.6.5 SecTRAPs

In addition to inhibition of TrxR1 enzymatic activity it was shown that specific electrophilic small molecule inhibitors of TrxR1 more selectively bind to the Sec-residue in its C-terminal active site, leading to the formation of selenium compromised thioredoxin reductase-derived apoptotic proteins (SecTRAPs) [198]. The inhibition of the Sec-containing active site prevents TrxR1 from reducing Trx1. However, the secondary active site, containing a FAD moiety and a redox active disulfide/dithiol motif), still has NADPH oxidase activity and remains able to redox cycle with substrates such as Juglone and other Quinones (Figure 7) [199]. A cellular SecTRAP substrate has however not been identified so far [198]. The NADPH oxidase activity combined with the lack of its normal redox-dependent antioxidant activity, should transform the SecTRAPs into a gain of function pro-oxidant protein [198]. This gain of function could

results in increased ROS production, thereby making TrxR1 a target for anticancer therapeutics with a unique additive effect to enhance apoptotic cell death.

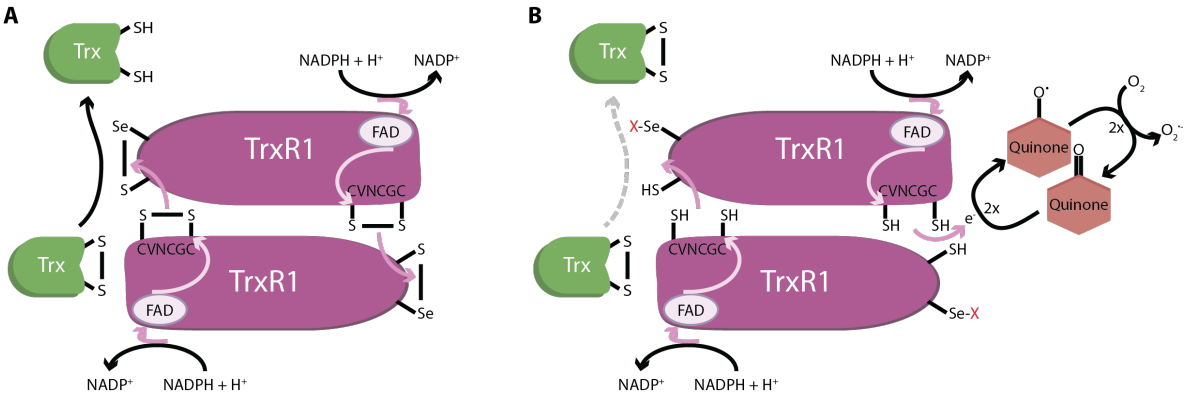

Figure 7. TrxR1 redox activity and SecTRAPs. A. The first active site of TrxR1 contains a FAD moiety that utilizes electrons from NADPH, which flow to the N-terminal disulfide of the same active site. Since TrxR1 resides in the cytosol as a dimer, the electrons can then be transferred to the C-terminal active site containing a selenosulfide. The reduced C-terminal active site can reduce Trx1 or other cellular substrates. **B.** Occupation of the Sec residue of the C-terminal active site by an electrophilic small molecule inhibitor leads to inactivation of that active site. Therefore, TrxR1 can no longer reduce Trx1. The N-terminal active site however remains active, which results in an activity similar to NADPH-oxidase and can actively redox-cycle with quinones generating superoxide [198,199].

1.7 REDOX REGULATED TRANSCRIPTION FACTORS

Different transcription factors contain specific residues that can be modified post-translationally, including phosphorylation, acetylation, and oxidation/reduction. Due to changes in the cellular redox balance, specific cysteine residues become either oxidized or reduced which determines the activity of these transcription factor. Here the redox regulation of the transcription factor systems Nrf2-Keap1, the NF-κB and STAT3 will be described.

1.7.1 Nrf2-Keap1

Several transcription factors have been described to be subject of redox regulation. The most well described and explored transcription factor is NF-E2 related factor 2 (Nrf2), which upon activation drives transcription of many cytoprotective genes and is one of the main regulators of the cellular oxidative stress response [200,201]. Under normal conditions Nrf2 resides in the cytoplasm in a complex with Kelch-like ECH associated protein 1 (Keap1), a ubiquitin E3 ligase which continuously targets Nrf2 for proteasomal degradation [202]. When cells are exposed to oxidative stresses or electrophilic attacks specific cysteine residues on Keap1 become oxidized disrupting the Nrf2-Keap1 complex [203]. Consequently, Nrf2 is stabilized and cellular levels rapidly increase. Nrf2 then translocates to the nucleus and induces

transcription of genes involved in cellular protection against oxidative and xenobiotic stresses [204]. These genes contain specific binding elements in their promoters, known as antioxidant response elements (ARE) or electrophilic response elements (EpRE) [204,205]. In cancer, somatic mutation in both NRF2 and KEAP1 genes often occur, which leads to constitutive activation of Nrf2 [203]. This facilitates tumor therapy resistance and driving disease progression [203].

1.7.2 NF-κB

The NF-κB pathway has been showed to play a central role in both immunity and cancer development. NF-κB consists of a heterodimer of p65 and p50, which are sequestered in the cytoplasm by IκB, regarded as the main gatekeeper of NF-κB activation [206]. Upon extracellular stimulation by inflammatory cytokines, e.g. TNF-α, IL-1 and Lipopolysaccharide (LPS), IκB is phosphorylated and targeted for proteasomal degradation [207]. Hereby, NF-κB gets released and translocates to the nucleus where it drives transcription of a plethora of genes, which regulate proinflammatory mediators, i.e. iNOS, COX-2, TNF-α, IL-1 and IL-8 [208]. Other target gene products promote cancer progression by inhibition of cell death, promotion of cell proliferation, and facilitation of epithelial-mesenchymal transition (EMT) [209]. Under oxidative stress NF-κB activation is impeded. Interestingly, the Trx system affects NF-κB in multiple ways. Trx overexpression was shown to stabilize IκB, while Trx can also reduce oxidized cysteine residues on nuclear NF-κB facilitating DNA binding affinity [210,211]. An additional inhibitory protein of NF-κB was found, which has to be dissociated from the cytosolic NF-κB complex in order to facilitate pathway activation. Dynein light chain LC8, a redox-sensitive interacting protein of IκB, contains a cysteine residue that in unstimulated conditions is reduced [212]. Two LC8 proteins reside in the cytosol together with IκB preventing NF-κB activation. However, upon binding of TNF-α, IL-1, or LPS to the TNF receptor 1 (TNFR1), NADPH oxidases (Nox) are activated and start the generation of intracellular ROS [213]. The ROS oxidizes the cysteines on LC8, inducing the formation of an LC8 homodimer, which dissociates from IκB [214]. In order to restore its inhibitory contribution the disulfide bond is reduced Trp14, which is subsequently reduced by TrxR1 [214].

1.7.3 STAT3

Both JAK and STAT3 phosphorylation can be regulated by alterations in redox homeostasis. H_2O_2 can increase JAK2 phosphorylation, due to activation of the tyrosine-protein kinase Fyn and perhaps in addition inhibition of redox sensitive PTPs [215]. Moreover, exposure of unstimulated cells to H_2O_2 can induce STAT3 activation through activation of both JAK2 and TYK2 [84]. These observations also involve the activity of Nox [216]. Also, it appears that in starved cells, ROS can activate JAK-STAT3 pathway leading to the increased expression of IL6, which might indicate the formation of an autocrine feed forward loop [217]. This loop could maintain STAT3 activity and possibly promote cancer cell survival.

In contrast, reports have also been made that ROS can inhibit JAK-STAT pathway activation [149,218,219]. This might be explained through a discrepancy in experimental setup, whereby

cytokines can no longer activate the signaling pathway due to direct oxidation of JAK and STAT proteins. In this respect, a potential mechanism of STAT3 inhibition is due to glutathionylation of specific cysteine residues, which impeded STAT3 phosphorylation, nuclear translocation and transcriptional activity [220,221]. The glutathionylation could be reversed by the addition of Grx1, however Grx1 overexpression did not significantly affect nuclear translocation or transcriptional activity of STAT3 by cytokine stimulation [220]. Moreover, cysteine residues in the JAK2 catalytic domain have been shown to be redox sensitive and can become oxidized, affecting kinase activity [222]. Similarly, STAT3 was shown to form dimers and oligomers by oxidization of specific cysteine thiols [149]. It was suggested that this oxidation is due to a peroxidase-based redox relay. H_2O_2 readily oxidizes active site cysteines on Prx2, which can transfer their oxidizing equivalents to several redox-regulated proteins. STAT3 was identified as one of the target proteins of Prx2 [149]. Moreover, cytokine stimulation was also able to induce Prx2 and STAT3 oxidation, and Prx2 knockdown led to an increased transcriptional activity of a cytokine-stimulated STAT3 [149]. These contradictory findings might be explained by discrepancies in measurement time points, where initial ROS induction might directly oxidize STAT3 and inactivate it, while longer exposure to ROS possibly leads to antioxidant effects which cause subsequent the reduction of STAT3, permitting the activation of the pathway. Indeed, the formation of disulfide-linked STAT3-Trx1 intermediates was observed after H_2O_2 exposure, indicating the ability of the Trx system to increase the reduced pool of STAT3 protein (Figure 6) [149]. Inhibition of the important cytosolic reductase in the Trx system, TrxR1, thereby also leads to inhibition of STAT3 activity [149]. Not only do TrxR1 inhibitors induce Prx2 oxidation, they might also limit the subsequent Trx1-mediated reduction of STAT3.

1.8 TARGETING STAT3 IN CANCER

Constitutive activation of STAT3 has been detected in many different human cancers. In addition, tissue-specific induced knockout of STAT3 does not overtly affect normal tissues *in vivo*. These two observations have led to the belief that STAT3 inhibition could be a very lucrative avenue for targeted anti-cancer therapy, specifically targeting the tumor that depends on STAT3 activation, while other healthy tissues should remain unaffected. This has led to the development of many different strategies to target STAT3 signaling [24,223]. Below, indirect targeting methods, as well as direct STAT3 targeting methods will be addressed.

1.8.1 Indirect STAT3 targeting approaches

Targeting the STAT3 signaling axis can be approached from targeting IL6, JAK, or STAT3. The first JAK targeted therapy that was approved by the FDA is Ruxolitinib [224,225]. An orally bioavailable drug with selective JAK1 and JAK2 inhibition showed clinical efficacy in the treatment of polycythemia vera and MPNs[224–227]. Its use in MPNs is largely to alleviate splenomegaly and other disease-related symptoms [227,228]. Tofacitinib is another orally administered drug with selective JAK1 and JAK3 inhibition, with lower JAK2 binding

affinity [229–231]. It has been approved for clinical use in rheumatoid arthritis, and is currently being clinically evaluated for treatment of several other inflammatory conditions [24]. Unfortunately, none of the JAK inhibitors have shown clinical antitumor efficacy [24]. With ongoing clinical trials showing some clinical benefit of combination treatment strategies, JAK inhibitors could possibly be used as anticancer therapeutics in the future.

Besides the other kinase targeting small molecules, many different and creative methods have been utilized to inhibit STAT3 in preclinical settings. Peptidomimetics derived from different origins targeting the STAT3 SH2 domains, despite having high affinity binding, did not affect STAT3 transcriptional activity in cells [232]. This might be due to their poor stability and cellular permeability, effectively negating their effect *in vivo* [232]. Another method is by effectively diverting phosphorylated STAT3 dimers by decoy oligonucleotides (ODN) containing the consensus STAT3 response element. Intratumoral injection of the ODN in head-and-neck cancer patients demonstrated efficacy through downregulation of STAT3 target genes [233]. Subsequently, a more stable cyclic form of ODNs was generated with improved ADME properties and no apparent toxicities at high doses [24]. Lastly, AstraZeneca has substantially invested in the development of another method of STAT3 targeting [234–236]. They are currently in early clinical trials with AZD9150, a second-generation antisense oligonucleotide targeting STAT3 mRNA [236]. After promising preclinical findings, AZD9150 demonstrated efficacy in patients with treatment-refractory lymphoma and non-small cell lung cancer [236,237]. Although initial promising results, there are major challenges with further clinical implementations of ODNs and AZD9150. Their high molecular weight could mean that efficient delivery is quite challenging, especially in contrast to small molecule inhibitors.

1.8.2 Direct small molecule inhibitors of STAT3

Different small molecule inhibitors have gone through preclinical development over the years and several have been tested in a clinical setting as anti-cancer drugs. Most of the designed small molecules were developed with the rationale to bind to the STAT3 SH2 domain. Through occupation of the binding site of the phosphorylated transactivation domain, STAT3 dimerization should effectively be impaired and transcriptional activity impeded [238,239]. Unfortunately, none of the tested inhibitors have been approved for clinical use, due to lack of therapeutic response or treatment related toxicities [24,26]. Some of the most widely used and most recently identified STAT3 small molecule inhibitors implemented in early clinical trials will be described.

1.8.2.1 STA-21

One of the first hits from a STAT3 SH2 domain virtual screening was STA-21 [240]. Approximately 429.000 small-molecule organic compounds were virtually screened. 100 out of the 200 best scoring small molecules were purchased and further evaluated. These 100 compounds were screened in a cellular STAT3-dependent luciferase assay, where it inhibited STAT3-dependent but not -independent luciferase activity. It was then shown to inhibit STAT3 DNA binding, induce cell death in STAT3-dependent cell lines, affect nuclear

translocation and dimerization [240]. A series of STA-21 derivatives have since been designed with more favorable pharmacokinetic properties. This LLL compound series was consequently patented and the compounds LLL-3 as well as LLL-12 were shown to inhibit STAT3 activity in different preclinical glioblastoma and leukemia models [241].

1.8.2.2 Stattic

In a fluorescence-based polarization assay of approximately 18.000 compounds, Stattic was identified [242]. Since its discovery has widely been used as a STAT3 inhibitor in preclinical STAT3 biology studies. Unfortunately, it has more recently come to light that Stattic is an electrophilic compound, which covalently bind to nucleophilic amino acids, like reduced (seleno-)cysteine residues (Figure 8A) [243]. The exact biological mechanism of STAT3 signaling inhibition has however not been completely elucidated. The original study that identified Stattic as a STAT3 inhibitor, however designed a validation pipeline that has also widely been utilized to identify other STAT3 small molecule inhibitors. This pipeline consists of: an *in silico* or *in vitro* binding assay, cellular reporter assays, EMSA for DNA binding, nuclear translocation by immunofluorescence, phosphorylation of STAT3, STAT1 and other pathway kinases by western blotting, and differential cytotoxicity on STAT3-dependent and –independent cell lines. Direct binding of the small molecule to STAT3 protein is shown *in silico* or *in vitro*, while STAT3 inhibition is measured through indirect methods of transcription factor activity in cells or *in vivo*.

1.8.2.3 S3I-201

In another STAT3 SH2 domain virtual screening campaign of approximately 150.000 small molecules, S3I-201 was found as a potent SH2 domain binder [244]. A different docking approach was utilized, where the STAT3 phosphopeptide crystal structure was docked into the SH2 domain, and small molecules were scored for binding into the predicted phosphopeptide binding site. The *in silico* results were verified by inhibition of STAT3 DNA binding by EMSAs, STAT3-dependent luciferase reporter, STAT3-dependent cell line cytotoxicity, target gene downregulation, and STAT3-dependent xenograft studies. More recently it was indicated that S3I-201 is also an electrophilic compound, which reacts with GSH and unspecifically alkylates different proteins in cells (Figure 8B) [245].

1.8.2.4 BP1-102

Many compounds have been derived from the structural backbone of S3I-201. One compound, BP1-102, was designed to more potently bind to the same SH2 domain binding site [246]. It bound with nanomolar affinity to STAT3. It had good activity in the STAT3 fluorescence polarization assay. Moreover, it also inhibited STAT3 DNA binding and phosphorylation, while not affecting phosphorylation of other kinases in the STAT3 pathway. Interestingly, it had good PK/PD properties, and was orally bioavailable, inducing potent antitumor efficacy in tumor-bearing mice. This compound was later also found to contain an electrophilic moiety, which covalently binds to GSH (Figure 8C) [247]. These properties were

deemed unfavorable and using medicinal chemistry another derivative was designed, which could be evaluated in further preclinical testing.

1.8.2.5 OPB compound series

Several different compounds from Otsuka pharmaceuticals have been tested in early clinical trial stages. Phase 1 clinical trials have been conducted with OPB-31121, OPB-51602 and OPB-111077, showing moderate efficacy in a subset of patients [248–254]. Only sparse preclinical findings have been published on these compounds, the mechanism of action of OPB-51602 has been more elucidated than the other two compounds. However, the other two compounds are claimed to have similar anticancer activity. Initial studies implicated that targeting of the STAT3 SH2 domain leads to mitochondrial dysfunction due to interference of the mitochondrial STAT3 pool [255]. In addition, OPB-51602 treatment leads to aggregation of STAT3 in autophagosomes, while also blocking late stage autophagy [255]. In contrast, another study published that STAT3 knockdown failed to rescue cells from compound cytotoxicity and inhibition of mitochondrial function [256]. Thereby showing that mitochondrial dysfunction is STAT3-independent, and that OPB-51602 is a potent and specific inhibitor of the mitochondrial complex 1. Through inhibition of oxidative phosphorylation, a widely observed mechanism of TKI resistance, cancer cells that are more dependent on the function electron transport chain are more sensitive to treatment with OPB-51602 [256].

Since many of the described STAT3 small molecule inhibitors were shown to not specifically target the STAT3 SH2 domain, the search for a potent and specific inhibitor of STAT3 signaling is still active [243,245,247]. Meanwhile, clinical trials for cancer treatment are also being conducted with FDA approved drugs that were found target STAT3 signaling indirectly. One example is Pyrimethamine, an anti-microbial used for treatment of toxoplasmosis and malaria [257,258]. It inhibits STAT3 activity at concentrations that are safe for patients. It is currently being tested in chronic and small lymphocytic leukemia [257,258].

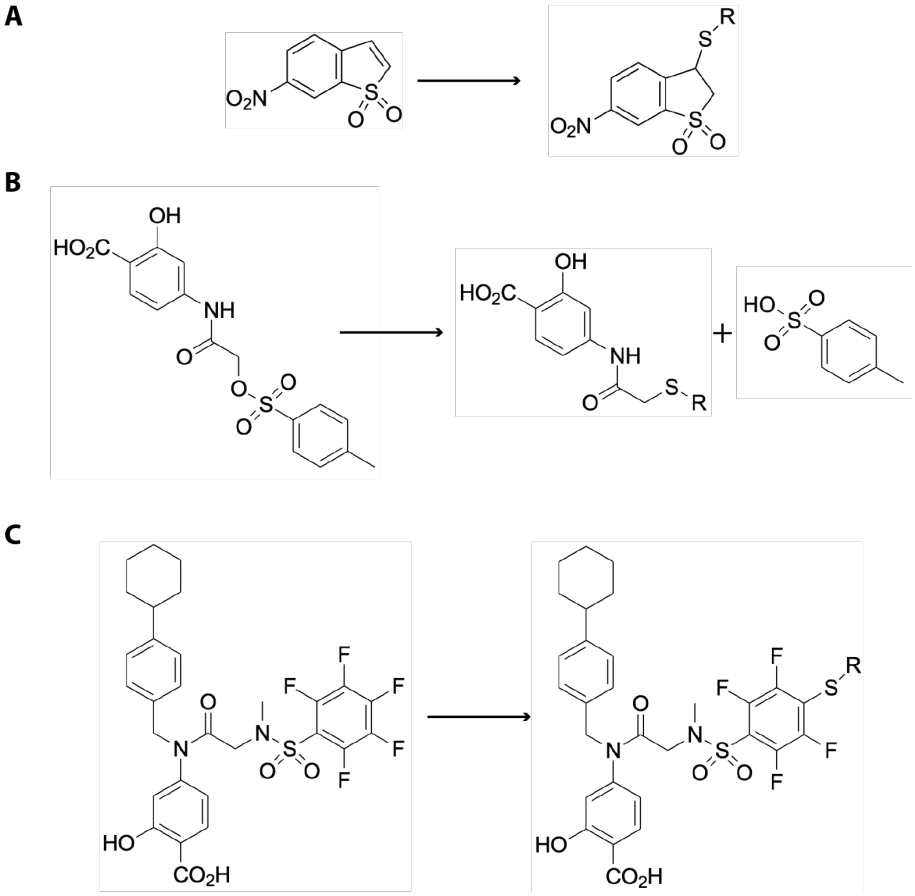

Figure 8. Electrophilic STAT3 small molecule reactivity with nucleophiles. A-C. Proposed mechanism of interaction of Stattic (A), S3I-201 (B) and BP1-102 (C) with cysteine thiols, interaction can also occur with other nucleophilic residues.

2 DOCTORAL THESIS

2.1 PURPOSE & AIMS

Many small molecule inhibitors of STAT3 have been described in the scientific literature. Several of them have also been tested in early clinical trials, but failed to show clinical benefit. **Therefore, the overall aim of this thesis was to elucidate the mechanism of action of known and novel small molecule inhibitors of STAT3, and in due process critically assess the methods used to identify direct targeting small molecule inhibitors of STAT3.**

The specific aims were as follows:

- Elucidate the mechanism of action of classical and novel inhibitors of STAT3 signaling.
- Develop novel methods that can be utilized to identify novel small molecules that specifically target the STAT3 SH2 domain.
- Develop novel methods that can be utilized to identify the cellular target of small molecule inhibitors of STAT3 signaling.

These aims were addressed in the three papers of this thesis, where we attempted to answer the following specific questions:

Paper I

- Is it possible to perform a high-throughput cell-based screening method for STAT3 inhibition?
- Are there any potent novel compounds that inhibit STAT3 function, without affecting upstream kinase activity?

Paper II

- Can differential scanning fluorimetry be utilized as a method for the identification novel small molecule inhibitors of STAT3?
- Do classical STAT3 small molecule inhibitors specifically target the STAT3 SH2 domain?

Paper III

- Is it possible to synthesize very potent small molecule inhibitors of STAT3 signaling?
- What is the cellular target of our identified electrophilic small molecule inhibitors?
- Why are electrophilic small molecules identified as STAT3 inhibitors?
- What is the mechanism of action of STAT3 transcriptional activity inhibition of electrophilic small molecules?

2.2 SCIENTIFIC METHODOLOGIES

2.2.1 Cellular STAT3 luciferase reporter assay (Paper I & III)

A reporter gene is a gene that can encode different identifiable characteristics, which often can be quantified. Commonly used reporter genes include fluorescent proteins, luciferases or other enzymes, like lacZ. The expression of the reporter gene is regulated by the activity of the gene of interest, by the introduction of a regulatory sequence in the promoter of the reporter. Thereby, activity of the gene of interest can be easily measured on the basis of the quantifiable amounts of the reporter protein or enzyme. In our setup we utilized a firefly luciferase reporter construct, which contained 4 STAT inducible elements (SIE) in front of a minimal promoter that drove the expression of the luciferase gene, which contained a protein destabilization sequence (Figure 9). Luciferase transcription is induced upon stimulation of the JAK/STAT pathway with IL6 or IFN-γ. Due to the utilization of stably transfected STAT3 knockout colorectal cancer cell lines (A4 and A4wt) we were able to distinguish specific STAT3-driven luciferase transcription, since these cytokines also activate STAT1, which also can bind to SIE elements and drive transcription. Next we utilized the pan-JAK inhibitor Pyr6 as a negative control, and Stattic, an electrophilic small molecule inhibitor of STAT3, as a positive control. These compounds were selected in order to prevent the selection of upstream kinase inhibitors. Moreover, the addition of the screening library compounds was done one hour after the addition of IL6, again to prevent the selection of upstream kinase inhibitors. The rationale behind being phosphatases should have inactivated JAK kinases after that time.

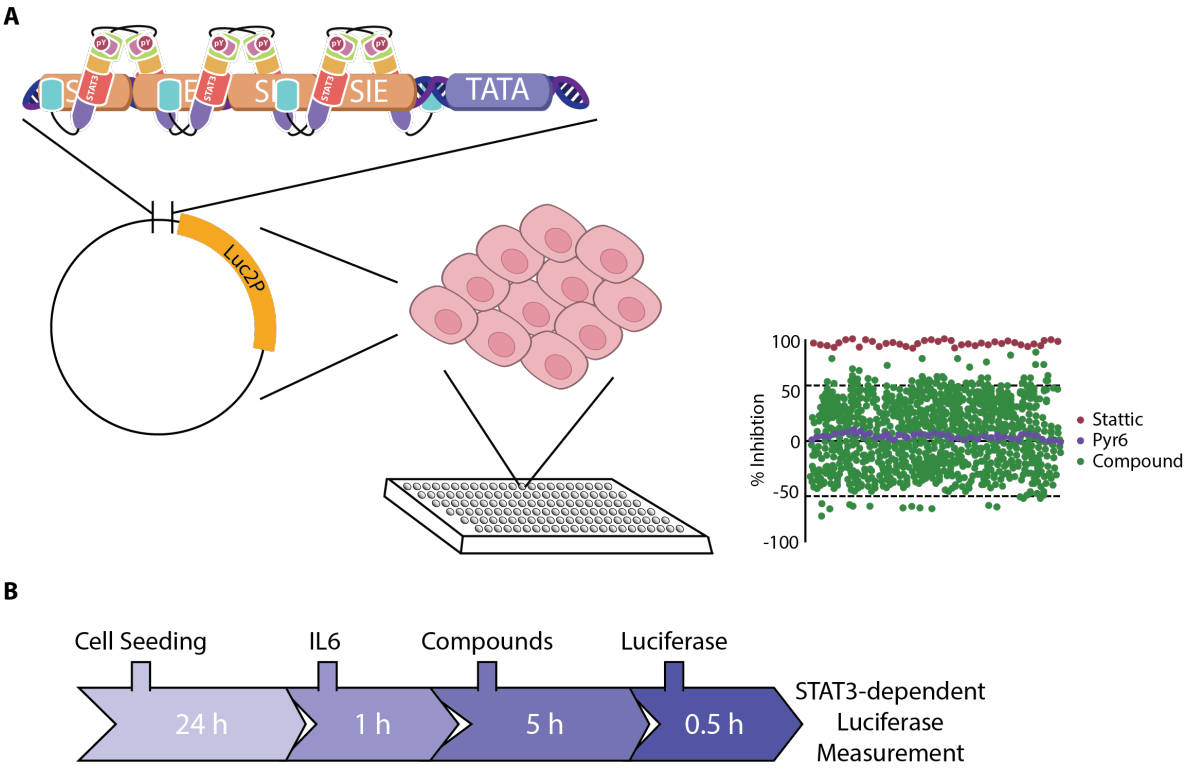

Figure 9. High throughput screening setup using cellular STAT3-dependent luciferase assay. A. Four SIE elements were cloned in front of a minimal promoter, which drives the

transcription of a destabilized luciferase enzyme. Stably transfected cells were then seeded in 384-well plates. Stattic was used as a positive control of STAT3 inhibition, while Pyr6 was used as a negative control in order to exclude upstream kinase inhibition **B.** Cells were stimulated with IL6 for one hour, compounds were added for five hours, followed by measurement of luciferase activity.

In **Paper I** and **Paper III** this assay was used to identify small molecule inhibitors of STAT3 (**Paper I**; Figure 1), and to optimize compound efficiency in the medicinal chemistry campaign (**Paper III**; Figure 1). The initial unforeseen complications with the assays became apparent during the course of **Paper III**. Cellular STAT3 biology appeared to be more intricate than was initially expected. STAT3 transcriptional activity was not only regulated through the activation of the canonical JAK/STAT pathway. It was elucidated that STAT3 activity was also constrained by changes in the cellular redox state, which affected STAT3 directly by oxidation of cysteine thiols on the protein. These findings would advocate for the utilization of *in vitro* experiments in high throughput screenings, in order to find direct inhibitors of STAT3. However, both *in vitro* and cellular assays have different drawbacks that should be regarded when conducting small molecule screening campaigns.

2.2.2 Differential scanning fluorimetry (Paper II)

The efficacy of a small molecule can be directly related with its ability to bind its target of interest. The interaction between small molecule and protein target can be assessed using different experimental setups. Differential scanning fluorimetry (DSF) utilizes the biophysical principle that ligand binding can increase the inherent thermal stability of a protein target. The protein unfolding, which occurs upon gradual heating of the sample can be measured with a nonspecific dye, such as Sypro Orange, which starts to fluoresce upon binding to hydrophobic surfaces, while in water the fluorescent signal is quenched. Measurement of fluorescence on a qRT-PCR, allows for real time tracking of protein unfolding (Figure 10). The inflection point of the melting curve is considered the melting temperature (T_m), which is used to compare different experimental conditions. After the protein has completely unfolded peak fluorescence is achieved upon which the protein starts to aggregate and fluorescence starts to decrease over time. Upon binding of a ligand or small molecule that binds to the protein of interest, the thermal stability should be increased causing a shift in the melting temperature of the protein (ΔT_m).

In **Paper II** this assay was used to identify the thermal stability of two different STAT3 protein truncations, in order to pinpoint specificity of phosphopeptides and small molecules binding to the STAT3 SH2 domain. However, there are still inherent drawbacks to the fundamental concept of the assay. Namely, some ligand-protein interactions do not affect thermal stability, which could lead to false negative results. In addition, the proper protein folding of recombinant protein is key in order to assess its thermal stability, therefore proper verification of protein function and folding using orthogonal methods is essential. Different

methods of protein synthesis and purification could affect protein folding and thereby thermal stability measurements.

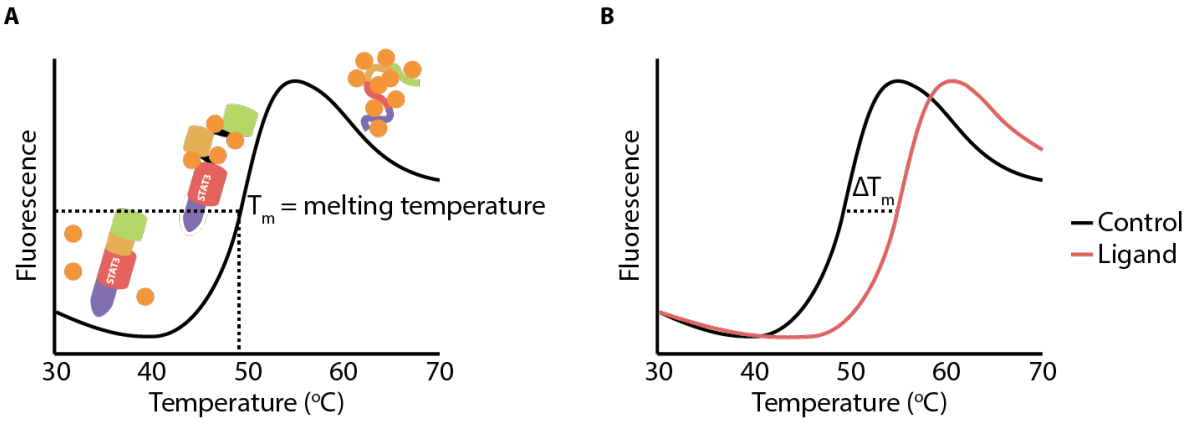

Figure 10. Illustration of DSF protocol A. Samples are heated in a qRT-PCR, where simultaneously fluorescence of Sypro Orange is measured. Temperature is gradually increased, which induces protein unfolding and Sypro Orange binding to its hydrophobic sites. **B.** The addition of a ligand leads to a shift in the ΔT_m of the protein of interest.

2.3 SUMMARY OF RESEARCH PAPERS

2.3.1 Identification of novel small molecules that inhibit STAT3-dependent transcription and function (Paper I)

The transcription factor STAT3 has been considered a lucrative target for anti-cancer therapy, especially since it is activated in many different cancer types. Moreover, it is activated by different cytokine and growth factor pathways. STAT3 is activated in the cytoplasm, through phosphorylation of tyrosine 705 [92]. It subsequently translocates to the nucleus where it initiates gene transcription by binding specific sequences in their promoter region. STAT3 target genes promote proliferation, survival, metastasis and immune evasion [28,99]. A limited amount of STAT3 inhibitors have been tested in early clinical trials, while none have made it to later stages [24]. Therefore, identification of new small molecule inhibitors could lead to their development towards being tested in the clinic.

STAT3 might be a difficult target due to its 3-dimensional structure. Since it is not a kinase, it lacks a commonly targeted ATP-binding pocket or kinase domain. This transcription factor mainly has rather big and flat surfaces unfit for small molecule docking [259]. It does however contain a quite structurally unique SH2 domain and it is believed that small molecules could potentially be designed to target this specific site of STAT3. Therefore, in this study, we ventured to identify and verify novel small molecules that inhibited STAT3 transcriptional activation.

In order to find novel small molecules, a cell-based high-throughput screening system was developed using derived cell lines from DLD1 colon carcinoma cells. The lines A4, with a

homozygous STAT3 deletion, and A4wt, A4 cells with reconstituted expression of STAT3 at physiological levels. A stable transfected line of A4wt cells expressing a STAT3-reporter construct was made. The construct contained 4 SIE binding elements, which drove the expression of a destabilized luciferase gene (Figure 9). Luciferase expression was driven by STAT3 activation through addition of the cytokine IL6. IL6 stimulated expression of luciferase was inhibited by the addition of a pan-JAK inhibitor, Pyr6, and the notorious STAT3 inhibitor Stattic. In order to exclude the potential identification of JAK inhibitors (or other upstream kinase inhibitors) compounds were added 1h after IL6 addition, which effectively prevented Pyr6 inhibition of luciferase expression.

In total 28.000 compounds were screened, of which 267 were identified hits. Of those, 86 did not affect the luciferase assay directly, and 67 were confirmed to have an efficient inhibitory dose response. Further chemical analysis and *in silico* STAT3 SH2 domain docking led to the selection of four lead compounds for further characterization in this study (Figure 11A). In order to test specific inhibition of STAT3 compared to STAT1, inhibition of IL6-induced luciferase by STAT3 was compared to IFN-γ-induced luciferase by STAT1 in respectively A4wt and A4. Inhibition of luciferase was similar for KI1, KI4 and KI12. However, KI16 showed higher propensity towards inhibition of STAT3-driven luciferase transcription. While no acute toxicities were noted after the completion of the luciferase assay (5 hours), KI1 and KI4 were quite cytotoxic to both A4 and A4wt when assessed after 48 hours. Since both cell lines do not depend on STAT3, cytotoxicity was considered an indication of off-target effects.

The quinazolone core structure of both KI1 and KI4 were found to also be present in the clinically used drugs Gefitinib, Erlotinib and Vandetanib (Figure 11B). Due to this similarity these compounds were suspected to target ATP binding pockets of kinases. It was later confirmed that both compounds inhibited phosphorylation of both upstream kinases JAK1 and JAK2 in A4wt cells (Figure 11C). Only KI16 was able to inhibit IL6-induced STAT3 phosphorylation in A4wt cells, while none of the compounds affected pSTAT1 either through IL6 or IFN-γ induction (Figure 11D). STAT3 mainly drives oncogenic processes though its transcriptional function. All four compounds inhibited IL6-driven STAT3 target gene expression, while affecting STAT1 target genes to a lesser extent especially for KI12 and KI16. Compounds were in general more cytotoxic towards STAT3-dependent compared to STAT3-independent cell lines. Interestingly, the compounds affected pSTAT3 levels differentially in two lines with constitutive STAT3 activation (MDA-MB468 & DU-145). They however did inhibit two target genes and affected migration ability in DU145 cells (Figure 11D-E).

Overall, these four novel compounds were identified using a cell-based high-throughput assay for STAT3 transcriptional activity. It was suggested that KI1 and KI4 target upstream kinases, while KI12 and KI16 directly target STAT3 by docking into the SH2 domain, thereby preventing STAT3 dimerization and transcriptional activity. Inhibition of STAT3

activity was further verified by assessment of STAT3 phosphorylation, target gene expression as well as STAT3-dependent migration.

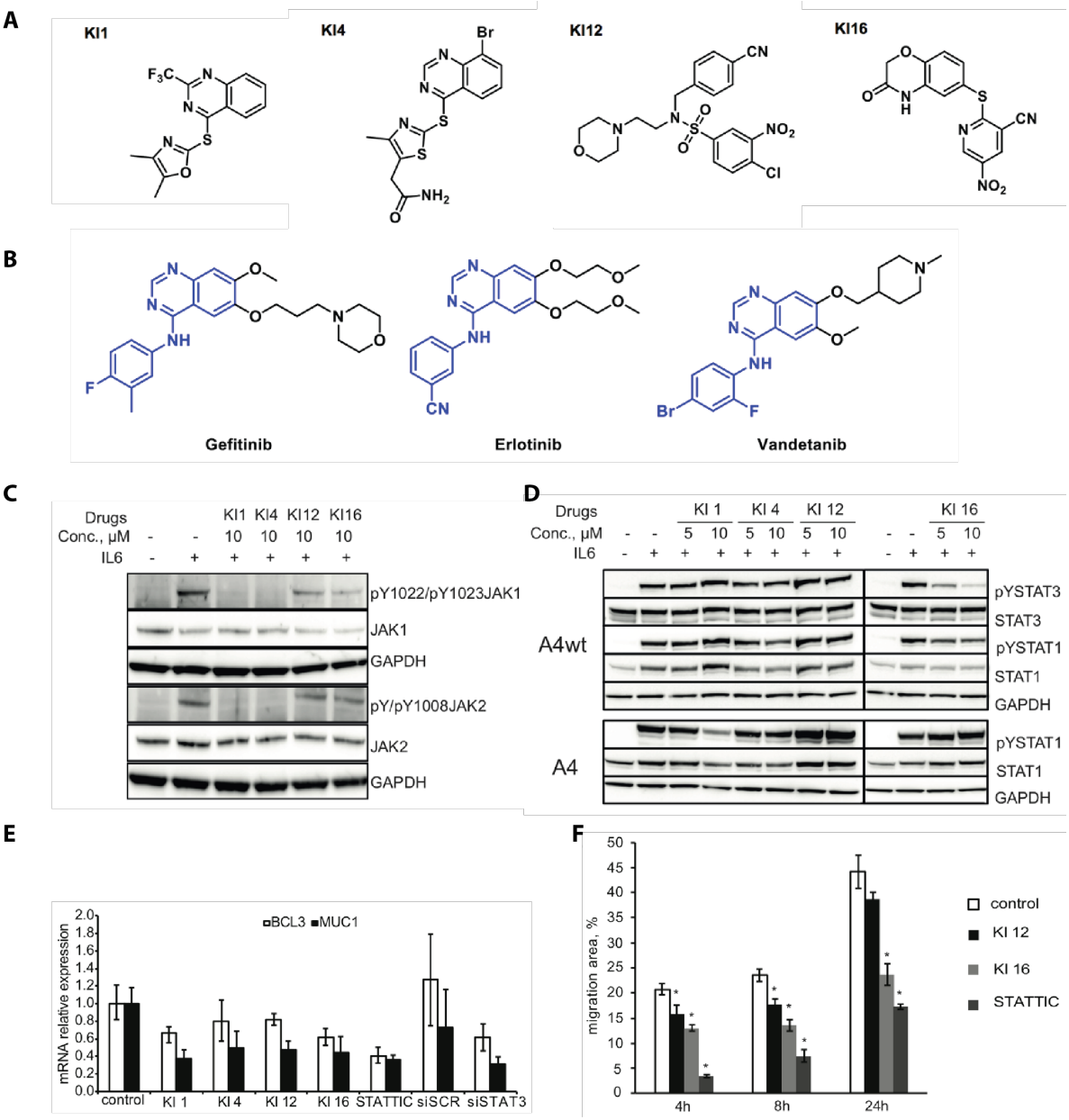

Figure 11. Four lead compounds have differential mechanisms of STAT3 inhibition. A. Compound structures of KI1, KI4, KI12 and KI16. **B.** The quinazolone core present in KI1 and KI4 is also found in different clinically approved kinase inhibitors Gefitinib, Erlotinib and Vandetanib. **C.** KI1 and KI4 inhibit phosphorylation of upstream JAK kinases, thereby inhibiting luciferase transcription. KI12 and KI16 do not inhibit upstream kinases and are therefore believed to directly inhibit STAT3. **D.** Only KI16 inhibits STAT3 phosphorylation, STAT1 phosphorylation is not affected by any of the compounds. **E.** STAT3 target gene expression is inhibited by all four compounds and Stattic in DU145 cells. STAT3 target gene expression was verified by STAT3 knockdown. **F.** KI12, KI16 and Stattic inhibit cellular migration of DU145 cells.

2.3.2 STAT3 differential scanning fluorimetry and differential scanning light scattering assays: Addressing a missing link in the characterization of STAT3 inhibitor interactions (Paper II)

Many therapeutic agents are under development and many papers continuously report on the identification of novel inhibitors of STAT3. These compounds are usually evaluated to be STAT3 inhibitors with a certain combination of *in silico, in vitro*, cellular and often *in vivo* experiments. Some of the established *in vitro* assays, such as the fluorescence polarization assay, electrophoretic mobility shift assay and ELISAs, are often utilized to assess direct interaction of an inhibitor with STAT3 protein [223,230,242,244,260]. Here we tried to highlight some of the shortcomings of some of these methods, through the development of DSF and differential scanning light scattering assays (DSLS) for STAT3 inhibitor evaluation.

First, the specificity of the method had to be evaluated. In order to do so, different STAT3 protein truncations were generated. $STAT3^{127-688}$ spanned from the coiled-coil to the SH2 domain, while $STAT3^{127-468}$ spanned from the coiled-coil to the DNA binding domain (Figure 12A). These truncations were selected for their stability and proper folding of the proteins, especially since full-length STAT3 protein was found to be very unstable and therefore unsuitable for DSF. More importantly, the apparent lack of the SH2 domain of $STAT3^{127-468}$ permitted the assessment of specific SH2 domain binders by comparison with $STAT3^{127-688}$. Using different phosphopeptides, gp130, LIFR and STAT3c, specific SH2 domain binding was assessed. These peptides were only able to bind to $STAT3^{127-688}$ or full-length STAT3 in fluorescence polarization assays (Figure 12B). Furthermore, specific binding to the SH2 domain was verified using hydrogen-deuterium exchange (HDX) mass spectrometry.

All phosphopeptides enhanced thermal stability of $STAT3^{127-688}$ in concentration-dependent manners, while $STAT3^{127-468}$ thermal stability was unaffected (Figure 12C). The thermal shift was determined by the binding affinity of the phosphopeptide, therefore gp130 caused the largest and STAT3c the smallest shift, similar to results found in the fluorescence polarization assay. Remarkably, four known and widely used STAT3 inhibitors, BP1-102, Stattic, STA-21 and S3I-201, either caused a negative thermal shift or did not affect thermal stability of both truncations (Figure 12D). All thermal stability results were lastly confirmed by nanoDSF and nanoDSLS.

These last results indicate that the STAT3 inhibitors that are widely used for preclinical assessment of STAT3 function might not target STAT3 through their supposed mechanism of action of SH2 domain binding. Other papers have also highlighted different mechanisms of action of BP1-102, Stattic and S3I-201, indicating that all three contain electrophilic properties that cause unspecific irreversible alkylation of protein cysteine residues [243,245,247]. The development of DSF for STAT3 protein with these two truncations furthermore allows for novel high-throughput screening campaigns to identify specific binders of the STAT3 SH2 domain, which could lead to the identification of novel compounds that might specifically target STAT3 function.

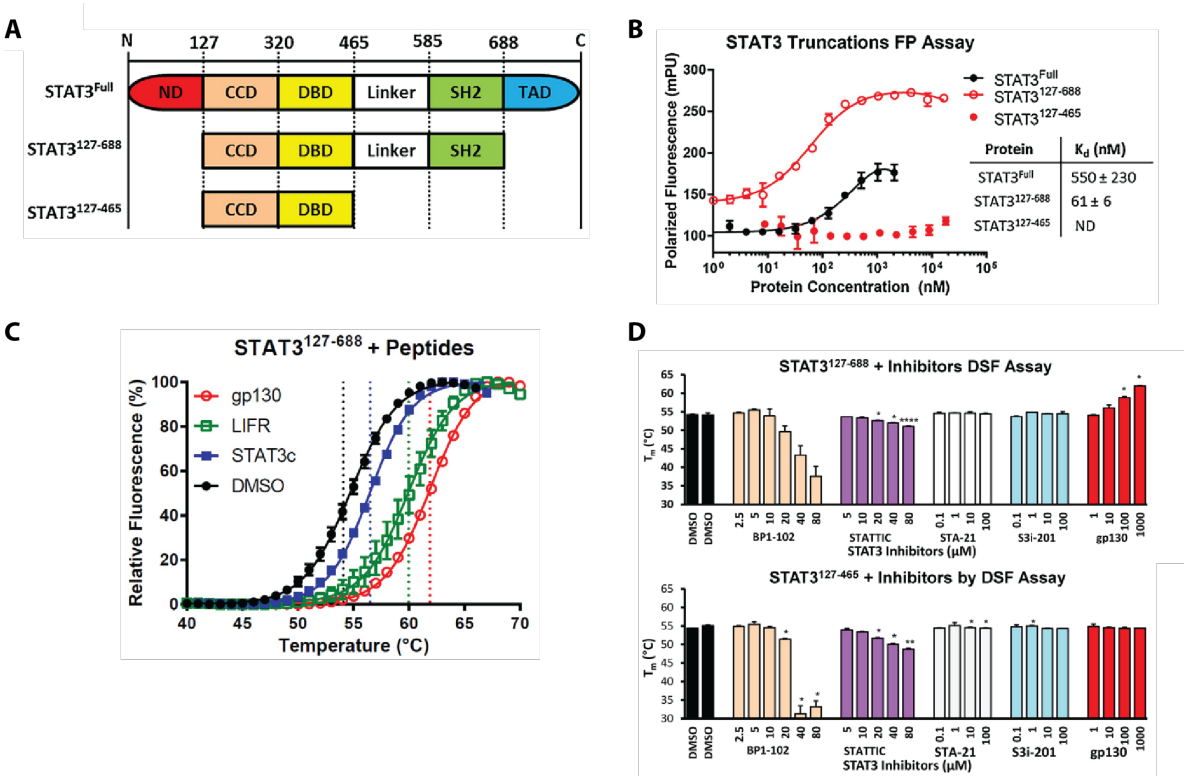

Figure 12. Utilization of two protein truncations allows the evaluation of specific STAT3 SH2 domain binders. A. The different domains that are present in the truncations used in this study. **B.** STAT3$^{127\text{-}468}$ was unable to bind a 5-FAM-gp130 peptide not causing any change in fluorescence polarization. The other two proteins contained an SH2 domain and were able to bind the fluorescent gp130 peptide. **C.** Different phosphopeptides cause a positive thermal shift of STAT3$^{127\text{-}688}$ while not affecting STAT3$^{127\text{-}468}$ thermal stability. **D.** Known STAT3 small molecule inhibitors caused a dose-dependent negative thermal shift of both STAT3$^{127\text{-}688}$ and STAT3$^{127\text{-}468}$ or did not affect thermal stability.

2.3.3 Disrupting oncogenic STAT3 activity by targeting TrxR1 with irreversible covalent inhibitors (Paper III)

A select series of compounds was identified in the high-throughput screening from **Paper I**. These 4,5-dichloropyridazanone compounds were further assessed in **Paper III**. In order to further explore these compounds we ventured to optimize activity through a structure-activity relationship (SAR) study, which led to the development of in total 70 analogues. Top compounds had sub 1 μM IC_{50} values in the STAT3-dependent luciferase assay also used in **Paper I** (Figure 9). Modifications to the "linker" and "tail" were well tolerated, however modifications to the 4,5-dichloropyridazanone moiety were not even slightly tolerated (Figure 13A). Counter-screens for luciferase enzyme inhibition or cytotoxicity revealed no false positive results with any of the tested analogues.

Four of the most potent compounds were then selected and further assessed (**DG-4** to **DG-7**). All compounds were cytotoxic to different cancer cell lines, while less affecting non-

cancerous cells. The lung adenocarcinoma A549 cells were however found to be the least sensitive. The discovery that several widely used STAT3 inhibitors turned out to be electrophilic alkylating agents, led us to also test the reactivity of these compounds. Which were found to react with GSH. It was feared that these compounds would also inhibit STAT3 in a more unspecific alkylation of protein cysteine residues, especially since the non-electrophilic analogues did not affect STAT3-dependent luciferase [243,245,247]. In order to pinpoint the mechanism of action of this series the "tail" of **DG-4** was substituted with a fluorescent dansyl moiety, **DG-8**. **DG-8** was able to interact with both STAT3 truncations generated in **Paper II**. However, cell lysate and cellular treatment with **DG-8** revealed that it specifically bound to a ~55kDa protein (Figure 13B). Therefore, we verified that even though our compounds could interact with STAT3, in a cellular setting this did not occur. Hence, we set out to discover the cellular protein target. Unfortunately, we were unable to exactly pinpoint the protein of interest, however scientific literature revealed a similar compound containing a 4,5-dichloropyridazinone group that was identified as a TrxR1 inhibitor. TrxR1 is a 55kDa protein, additionally containing a highly nucleophilic Sec residue in its active site that is very prone to interact with electrophilic moieties. **DG-8** was able to bind to reduced recombinant TrxR1, and promotion of cysteine insertion in the place of Sec also decreased **DG-8** binding affinity to TrxR1 in cells. Competition assays with top compounds and identified TrxR1 inhibitors showed the ability of **DG-4, DG-5, DG-6, TRi-1,** Stattic and Auranofin to potently outcompete **DG-8** in cells. The less potent compound **DG-7** as well as **TRi-3** was only able to outcompete **DG-8** at high concentrations. Interestingly, **TRi-2**, which is believed to not target the Sec residue but rather the FAD moiety of TrxR1, was unable to outcompete **DG-8** further confirming the specific targeting of the Sec residue by the **DG** compounds.

Next, we explored the inhibitory effects of the compounds on TrxR1 function. Compounds **DG-4** and **DG-5** were potent inhibitors of cellular TrxR1, while **DG-6** and Stattic inhibited approximately 40% of total TrxR1 activity (Figure 13C). Lastly, **DG-7** and BP1-102 did not affect cellular TrxR1 activity. Interestingly, the ability to inhibit cellular TrxR1 corresponded to the ability of the compounds to outcompete **DG-8**. All compounds were unable to inhibit TrxR1 function in the absence of NADPH, similar to the inability of **DG-8** to bind to oxidized TrxR1. Moreover, compound binding to the TrxR1 Sec residue led to the formation of SecTRAPs, which are known to create additional intracellular reactive oxygen species. In addition, we were able to show that four known TrxR1 inhibitors were also able to inhibit STAT3-dependent luciferase.

Interestingly, compound treatment caused Prx2 and STAT3 oxidation (Figure 13D). This was previously shown to be a mechanism of STAT3 transcriptional inactivation [149]. The induction of oxidative stress caused by compound treatment leads to oxidation of Prx2 that can relay oxidative equivalents to STAT3 (Figure 6). TrxR1 is the reductase that keeps Prx2 and STAT3 reduced by consuming NADPH. Our compounds however inhibit TrxR1 leading to a halt in the reduction of the relay, thereby causing Prx2 and STAT3 oxidation, which is further exacerbated by SecTRAP formation. Lastly, compound cytotoxicity was potentiated

approximately 5-fold by buthionine sulfoximine (BSO) treatment. BSO inhibits the synthesis of GSH, leading to cellular GSH depletion. This indicated that cytotoxicity was caused by induction of oxidative stress.

Altogether, our data suggest that the top **DG** compounds are potent inhibitors of TrxR1, and TrxR1 inhibition could cause additional cellular ROS generation, which in turn oxidizes Prx2 and STAT3 inhibiting its transcriptional activity. These results were strengthened by the observation that other widely used STAT3 inhibitors also inhibited TrxR1 function, while several known TrxR1 inhibitors were also able to inhibit STAT3 transcriptional activity.

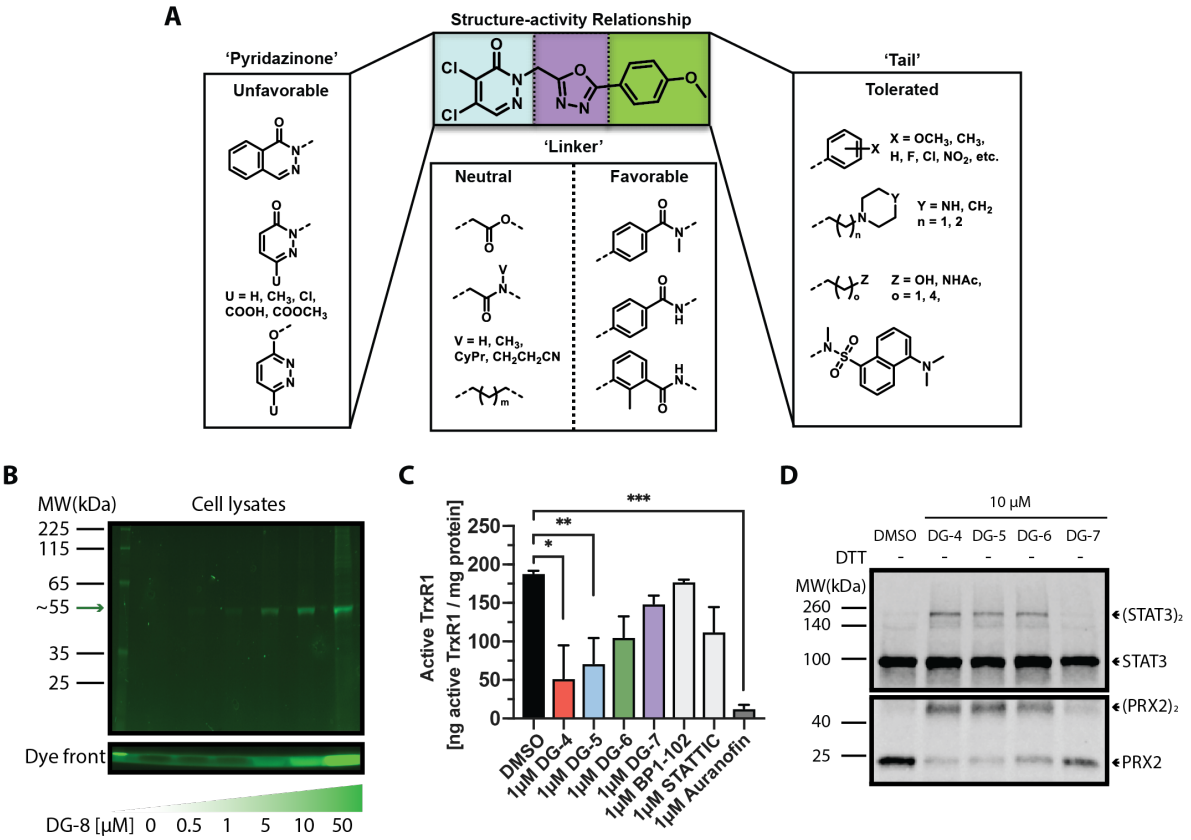

Figure 13. 4,5-dichloropyridazinone compounds inhibit STAT3 through inhibition of TrxR1 causing STAT3 inactivation through its oxidation A. Summary of SAR study, modifications to the dichloropyridazinone were found to be generally unfavorable and led to the loss of STAT3 inhibition. Modifications to the "linker" were able to increase compound potency, especially by the introduction of electron-withdrawing groups to the nitrogen at the two position of the pyridazinone ring. Modifications to the "tail" were well tolerated and allowed for the appendage of a dansyl fluorescent group to the compound, without significant loss of cellular permeability and inhibition potency. **B.** Instead of binding to STAT3 in a cellular setting, the fluorescent **DG-8** bound a singular 55kDa protein in A549 cells. This protein was confirmed to be TrxR1. **C.** Top compounds are potent inhibitors of cellular TrxR1. **D.** Top compounds cause oxidation of both Prx2 and STAT3, causing STAT3 to no longer be transcriptionally active and drive expression of its target genes.

2.4 DISCUSSION & IMPLICATIONS

The papers enclosed in this thesis are purely dedicated to the STAT3 small molecule inhibitor field. This was initiated by the observations and conclusions we made in **Paper I**. We were able to identify novel small molecule inhibitors of STAT3 function, and could provide both *in vitro* as well as cellular data to confirm STAT3 inhibition. However, we lacked target engagement experiments in a cellular setting. Therefore, we tried to highlight the importance of result translation from *in vitro* to cellular data in **Paper II** & **Paper III**. We focused on novel *in vitro* method development in **Paper II**, which could be utilized for the development of novel direct STAT3 inhibitors. In **Paper III** we focused on compound target and mechanism of action elucidation, where we were able to pinpoint a target in a cellular setting that could directly affect STAT3 function. The cellular function of our target, TrxR1, and its effects on other cellular functions could also be explored further. Future work could focus on two main areas. The further development and identification of novel direct STAT3 inhibitors, more specifically SH2 domain binders of STAT3. On the other hand, the TrxR1 or Trx pathway function and its effects on STAT3, other transcription factors and cellular functions could be further assessed.

Several novel methods have been developed in recent years to explore protein target engagement. One of the more repeatedly used is the cellular thermal shift assay (CETSA). CETSA is based on the same concept as DSF that ligand or small molecule binding can shift protein thermal stability (**Paper II**). The clear advantage of CETSA is that target engagement can be assessed in cells, *in vivo* and the method has been optimized to also be performed in high throughput [261–263]. It has further been combined with mass spectrometry in order to analyze thermal stability of many different cellular proteins in the same biological sample [264]. These different methods could be readily used to determine the protein target and specificity of the KI compounds of **Paper I**, and could further explain the differences in mechanism of action. The potential drawback to this method could be that different post-translational modifications indirectly caused by small molecule compounds potentially also influence protein thermal stability. This could convolute the results, especially when the target protein is unknown.

STAT3 is not the only protein that becomes oxidized during cellular oxidative stress. As described before, the transcription factor pathways of Nrf2 and NF-κB are also redox regulated through different mechanisms [200,204,214]. A comprehensive list of proteins that are oxidized by different Prxs during H_2O_2 exposure was recently published, where oxidized proteins were captured using a recombinant human Trx1 trapping mutants [150]. ASK1, annexin A2, cystathionine β-synthase, STAT3 and different heat shock proteins among approximately 40 proteins were identified as substrates of Prx1, Prx2 and Trx1 by tryptic peptide mass spectrometry. This is an indication that only a select group of proteins is prone to oxidation. However, direct oxidation of proteins should not be disregarded. These proteins might not have been identified using this method. An overall decrease in protein thiol oxidation however was observed in dual Prx1/Prx2 knockout and knockdown cell lines. The effects of the **DG** compounds (**Paper III**) on the transcriptional function of Nrf2 and NF-κB should be

tested. And the function of the other identified protein substrates of Prxs should also be assessed for their importance in cancer development and potential off-target effects. Perhaps specific targeting of TrxR1 is the way forward to inhibit STAT3 in tumor cells. TRi compounds are actively being optimized for clinical application, and therefore time will tell if they also can be used as clinical drugs. There might be a role for TrxR1 inhibitors in the clinic, very recently APR-246 was FDA approved for treatment of patients with TP53 mutant MPN. APR-246 has been described to reactive mutant p53 and trigger apoptosis in tumor cells, however it was also shown to inhibit TrxR1 irrespective of p53 status [265,266].

At the start of this thesis the importance of cellular redox signaling for STAT3 function had just become apparent [149]. Several STAT3 small molecule inhibitor papers acknowledge the effects their compounds have on cellular redox balance, and often highlight the importance of STAT3 for mitochondrial function and therefore direct inhibition of STAT3 might affect the cellular redox balance [239,255]. One recent study however focused on cancer cell metabolism and mitochondrial function [256]. The authors highlighted the importance of a metabolic switch to oxidative phosphorylation as a key driver for cancer cells to acquire resistance to a wide range of oncogene-targeted therapies, which has moreover been shown to cause STAT3 activation [100–105]. More interestingly is that they identified OPB-51602, one of the most recent and promising STAT3 inhibitors in early clinical trials, as a specific inhibitor of oxidative phosphorylation. OPB-51602 was identified as a specific inhibitor of mitochondrial complex I activity at nanomolar concentration. Other oxidative phosphorylation inhibitors have also been identified as inhibitors of STAT3 activity. Analysis of a 12-gene STAT3 activation signature, revealed Atovaquone as a potent inhibitor of STAT3 transcriptional activity [267]. Atovaquone is an aromatic compound known as a quinone, which are used as antimicrobials and also have antitumor activity. Many quinones were shown to also inhibit TrxR1 and cause the formation of SecTRAPs [199]. These observations draw a link between the effects of cellular redox, metabolism and STAT3 function. It would be very interesting to further explore the cellular targets of these compounds, and clearly delineate the causes of the observed effects of many electrophilic and other compounds.

The intricacies of STAT3 biology are becoming more and more apparent over the years, and especially with the number of published small molecule inhibitors of STAT3. The shear number of claimed STAT3 inhibitors that are being continuously published could however counteract the potential of one being selected and pushed towards clinical trial. It is painful to see that none have made it to a clinical application and it is testament to the degree of difficulty of specific STAT3 targeting. This might unfortunately also deter potential new exploits of reaching this esteemed goal. However, several academic and commercial ventures are not deterred and keep working towards reaching it.

3 ACKNOWLEDGEMENTS

My PhD has rather unconventionally led me through different groups (Grandér, Page & Arnér), buildings (CCK, SciLife & Biomedicum), and departments (Onk-Pat & MBB). I would like to thank all who made these four years as smooth and pleasant as possible.

Brent, my supervisor extraordinaire, I never would have been where I am now if you had not invested your faith in me. You took me in when I really needed it. Your one on one talks in the lab and elaborate schematic drawings on the chemical hood glass really helped the project advance at a rapid pace. Thank you for all the time you have invested and your excellent supervision, you are and will be a great PI to many PhD students to come.

Elias, first time we met you immediately hit the nail on the head. "That band has to be Thioredoxin reductase!" The protein target was identified in the blink of an eye. Thank you for all your help and supervision, even when you were not my supervisor. You showed me how to really dissect the story that is hidden in experimental data and results. And your kind and positive supervision was the perfect way of getting me acquainted with the world of redox biology. Thank you for taking me in and putting your faith in a nomad PhD student.

Arne, you are the epitome of what a lifetime in academic research can give to a person, which is very inspiring. As a pioneer of the redox field your knowledge and input is always insightful and interesting. Thank you for sharing your amazing stories on the many interesting scientists and Hollywood stars you have had the pleasure to be acquainted with.

Dan, you were the one that gave me this opportunity. Adam Sierakowiak pulled you aside and emphasized that you had to meet this Dutch student working on fMRI analysis of an animal model of Parkinson's disease. I remember our first meeting, you asked me to explain my findings of one of my projects, and I couldn't even remember if my results were up or down. Regardless, you put your trust in me during my very early stages of my scientific career. I hope I was able to convince you that you made the right decision. It is bitter that everything went as it did, and you were unable to guide me through the second part of my PhD. Your kindness, patience and ability to listen to whatever (non-) sense I had to share I will forever remember.

My mentor **Niko**, thank you for your company, for sharing your experiences, and your insightful advice during our evening meetings over beers and a fläsknoisette. Even though meeting became more difficult after you moved to Germany, I really appreciate your support from the time of my internship in the Svenningsson lab until now.

From CCK, **Muyi**, always available for a last-minute lunch date, or playing seemingly endless games of HORSE no matter the weather. **Pablo, Pedro, Ioannis, Lilly, Julia & Nikos,** thank you for all the fun times, and for creating a nice atmosphere.

From SciLife, **Sanaz**, Brent hit the jackpot when he decided to hire you. You made STAT3 your own in a flash! **Nick, Simin, Sean, Anna & Flor**, thank you for your kindness and help

in the lab and your enthusiasm for science. **Carlos & Edwin**, thank you for the football games and good times!

From the Biochemistry division, **Belen, Chuying, Dorian, Lucia, Madhu, Markus, Pradeep, Radosveta, Ren, Qing, Yuan & Åse,** I never knew a lab could run so smoothly. You all have created a very nice and pleasant work environment for each other. Thanks to all of you I was generating results in just a couple of days, a special thanks to **Belen & Qing** for showing me the ropes on everything Biochemistry!

Peter, thanks for all the late night gaming sessions and being a great friend. Thank God that our internet connections were always quite smooth so we could stay in touch and bullshit. I am sorry that I was the one that kept moving away right at the moments you were able to move closer to me.

My parents, for being understanding during these years I have dedicated to this PhD. Thank you for all your support and help, mentally as well as with painting and building in our apartment!

My siblings, **Laura & Stefan** (**Emi & Yoshi)**, thank you for your support. Sorry for being MIA on multiple occasions back in the Netherlands.

Christina, you are the best partner anyone could wish for. You always know how to support me whatever my mindset and mood. All the words are pale in comparison to how I feel about you. I will try to give you my best self, every single day.

Ianthe, thank you for waiting to come into our lives until I finished including all the references in this thesis.

4 REFERENCES

1. Trigos, A. S., Pearson, R. B., Papenfuss, A. T. & Goode, D. L. How the evolution of multicellularity set the stage for cancer. *Br. J. Cancer* **118,** 145–152 (2018).

2. Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: The next generation. *Cell* **144,** 646–674 (2011).

3. Gilman, A. The initial clinical trial of nitrogen mustard. - PubMed - NCBI. *Am J Surg* **105,** 574–578 (1963).

4. DeVita, V. T. & Chu, E. A history of cancer chemotherapy. *Cancer Res.* **68,** 8643–8653 (2008).

5. Robbins, R. J. & Schlumberger, M. J. The evolving role of (131)I for the treatment of differentiated thyroid carcinoma. *J. Nucl. Med.* **46 Suppl 1,** 28S–37S (2005).

6. Butt, W. R., Lynch, S. S. & Rudd, B. T. Recurrent breast cancer treated with the antioestrogen tamoxifen: Correlation between hormonal changes and clinical course. *Br. Med. J.* **1,** 425–428 (1977).

7. Druker, B. J. *et al.* Efficacy and Safety of a Specific Inhibitor of the Bcr-Abl Tyrosine Kinase in Chronic Myeloid Leukemia. *N. Engl. J. Med.* **344,** 1031–1037 (2001).

8. Talpaz, M. *et al.* Imatinib induces durable hematologic and cytogenetic responses in patients with accelerated phase chronic myeloid leukemia: results of a phase 2 study. *Blood* **99,** 1928–37 (2002).

9. Liou, G.-Y. & Storz, P. Reactive oxygen species in cancer. *Free Radic. Res.* **44,** 479–496 (2010).

10. Rhee, S. G. H2O2 , a Necessary Evil for Cell Signaling. *Science (80-.).* **312,** 1882–1883 (2006).

11. Waris, G. & Ahsan, H. Reactive oxygen species: role in the development of cancer and various chronic conditions. *J. Carcinog.* **8,** 1–8 (2006).

12. Trachootham, D., Alexandre, J. & Huang, P. Targeting cancer cells by ROS-mediated mechanisms: A radical therapeutic approach? *Nat. Rev. Drug Discov.* **8,** 579–591 (2009).

13. Conklin, K. A. Chemotherapy-associated oxidative stress: Impact on chemotherapeutic effectiveness. *Integr. Cancer Ther.* **3,** 294–300 (2004).

14. Sonveaux, P. ROS and radiotherapy: more we care. *Oncotarget* **8,** 35482–35483 (2017).

15. Fuller, G. M., Otto, J. M., Woloski, B. M., McGary, C. T. & Adams, M. A. The effects of hepatocyte stimulating factor on fibrinogen biosynthesis in hepatocyte monolayers. *J. Cell Biol.* **101,** 1481–1486 (1985).

16. Nishi, N. *et al.* A human prostatic growth factor (hPGF): partial purification and characterization. *Biochem. Biophys. Res. Commun.* **132,** 1103–9 (1985).

17. Hirano, T. *et al.* Purification to homogeneity and characterization of human B-cell differentiation factor (BCDF or BSFp-2). *Proc. Natl. Acad. Sci. U. S. A.* **82,** 5490–4 (1985).

18. Andus, T. *et al.* Recombinant human B cell stimulatory factor 2 (BSF-2/IFN-β2) regulates β-fibrinogen and albumin mRNA levels in Fao-9 cells. *FEBS Lett.* **221,** 18–22 (1987).

19. Gauldie, J., Richards, C., Harnish, D., Lansdorp, P. & Baumann, H. Interferon beta 2/B-cell stimulatory factor type 2 shares identity with monocyte-derived hepatocyte-stimulating factor and regulates the major acute phase protein. *Proc. ...* **84,** 7251–7255 (1987).

20. Yamasaki, K. *et al.* Cloning and expression of the human interleukin-6 (BSF-2/IFNβ 2) receptor. *Science (80-.).* **241,** 825–828 (1988).

21. Bromberg, J. F. *et al.* Stat3 as an oncogene. *Cell* **98,** 295–303 (1999).

22. Darnell, J., Kerr, I. & Stark, G. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science (80-.).* **264,** 1415–1421 (1994).

23. Hirano, T. & Kishimoto, T. Interleukin-6: possible implications in human diseases. *Ric. Clin. Lab.* **19,** 1–10 (1989).

24. Johnson, D. E., O'Keefe, R. A. & Grandis, J. R. Targeting the IL-6/JAK/STAT3 signalling axis in cancer. *Nature Reviews Clinical Oncology* **15,** 234–248 (2018).

25. O'Shea, J. J. *et al.* The JAK-STAT Pathway: Impact on Human Disease and Therapeutic Intervention. *Annu. Rev. Med.* **66,** 311–328 (2015).

26. Wake, M. S. & Watson, C. J. STAT3 the oncogene - Still eluding therapy? *FEBS J.* **282,** 2600–2611 (2015).

27. Munoz, J., Dhillon, N., Janku, F., Watowich, S. S. & Hong, D. S. STAT3 Inhibitors: Finding a Home in Lymphoma and Leukemia. *Oncologist* **19,** 536–544 (2014).

28. Carpenter, R. L. & Lo, H. W. STAT3 target genes relevant to human cancers. *Cancers (Basel)*. **6,** 897–925 (2014).

29. Akira, S. *et al.* Molecular cloning of APRF, a novel IFN-stimulated gene factor 3 p91-related transcription factor involved in the gp130-mediated signaling pathway. *Cell* **77,** 63–71 (1994).

30. Levy, D. E. & Darnell, J. E. STATs: Transcriptional control and biological impact. *Nat. Rev. Mol. Cell Biol.* **3,** 651–662 (2002).

31. Abroun, S. *et al.* STATs: An Old Story, Yet Mesmerizing. *Cell J.* **17,** 395–411 (2015).

32. Decker, T. & Kovarik, P. Serine phosphorylation of STATs. *Oncogene* **19,** 2628–2637 (2000).

33. Gough, D. J. *et al.* Mitochondrial STAT3 supports RasDependent oncogenic transformation. *Science (80-.).* **324,** 1713–1716 (2009).

34. Wegrzyn, J. *et al.* Function of mitochondrial Stat3 in cellular respiration. *Science (80-.).* **323,** 793–797 (2009).

35. Becker, S., Groner, B. & Müller, C. W. Three-dimensional structure of the Stat3β homodimer bound to DNA. *Nature* **394,** 145–151 (1998).

36. Nkansah, E. *et al.* Observation of unphosphorylated STAT3 core protein binding to target dsDNA by PEMSA and X-ray crystallography. *FEBS Lett.* **587,** 833–839 (2013).

37. Hu, T. *et al.* Impact of the N-Terminal Domain of STAT3 in STAT3-Dependent Transcriptional Activity. *Mol. Cell. Biol.* **35,** 3284–3300 (2015).

38. Yu, H. & Jove, R. The STATs of cancer — new molecular targets come of age. *Nat. Rev. Cancer* **4,** 97–105 (2004).

39. Linher-Melville, K. & Singh, G. The complex roles of STAT3 and STAT5 in maintaining redox balance: Lessons from STAT-mediated xCT expression in cancer cells. *Mol. Cell. Endocrinol.* **451,** 40–52 (2017).

40. Bromberg, J. F., Horvath, C. M., Wen, Z., Schreiber, R. D. & Darnell, J. E. Transcriptionally active Stat1 is required for the antiproliferative effects of both interferon alpha and interferon gamma. *Proc. Natl. Acad. Sci. U. S. A.* **93,** 7673–8 (1996).

41. Schoenborn, J. R. & Wilson, C. B. Regulation of Interferon-γ During Innate and Adaptive Immune Responses. *Adv. Immunol.* **96,** 41–101 (2007).

42. Sadler, A. J. & Williams, B. R. G. Interferon-inducible antiviral effectors. *Nat. Rev. Immunol.* **8,** 559–568 (2008).

43. Mogensen, T. H. IRF and STAT Transcription Factors - From Basic Biology to Roles in Infection, Protective Immunity, and Primary Immunodeficiencies. *Front. Immunol.* **9,** 3047 (2018).

44. Durbin, J. E., Hackenmiller, R., Simon, M. C. & Levy, D. E. Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. *Cell* **84,** 443–50 (1996).

45. Meraz, M. A. *et al.* Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. *Cell* **84,** 431–42 (1996).

46. Meissl, K., Macho-Maschler, S., Müller, M. & Strobl, B. The good and the bad faces of STAT1 in solid tumours. *Cytokine* **89,** 12–20 (2017).

47. Kaplan, D. H. *et al.* Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice. *Proc. Natl. Acad. Sci. U. S. A.* **95,** 7556–61 (1998).

48. Klover, P. J. *et al.* Loss of STAT1 from Mouse Mammary Epithelium Results in an Increased Neu-Induced Tumor Burden. *Neoplasia* **12,** 899–905 (2010).

49. Chan, S. R. *et al.* STAT1-deficient mice spontaneously develop estrogen receptor α-positive luminal mammary carcinomas. *Breast Cancer Res.* **14,** R16 (2012).

50. Battle, T. E. & Frank, D. A. The role of STATs in apoptosis. *Curr. Mol. Med.* **2,** 381–92 (2002).

51. Ramana, C. V *et al.* Regulation of c-myc expression by IFN-γ through Stat1-dependent and -independent pathways. *EMBO J.* **19,** 263–272 (2000).

52. Xiao, S. *et al.* RIG-G as a key mediator of the antiproliferative activity of interferon-related pathways through enhancing p21 and p27 proteins. *Proc. Natl. Acad. Sci. U. S.*

A. **103,** 16448–53 (2006).

53. Dimco, G., Knight, R. A., Latchman, D. S. & Stephanou, A. STAT1 interacts directly with cyclin D1/Cdk4 and mediates cell cycle arrest. *Cell Cycle* **9,** 4638–4649 (2010).

54. Tymoszuk, P. *et al.* High STAT1 mRNA levels but not its tyrosine phosphorylation are associated with macrophage infiltration and bad prognosis in breast cancer. *BMC Cancer* **14,** 257 (2014).

55. Hix, L. M. *et al.* Tumor STAT1 transcription factor activity enhances breast tumor growth and immune suppression mediated by myeloid-derived suppressor cells. *J. Biol. Chem.* **288,** 11676–88 (2013).

56. Simpson, J. A. D. *et al.* Intratumoral T cell infiltration, MHC class I and STAT1 as biomarkers of good prognosis in colorectal cancer. *Gut* **59,** 926–933 (2010).

57. Sun, Y., Yang, S., Sun, N. & Chen, J. Differential expression of STAT1 and p21 proteins predicts pancreatic cancer progression and prognosis. *Pancreas* **43,** 619–23 (2014).

58. Osborn, J. L. & Greer, S. F. Metastatic melanoma cells evade immune detection by silencing STAT1. *Int. J. Mol. Sci.* **16,** 4343–4361 (2015).

59. Chen, G., Wang, H., Xie, S., Ma, J. & Wang, G. STAT1 negatively regulates hepatocellular carcinoma cell proliferation. *Oncol. Rep.* **29,** 2303–2310 (2013).

60. Slattery, M. L., Lundgreen, A., Kadlubar, S. A., Bondurant, K. L. & Wolff, R. K. JAK/STAT/SOCS-signaling pathway and colon and rectal cancer. *Mol. Carcinog.* **52,** 155–166 (2013).

61. Khodarev, N. N. *et al.* STAT1 is overexpressed in tumors selected for radioresistance and confers protection from radiation in transduced sensitive cells. *Proc. Natl. Acad. Sci. U. S. A.* **101,** 1714–9 (2004).

62. Weichselbaum, R. R. *et al.* An interferon-related gene signature for DNA damage resistance is a predictive marker for chemotherapy and radiation for breast cancer. *Proc. Natl. Acad. Sci. U. S. A.* **105,** 18490–5 (2008).

63. Wilson, G. D. *et al.* Gene expression changes during repopulation in a head and neck cancer xenograft. *Radiother. Oncol.* **113,** 139–145 (2014).

64. Duarte, C. W. *et al.* Expression signature of IFN/STAT1 signaling genes predicts poor survival outcome in glioblastoma multiforme in a subtype-specific manner. *PLoS One* **7,** e29653 (2012).

65. Kaewpiboon, C. *et al.* Upregulation of Stat1-HDAC4 confers resistance to etoposide through enhanced multidrug resistance 1 expression in human A549 lung cancer cells. *Mol. Med. Rep.* **11,** 2315–2321 (2015).

66. Stronach, E. A. *et al.* HDAC4-Regulated STAT1 Activation Mediates Platinum Resistance in Ovarian Cancer. *Cancer Res.* **71,** 4412–4422 (2011).

67. Sasidharan Nair, V., Toor, S. M., Ali, B. R. & Elkord, E. Dual inhibition of STAT1 and STAT3 activation downregulates expression of PD-L1 in human breast cancer cells. *Expert Opin. Ther. Targets* **22,** 547–557 (2018).

68. Qing, Y. & Stark, G. R. Alternative Activation of STAT1 and STAT3 in Response to

Interferon-. *J. Biol. Chem.* **279,** 41679–41685 (2004).

69. Akira, S. Functional roles of STAT family proteins: lessons from knockout mice. *Stem Cells* **17,** 138–46 (1999).

70. Yu, H., Kortylewski, M. & Pardoll, D. Crosstalk between cancer and immune cells: role of STAT3 in the tumour microenvironment. *Nat. Rev. Immunol.* **7,** 41–51 (2007).

71. Holland, S. M. *et al.* STAT3 mutations in the hyper-IgE syndrome. *N. Engl. J. Med.* **357,** 1608–19 (2007).

72. Levy, D. E. & Loomis, C. A. STAT3 signaling and the hyper-IgE syndrome. *N. Engl. J. Med.* **357,** 1655–8 (2007).

73. Bournazou, E. & Bromberg, J. Targeting the tumor microenvironment. *Jak-Stat* **2,** e23828 (2013).

74. Kortylewski, M. *et al.* Inhibiting Stat3 signaling in the hematopoietic system elicits multicomponent antitumor immunity. *Nat. Med.* **11,** 1314–1321 (2005).

75. Chen, J., Jiang, C. C., Jin, L. & Zhang, X. D. Regulation of PD-L1: a novel role of pro-survival signalling in cancer. *Ann. Oncol.* **27,** 409–416 (2016).

76. Horlad, H. *et al.* An IL-27/Stat3 axis induces expression of programmed cell death 1 ligands (PD-L1/2) on infiltrating macrophages in lymphoma. *Cancer Sci.* **107,** 1696–1704 (2016).

77. Bu, L. L. *et al.* STAT3 Induces Immunosuppression by Upregulating PD-1/PD-L1 in HNSCC. *J. Dent. Res.* **96,** 1027–1034 (2017).

78. Song, T. L. *et al.* Oncogenic activation of the STAT3 pathway drives PD-L1 expression in natural killer/T-cell lymphoma. *Blood* **132,** 1146–1158 (2018).

79. Kondo, K. *et al.* Ibrutinib modulates the immunosuppressive CLL microenvironment through STAT3-mediated suppression of regulatory B-cell function and inhibition of the PD-1/PD-L1 pathway. *Leukemia* **32,** 960–970 (2018).

80. Woods, D. M. *et al.* Decreased Suppression and Increased Phosphorylated STAT3 in Regulatory T Cells are Associated with Benefit from Adjuvant PD-1 Blockade in Resected Metastatic Melanoma. *Clin. Cancer Res.* **24,** 6236–6247 (2018).

81. Hocke, G. M., Cui, M. Z. & Fey, G. H. The LIF response element of the alpha 2 macroglobulin gene confers LIF-induced transcriptional activation in embryonal stem cells. *Cytokine* **7,** 491–502 (1995).

82. Chen, H. *et al.* Reinforcement of STAT3 activity reprogrammes human embryonic stem cells to naive-like pluripotency. *Nat. Commun.* **6,** 7095 (2015).

83. Raz, R., Lee, C. K., Cannizzaro, L. A., D'Eustachio, P. & Levy, D. E. Essential role of STAT3 for embryonic stem cell pluripotency. *Proc. Natl. Acad. Sci. U. S. A.* **96,** 2846–51 (1999).

84. Simon, A. R., Rai, U., Fanburg, B. L. & Cochran, B. H. Activation of the JAK-STAT pathway by reactive oxygen species. *Am. J. Physiol.* **275,** C1640-52 (1998).

85. Li, L., Cheung, S. -h., Evans, E. L. & Shaw, P. E. Modulation of Gene Expression and Tumor Cell Growth by Redox Modification of STAT3. *Cancer Res.* **70,** 8222–8232

(2010).

86. Szczepanek, K., Chen, Q., Larner, A. C. & Lesnefsky, E. J. Cytoprotection by the modulation of mitochondrial electron transport chain: The emerging role of mitochondrial STAT3. *Mitochondrion* **12,** 180–189 (2012).

87. Yamauchi-Takihara, K. & Kishimoto, T. A novel role for STAT3 in cardiac remodeling. *Trends Cardiovasc. Med.* **10,** 298–303 (2000).

88. Funamoto, M. *et al.* Signal transducer and activator of transcription 3 is required for glycoprotein 130-mediated induction of vascular endothelial growth factor in cardiac myocytes. *J. Biol. Chem.* **275,** 10561–6 (2000).

89. Boengler, K., Hilfiker-Kleiner, D., Heusch, G. & Schulz, R. Inhibition of permeability transition pore opening by mitochondrial STAT3 and its role in myocardial ischemia/reperfusion. *Basic Res. Cardiol.* **105,** 771–785 (2010).

90. Haghikia, A., Ricke-Hoch, M., Stapel, B., Gorst, I. & Hilfiker-Kleiner, D. STAT3, a key regulator of cell-to-cell communication in the heart. *Cardiovasc. Res.* **102,** 281–289 (2014).

91. Haghikia, A. *et al.* Signal transducer and activator of transcription 3-mediated regulation of miR-199a-5p links cardiomyocyte and endothelial cell function in the heart: a key role for ubiquitin-conjugating enzymes. *Eur. Heart J.* **32,** 1287–1297 (2011).

92. Levy, D. E. & Lee, C. K. What does Stat3 do? *J. Clin. Invest.* **109,** 1143–1148 (2002).

93. Sargeant, T. J. *et al.* Stat3 controls cell death during mammary gland involution by regulating uptake of milk fat globules and lysosomal membrane permeabilization. *Nat. Cell Biol.* **16,** 1057–1068 (2014).

94. Chapman, R. S. *et al.* Suppression of epithelial apoptosis and delayed mammary gland involution in mice with a conditional knockout of Stat3. *Genes Dev.* **13,** 2604–16 (1999).

95. Kreuzaler, P. A. *et al.* Stat3 controls lysosomal-mediated cell death in vivo. *Nat. Cell Biol.* **13,** 303–309 (2011).

96. Adam-Vizi, V. & Starkov, A. A. Calcium and mitochondrial reactive oxygen species generation: how to read the facts. *J. Alzheimers. Dis.* **20 Suppl 2,** S413-26 (2010).

97. Gough, D. J., Koetz, L. & Levy, D. E. The MEK-ERK Pathway Is Necessary for Serine Phosphorylation of Mitochondrial STAT3 and Ras-Mediated Transformation. *PLoS One* **8,** e83395 (2013).

98. Turkson, J. & Jove, R. STAT proteins: novel molecular targets for cancer drug discovery. *Oncogene* **19,** 6613–26 (2000).

99. Ernst, M. & Putoczki, T. L. Stat3: Linking inflammation to (gastrointestinal) tumourigenesis. *Clin. Exp. Pharmacol. Physiol.* **39,** 711–718 (2012).

100. Liu, F. *et al.* Stat3-targeted therapies overcome the acquired resistance to vemurafenib in melanomas. *J. Invest. Dermatol.* **133,** 2041–2049 (2013).

101. Lee, H. J. *et al.* Drug resistance via feedback activation of stat3 in oncogene-addicted cancer cells. *Cancer Cell* **26,** 207–221 (2014).

102. Li, G. *et al.* Feedback activation of STAT3 mediates trastuzumab resistance via upregulation of MUC1 and MUC4 expression. *Oncotarget* **5,** 8317–8329 (2014).

103. Koppikar, P. *et al.* Heterodimeric JAK-STAT activation as a mechanism of persistence to JAK2 inhibitor therapy. *Nature* **489,** 155–159 (2012).

104. Fiskus, W. *et al.* Dual PI3K/AKT/mTOR Inhibitor BEZ235 Synergistically Enhances the Activity of JAK2 Inhibitor against Cultured and Primary Human Myeloproliferative Neoplasm Cells. *Mol. Cancer Ther.* **12,** 577–588 (2013).

105. Eiring, A. M. *et al.* Combined STAT3 and BCR-ABL1 inhibition induces synthetic lethality in therapy-resistant chronic myeloid leukemia. *Leukemia* **29,** 586–597 (2015).

106. Grimley, P. M., Dong, F. & Rui, H. Stat5a and Stat5b: fraternal twins of signal transduction and transcriptional activation. *Cytokine Growth Factor Rev.* **10,** 131–57 (1999).

107. Mao, X. *et al.* Structural bases of unphosphorylated STAT1 association and receptor binding. *Mol. Cell* **17,** 761–771 (2005).

108. Yuan, Z. L., Guan, Y. J., Chatterjee, D. & Chin, Y. E. Stat3 dimerization regulated by reversible acetylation of a single lysine residue. *Science (80-.).* **307,** 269–273 (2005).

109. Neculai, D. *et al.* Structure of the unphosphorylated STAT5a dimer. *J. Biol. Chem.* **280,** 40782–40787 (2005).

110. Birkenkamp, K. U., Geugien, M., Lemmink, H. H., Kruijer, W. & Vellenga, E. Regulation of constitutive STAT5 phosphorylation in acute myeloid leukemia blasts. *Leukemia* **15,** 1923–31 (2001).

111. Wagner, K. & Rui, H. Jak2/Stat5 Signaling in Mammogenesis, Breast Cancer Initiation and Progression. *J. Mammary Gland Biol. Neoplasia* **13,** 93–103 (2008).

112. Hantschel, O. *et al.* BCR-ABL uncouples canonical JAK2-STAT5 signaling in chronic myeloid leukemia. *Nat. Chem. Biol.* **8,** 285–293 (2012).

113. Walker, S. R., Xiang, M. & Frank, D. A. Distinct roles of STAT3 and STAT5 in the pathogenesis and targeted therapy of breast cancer. *Mol. Cell. Endocrinol.* **382,** 616–621 (2014).

114. Horita, M. *et al.* Blockade of the Bcr-Abl kinase activity induces apoptosis of chronic myelogenous leukemia cells by suppressing signal transducer and activator of transcription 5-dependent expression of Bcl-xL. *J. Exp. Med.* **191,** 977–84 (2000).

115. Fernandez-Luna, J. L. Bcr-Abl and inhibition of apoptosis in chronic myelogenous leukemia cells. *Apoptosis* **5,** 315–8 (2000).

116. Hayakawa, F. *et al.* Tandem-duplicated Flt3 constitutively activates STAT5 and MAP kinase and introduces autonomous cell growth in IL-3-dependent cell lines. *Oncogene* **19,** 624–31 (2000).

117. Tefferi, A. & Spivak, J. L. Polycythemia Vera: Scientific Advances and Current Practice. *Semin. Hematol.* **42,** 206–220 (2005).

118. Danovaro, R. *et al.* The first metazoa living in permanently anoxic conditions. *BMC Biol.* **8,** 30 (2010).

119. Riley, P. A. Free Radicals in Biology: Oxidative Stress and the Effects of Ionizing Radiation. *Int. J. Radiat. Biol.* **65,** 27–33 (1994).

120. Esposito, L. A., Melov, S., Panov, A., Cottrell, B. A. & Wallace, D. C. Mitochondrial disease in mouse results in increased oxidative stress. *Proc. Natl. Acad. Sci. U. S. A.* **96,** 4820–5 (1999).

121. Balaban, R. S., Nemoto, S. & Finkel, T. Mitochondria, oxidants, and aging. *Cell* **120,** 483–95 (2005).

122. Malhotra, J. D. & Kaufman, R. J. Endoplasmic Reticulum Stress and Oxidative Stress: A Vicious Cycle or a Double-Edged Sword? *Antioxid. Redox Signal.* **9,** 2277–2294 (2007).

123. Schrader, M. & Fahimi, H. D. Peroxisomes and oxidative stress. *Biochim. Biophys. Acta - Mol. Cell Res.* **1763,** 1755–1766 (2006).

124. Dagnell, M., Schmidt, E. E. & Arnér, E. S. J. The A to Z of modulated cell patterning by mammalian thioredoxin reductases. *Free Radic. Biol. Med.* **115,** 484–496 (2018).

125. Lo, Y. Y. & Cruz, T. F. Involvement of reactive oxygen species in cytokine and growth factor induction of c-fos expression in chondrocytes. *J. Biol. Chem.* **270,** 11727–30 (1995).

126. Lo, Y. Y., Wong, J. M. & Cruz, T. F. Reactive oxygen species mediate cytokine activation of c-Jun NH2-terminal kinases. *J. Biol. Chem.* **271,** 15703–7 (1996).

127. Thannickal, V. J. & Fanburg, B. L. Reactive oxygen species in cell signaling. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **279,** L1005-28 (2000).

128. Chiarugi, P. & Cirri, P. Redox regulation of protein tyrosine phosphatases during receptor tyrosine kinase signal transduction. *Trends Biochem. Sci.* **28,** 509–514 (2003).

129. Kamata, T. Roles of Nox1 and other Nox isoforms in cancer development. *Cancer Sci.* **100,** 1382–1388 (2009).

130. Dagnell, M. *et al.* Selective activation of oxidized PTP1B by the thioredoxin system modulates PDGF- receptor tyrosine kinase signaling. *Proc. Natl. Acad. Sci.* **110,** 13398–13403 (2013).

131. DeNicola, G. M. *et al.* Oncogene-induced Nrf2 transcription promotes ROS detoxification and tumorigenesis. *Nature* **475,** 106–109 (2011).

132. Sabharwal, S. S. & Schumacker, P. T. Mitochondrial ROS in cancer: initiators, amplifiers or an Achilles' heel? *Nat. Rev. Cancer* **14,** 709–721 (2014).

133. Arnér, E. S. J. & Holmgren, A. The thioredoxin system in cancer. *Semin. Cancer Biol.* **16,** 420–426 (2006).

134. Lillig, C. H., Berndt, C. & Holmgren, A. Glutaredoxin systems. *Biochim. Biophys. Acta - Gen. Subj.* **1780,** 1304–1317 (2008).

135. Lu, J. & Holmgren, A. The thioredoxin antioxidant system. *Free Radic. Biol. Med.* **66,** 75–87 (2014).

136. Griffith, O. W. Mechanism of action, metabolism, and toxicity of buthionine sulfoximine and its higher homologs, potent inhibitors of glutathione synthesis. *J. Biol.*

Chem. **257,** 13704–12 (1982).

137. Lu, S. C. Glutathione synthesis. *Biochim. Biophys. Acta - Gen. Subj.* **1830,** 3143–3153 (2013).

138. Zhang, J., Ye, Z., Singh, S., Townsend, D. M. & Tew, K. D. An evolving understanding of the S-glutathionylation cycle in pathways of redox regulation. *Free Radic. Biol. Med.* **120,** 204–216 (2018).

139. Dickinson, D. A. & Forman, H. J. Glutathione in defense and signaling: lessons from a small thiol. *Ann. N. Y. Acad. Sci.* **973,** 488–504 (2002).

140. Leone, A., Roca, M. S., Ciardiello, C., Costantini, S. & Budillon, A. Oxidative Stress Gene Expression Profile Correlates with Cancer Patient Poor Prognosis: Identification of Crucial Pathways Might Select Novel Therapeutic Approaches. *Oxid. Med. Cell. Longev.* **2017,** (2017).

141. Mariotti, M. *et al.* Composition and Evolution of the Vertebrate and Mammalian Selenoproteomes. *PLoS One* **7,** e33066 (2012).

142. Rocher, C., Lalanne, J. L. & Chaudière, J. Purification and properties of a recombinant sulfur analog of murine selenium-glutathione peroxidase. *Eur. J. Biochem.* **205,** 955–60 (1992).

143. Winterbourn, C. C. & Hampton, M. B. Thiol chemistry and specificity in redox signaling. *Free Radic. Biol. Med.* **45,** 549–61 (2008).

144. Imai, H. & Nakagawa, Y. Biological significance of phospholipid hydroperoxide glutathione peroxidase (PHGPx, GPx4) in mammalian cells. *Free Radic. Biol. Med.* **34,** 145–69 (2003).

145. Yang, W. S. *et al.* Regulation of Ferroptotic Cancer Cell Death by GPX4. *Cell* **156,** 317–331 (2014).

146. Yang, W. S. *et al.* Peroxidation of polyunsaturated fatty acids by lipoxygenases drives ferroptosis. *Proc. Natl. Acad. Sci.* **113,** E4966–E4975 (2016).

147. Hanschmann, E. *et al.* Both Thioredoxin 2 and Glutaredoxin 2 Contribute to the Reduction of the Mitochondrial 2-Cys Peroxiredoxin Prx3. *J. Biol. Chem.* **285,** 40699–40705 (2010).

148. Du, Y., Zhang, H., Lu, J. & Holmgren, A. Glutathione and Glutaredoxin Act as a Backup of Human Thioredoxin Reductase 1 to Reduce Thioredoxin 1 Preventing Cell Death by Aurothioglucose. *J. Biol. Chem.* **287,** 38210–38219 (2012).

149. Sobotta, M. C. *et al.* Peroxiredoxin-2 and STAT3 form a redox relay for H2O2 signaling. *Nat. Chem. Biol.* **11,** 64–70 (2015).

150. Stöcker, S., Maurer, M., Ruppert, T. & Dick, T. P. A role for 2-Cys peroxiredoxins in facilitating cytosolic protein thiol oxidation. *Nat. Chem. Biol.* **14,** 148–155 (2018).

151. Fujii, J. & Ikeda, Y. Advances in our understanding of peroxiredoxin, a multifunctional, mammalian redox protein. *Redox Rep.* **7,** 123–130 (2002).

152. Langford, T. F., Deen, W. M. & Sikes, H. D. A mathematical analysis of Prx2-STAT3 disulfide exchange rate constants for a bimolecular reaction mechanism. *Free Radic. Biol. Med.* **120,** 239–245 (2018).

153. Miranda-Vizuete, A. *et al.* Characterization of Sptrx, a Novel Member of the Thioredoxin Family Specifically Expressed in Human Spermatozoa. *J. Biol. Chem.* **276,** 31567–31574 (2001).

154. Arnér, E. S. J. Focus on mammalian thioredoxin reductases - Important selenoproteins with versatile functions. *Biochim. Biophys. Acta - Gen. Subj.* **1790,** 495–526 (2009).

155. Zhang, H., Du, Y., Zhang, X., Lu, J. & Holmgren, A. Glutaredoxin 2 Reduces Both Thioredoxin 2 and Thioredoxin 1 and Protects Cells from Apoptosis Induced by Auranofin and 4-Hydroxynonenal. *Antioxid. Redox Signal.* **21,** 669–681 (2014).

156. Kwon, M., Yoon, C., Jeong, W., Rhee, S. G. & Waisman, D. M. Annexin A2-S100A10 Heterotetramer, a Novel Substrate of Thioredoxin. *J. Biol. Chem.* **280,** 23584–23592 (2005).

157. Lee, S.-R. *et al.* Reversible Inactivation of the Tumor Suppressor PTEN by H 2 O 2. *J. Biol. Chem.* **277,** 20336–20342 (2002).

158. Ueno, M. *et al.* Thioredoxin-dependent redox regulation of p53-mediated p21 activation. *J. Biol. Chem.* **274,** 35809–15 (1999).

159. Saccoccia, F. *et al.* Thioredoxin reductase and its inhibitors. *Curr. Protein Pept. Sci.* **15,** 621–46 (2014).

160. Huber, R. E. J. & Criddle, R. S. Comparison and of the Chemical Selenocystine Properties Their Sulfur of Selenocysteine Analogs ' with their chemical properties , and differ only slightly in properties such as ionic radius , several attempts have been made to replace the sulfur needed fo. *Public Health* (1967).

161. Urig, S. & Becker, K. On the potential of thioredoxin reductase inhibitors for cancer therapy. *Semin. Cancer Biol.* **16,** 452–465 (2006).

162. Xu, J. *et al.* The conserved Trp114 residue of thioredoxin reductase 1 has a redox sensor-like function triggering oligomerization and crosslinking upon oxidative stress related to cell death. *Cell Death Dis.* **6,** e1616–e1616 (2015).

163. Cheng, Q., Sandalova, T., Lindqvist, Y. & Arnér, E. S. J. Crystal structure and catalysis of the selenoprotein thioredoxin reductase 1. *J. Biol. Chem.* **284,** 3998–4008 (2009).

164. Arnér, E. S. J. Selenoproteins—What unique properties can arise with selenocysteine in place of cysteine? *Exp. Cell Res.* **316,** 1296–1303 (2010).

165. Kryukov, G. V *et al.* Characterization of mammalian selenoproteomes. *Science* **300,** 1439–43 (2003).

166. Rackham, O. *et al.* Substrate and inhibitor specificities differ between human cytosolic and mitochondrial thioredoxin reductases: Implications for development of specific inhibitors. *Free Radic. Biol. Med.* **50,** 689–699 (2011).

167. Arnér, E. S. J. in *Advances in Cancer Research* **136,** 139–151 (2017).

168. Bhatia, M. *et al.* The thioredoxin system in breast cancer cell invasion and migration. *Redox Biol.* **8,** 68–78 (2016).

169. Soini, Y. *et al.* Widespread expression of thioredoxin and thioredoxin reductase in non-small cell lung carcinoma. *Clin. Cancer Res.* **7,** 1750–7 (2001).

170. Peters, U. *et al.* Variation in the selenoenzyme genes and risk of advanced distal colorectal adenoma. *Cancer Epidemiol. Biomarkers Prev.* **17,** 1144–54 (2008).

171. Lincoln, D. T. *et al.* Thioredoxin and thioredoxin reductase expression in thyroid cancer depends on tumour aggressiveness. *Anticancer Res.* **30,** 767–75 (2010).

172. Iwasawa, S. *et al.* Upregulation of thioredoxin reductase 1 in human oral squamous cell carcinoma. *Oncol. Rep.* **25,** 637–44 (2011).

173. Cassidy, P. B. *et al.* The role of thioredoxin reductase 1 in melanoma metabolism and metastasis. *Pigment Cell Melanoma Res.* **28,** 685–95 (2015).

174. Kahlos, K. *et al.* Up-regulation of thioredoxin and thioredoxin reductase in human malignant pleural mesothelioma. *Int. J. cancer* **95,** 198–204 (2001).

175. Raffel, J. *et al.* Increased expression of thioredoxin-1 in human colorectal cancer is associated with decreased patient survival. *J. Lab. Clin. Med.* **142,** 46–51 (2003).

176. Mau, B. L. & Powis, G. Inhibition of cellular thioredoxin reductase by diaziquone and doxorubicin. Relationship to the inhibition of cell proliferation and decreased ribonucleotide reductase activity. *Biochem. Pharmacol.* **43,** 1621–7 (1992).

177. Saitoh, M. *et al.* Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. *EMBO J.* **17,** 2596–606 (1998).

178. Welsh, S. J., Bellamy, W. T., Briehl, M. M. & Powis, G. The redox protein thioredoxin-1 (Trx-1) increases hypoxia-inducible factor 1alpha protein expression: Trx-1 overexpression results in increased vascular endothelial growth factor production and enhanced tumor angiogenesis. *Cancer Res.* **62,** 5089–95 (2002).

179. Farina, A. R. *et al.* Thioredoxin stimulates MMP-9 expression , de-regulates the MMP-9 / TIMP-1 equilibrium and promotes MMP-9 dependent invasion in human MDA-MB-231 breast cancer cells. *FEBS Lett.* **585,** 3328–3336 (2011).

180. Prigge, J. R. *et al.* Hepatocyte DNA replication in growing liver requires either glutathione or a single allele of txnrd1. *Free Radic. Biol. Med.* **52,** 803–810 (2012).

181. Eriksson, S., Prigge, J. R., Talago, E. A., Arnér, E. S. J. & Schmidt, E. E. Dietary methionine can sustain cytosolic redox homeostasis in the mouse liver. *Nat. Commun.* **6,** 1–9 (2015).

182. Abruzzo, J. L. Auranofin: a new drug for rheumatoid arthritis. *Ann. Intern. Med.* **105,** 274–6 (1986).

183. George, B. *et al.* Treatment of children with newly diagnosed acute promyelocytic leukemia with arsenic trioxide: a single center experience. *Leukemia* **18,** 1587–1590 (2004).

184. Alimoghaddam, K. A review of arsenic trioxide and acute promyelocytic leukemia. *Int. J. Hematol. stem cell Res.* **8,** 44–54 (2014).

185. Roder, C. & Thomson, M. J. Auranofin: Repurposing an Old Drug for a Golden New Age. *Drugs R. D.* **15,** 13–20 (2015).

186. Onodera, T., Momose, I. & Kawada, M. Potential Anticancer Activity of Auranofin. *Chem. Pharm. Bull. (Tokyo).* **67,** 186–191 (2019).

187. Prast-Nielsen, S., Cebula, M., Pader, I. & Arnér, E. S. J. Noble metal targeting of thioredoxin reductase — covalent complexes with thioredoxin and thioredoxin-related protein of 14kDa triggered by cisplatin. *Free Radic. Biol. Med.* **49,** 1765–1778 (2010).

188. Dasari, S. & Bernard Tchounwou, P. Cisplatin in cancer therapy: Molecular mechanisms of action. *Eur. J. Pharmacol.* **740,** 364–378 (2014).

189. Yamada, M., Tomida, A., Yoshikawa, H., Taketani, Y. & Tsuruo, T. Increased expression of thioredoxin/adult T-cell leukemia-derived factor in cisplatin-resistant human cancer cell lines. *Clin. Cancer Res.* **2,** 427–32 (1996).

190. Stordal, B. & Davey, M. Understanding cisplatin resistance using cellular models. *IUBMB Life* **59,** 696–9 (2007).

191. Fang, J., Lu, J. & Holmgren, A. Thioredoxin Reductase Is Irreversibly Modified by Curcumin. *J. Biol. Chem.* **280,** 25284–25290 (2005).

192. Tomeh, M. A., Hadianamrei, R. & Zhao, X. A Review of Curcumin and Its Derivatives as Anticancer Agents. *Int. J. Mol. Sci.* **20,** (2019).

193. LIU, Z., DU, Z.-Y., HUANG, Z.-S., LEE, K.-S. & GU, L.-Q. Inhibition of Thioredoxin Reductase by Curcumin Analogs. *Biosci. Biotechnol. Biochem.* **72,** 2214–2218 (2008).

194. Feng, C. *et al.* Curcumin analog L48H37 induces apoptosis through ROS-mediated endoplasmic reticulum stress and STAT3 pathways in human lung cancer cells. *Mol. Carcinog.* **56,** 1765–1777 (2017).

195. Zhang, W. *et al.* Discovery of monocarbonyl curcumin-BTP hybrids as STAT3 inhibitors for drug-sensitive and drug-resistant breast cancer therapy. *Sci. Rep.* **7,** 46352 (2017).

196. Zhang, B. *et al.* Thioredoxin reductase inhibitors: a patent review. *Expert Opin. Ther. Pat.* **27,** 547–556 (2017).

197. Stafford, W. C. *et al.* Irreversible inhibition of cytosolic thioredoxin reductase 1 as a mechanistic basis for anticancer therapy. *Sci. Transl. Med.* **10,** 1–14 (2018).

198. Anestål, K., Prast-Nielsen, S., Cenas, N. & Arnér, E. S. J. Cell Death by SecTRAPs: Thioredoxin Reductase as a Prooxidant Killer of Cells. *PLoS One* **3,** e1846 (2008).

199. Cenas, N. *et al.* Interactions of quinones with thioredoxin reductase: A challenge to the antioxidant role of the mammalian selenoprotein. *J. Biol. Chem.* **279,** 2583–2592 (2004).

200. Itoh, K., Igarashi, K., Hayashi, N., Nishizawa, M. & Yamamoto, M. Cloning and characterization of a novel erythroid cell-derived CNC family transcription factor heterodimerizing with the small Maf family proteins. *Mol. Cell. Biol.* **15,** 4184–93 (1995).

201. Itoh, K. *et al.* An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochem. Biophys. Res. Commun.* **236,** 313–22 (1997).

202. Itoh, K. *et al.* Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev.* **13,** 76–86

(1999).

203. Dinkova-Kostova, A. T. *et al.* Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proc. Natl. Acad. Sci. U. S. A.* **99,** 11908–13 (2002).

204. Yamamoto, M., Kensler, T. W. & Motohashi, H. The KEAP1-NRF2 System: a Thiol-Based Sensor-Effector Apparatus for Maintaining Redox Homeostasis. *Physiol. Rev.* **98,** 1169–1203 (2018).

205. Mitsuishi, Y., Motohashi, H. & Yamamoto, M. The Keap1–Nrf2 system in cancers: stress response and anabolic metabolism. *Front. Oncol.* **2,** 1–13 (2012).

206. Baldwin, A. S. The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu. Rev. Immunol.* **14,** 649–83 (1996).

207. Chen, Z. *et al.* Signal-induced site-specific phosphorylation targets I kappa B alpha to the ubiquitin-proteasome pathway. *Genes Dev.* **9,** 1586–97 (1995).

208. Surh, Y.-J., Kundu, J. K., Na, H.-K. & Lee, J.-S. Redox-sensitive transcription factors as prime targets for chemoprevention with anti-inflammatory and antioxidative phytochemicals. *J. Nutr.* **135,** 2993S–3001S (2005).

209. Xia, Y., Shen, S. & Verma, I. M. NF-kappaB, an Active Player in Human Cancers. *Cancer Immunol. Res.* **2,** 823–830 (2014).

210. Toledano, M. B. & Leonard, W. J. Modulation of transcription factor NF-kappa B binding activity by oxidation-reduction in vitro. *Proc. Natl. Acad. Sci. U. S. A.* **88,** 4328–32 (1991).

211. Meyer, M., Schreck, R. & Baeuerle, P. A. H2O2 and antioxidants have opposite effects on activation of NF-kappa B and AP-1 in intact cells: AP-1 as secondary antioxidant-responsive factor. *EMBO J.* **12,** 2005–15 (1993).

212. Jeong, W., Chang, T., Boja, E. S., Fales, H. M. & Rhee, S. G. Roles of TRP14, a Thioredoxin-related Protein in Tumor Necrosis Factor-α Signaling Pathways. *J. Biol. Chem.* **279,** 3151–3159 (2004).

213. Yazdanpanah, B. *et al.* Riboflavin kinase couples TNF receptor 1 to NADPH oxidase. *Nature* **460,** 1159–1163 (2009).

214. Jung, Y., Kim, H., Min, S. H., Rhee, S. G. & Jeong, W. Dynein Light Chain LC8 Negatively Regulates NF-κB through the Redox-dependent Interaction with IκBα. *J. Biol. Chem.* **283,** 23863–23871 (2008).

215. Abe, J. & Berk, B. C. Fyn and JAK2 Mediate Ras Activation by Reactive Oxygen Species. *J. Biol. Chem.* **274,** 21003–21010 (1999).

216. Gao, X. *et al.* RNAi-mediated silencing of NOX4 inhibited the invasion of gastric cancer cells through JAK2/STAT3 signaling. *Am. J. Transl. Res.* **9,** 4440–4449 (2017).

217. Yoon, S. *et al.* STAT3 transcriptional factor activated by reactive oxygen species induces IL6 in starvation-induced autophagy of cancer cells. *Autophagy* **6,** 1125–1138 (2010).

218. Kaur, N., Lu, B., Ward, S. M. & Halvorsen, S. W. Inducers of oxidative stress block ciliary neurotrophic factor activation of Jak/STAT signaling in neurons. *J. Neurochem.*

92, 1521–1530 (2005).

219. Di Bona, D. *et al.* Oxidative stress inhibits IFN-α-induced antiviral gene expression by blocking the JAK–STAT pathway. *J. Hepatol.* **45,** 271–279 (2006).

220. Xie, Y., Kole, S., Precht, P., Pazin, M. J. & Bernier, M. S-Glutathionylation impairs signal transducer and activator of transcription 3 activation and signaling. *Endocrinology* **150,** 1122–1131 (2009).

221. Butturini, E. *et al.* S-glutathionylation at Cys328 and Cys542 impairs STAT3 phosphorylation. *ACS Chem. Biol.* **9,** 1885–1893 (2014).

222. Smith, J. K. *et al.* Identification of a redox-sensitive switch within the JAK2 catalytic domain. *Free Radic. Biol. Med.* **52,** 1101–1110 (2012).

223. Lai, P.-S. *et al.* A STAT inhibitor patent review: progress since 2011. *Expert Opin. Ther. Pat.* **25,** 1397–1421 (2015).

224. Vannucchi, A. M. & Harrison, C. N. Emerging treatments for classical myeloproliferative neoplasms. *Blood* **129,** 693–703 (2017).

225. Hobbs, G. S., Rozelle, S. & Mullally, A. The Development and Use of Janus Kinase 2 Inhibitors for the Treatment of Myeloproliferative Neoplasms. *Hematol. Oncol. Clin. North Am.* **31,** 613–626 (2017).

226. Arana Yi, C., Tam, C. S. & Verstovsek, S. Efficacy and safety of ruxolitinib in the treatment of patients with myelofibrosis. *Future Oncol.* **11,** 719–33 (2015).

227. Choong, M. L. *et al.* Combination treatment for myeloproliferative neoplasms using JAK and pan-class I PI3K inhibitors. *J. Cell. Mol. Med.* **17,** 1397–1409 (2013).

228. Zhan, H., Lin, C. H. S., Segal, Y. & Kaushansky, K. The JAK2V617F-bearing vascular niche promotes clonal expansion in myeloproliferative neoplasms. *Leukemia* 1–8 (2017). doi:10.1038/leu.2017.233

229. Meyer, S. C. & Levine, R. L. Molecular pathways: Molecular basis for sensitivity and resistance to JAK kinase inhibitors. *Clin. Cancer Res.* **20,** 2051–2059 (2014).

230. Furtek, S. L., Backos, D. S., Matheson, C. J. & Reigan, P. Strategies and Approaches of Targeting STAT3 for Cancer Treatment. *ACS Chem. Biol.* **11,** 308–318 (2016).

231. O'Shea, J. J., Kontzias, A., Yamaoka, K., Tanaka, Y. & Laurence, A. Janus kinase inhibitors in autoimmune diseases. *Ann. Rheum. Dis.* **72,** ii111-ii115 (2013).

232. Szelag, M., Wesoly, J. & Bluyssen, H. A. R. Advances in peptidic and peptidomimetic-based approaches to inhibit STAT signaling in human diseases. *Curr. Protein Pept. Sci.* **17,** 135–46 (2016).

233. Sen, M. *et al.* First-in-Human Trial of a STAT3 Decoy Oligonucleotide in Head and Neck Tumors: Implications for Cancer Therapy. *Cancer Discov.* **2,** 694–705 (2012).

234. Yan, S., Li, Z. & Thiele, C. J. Inhibition of STAT3 with orally active JAK inhibitor, AZD1480, decreases tumor growth in Neuroblastoma and Pediatric Sarcomas In vitro and In vivo. *Oncotarget* **4,** 433–445 (2013).

235. Suryani, S. *et al.* Evaluation of the In Vitro and In Vivo Efficacy of the JAK Inhibitor AZD1480 against JAK-Mutated Acute Lymphoblastic Leukemia. *Mol. Cancer Ther.*

14, 364–374 (2015).

236. Hong, D. *et al.* AZD9150, a next-generation antisense oligonucleotide inhibitor of STAT3 with early evidence of clinical activity in lymphoma and lung cancer. *Sci. Transl. Med.* **7,** 314ra185-314ra185 (2015).

237. Reilley, M. J. *et al.* STAT3 antisense oligonucleotide AZD9150 in a subset of patients with heavily pretreated lymphoma: results of a phase 1b trial. *J. Immunother. cancer* **6,** 119 (2018).

238. Desroses, M. *et al.* STAT3 differential scanning fluorimetry and differential scanning light scattering assays: Addressing a missing link in the characterization of STAT3 inhibitor interactions. *J. Pharm. Biomed. Anal.* **160,** 80–88 (2018).

239. Brambilla, L. *et al.* Hitting the right spot: Mechanism of action of OPB-31121, a novel and potent inhibitor of the Signal Transducer and Activator of Transcription 3 (STAT3). *Mol. Oncol.* **9,** 1194–1206 (2015).

240. Song, H., Wang, R., Wang, S. & Lin, J. A low-molecular-weight compound discovered through virtual database screening inhibits Stat3 function in breast cancer cells. *Proc. Natl. Acad. Sci.* **102,** 4700–4705 (2005).

241. Zuo, M., Li, C., Lin, J. & Javle, M. LLL12, a novel small inhibitor targeting STAT3 for hepatocellular carcinoma therapy. *Oncotarget* **6,** 10940–10949 (2015).

242. Schust, J., Sperl, B., Hollis, A., Mayer, T. U. & Berg, T. Stattic: A Small-Molecule Inhibitor of STAT3 Activation and Dimerization. *Chem. Biol.* **13,** 1235–1242 (2006).

243. Heidelberger, S. *et al.* Investigation of the protein alkylation sites of the STAT3:STAT3 inhibitor Stattic by mass spectrometry. *Bioorganic Med. Chem. Lett.* **23,** 4719–4722 (2013).

244. Siddiquee, K. *et al.* Selective chemical probe inhibitor of Stat3, identified through structure-based virtual screening, induces antitumor activity. *Proc. Natl. Acad. Sci.* **104,** 7391–7396 (2007).

245. Ball, D. P. *et al.* Signal transducer and activator of transcription 3 (STAT3) inhibitor, S3I-201, acts as a potent and non-selective alkylating agent. *Oncotarget* **7,** (2016).

246. Zhang, X. *et al.* Orally bioavailable small-molecule inhibitor of transcription factor Stat3 regresses human breast and lung cancer xenografts. *Proc. Natl. Acad. Sci.* **109,** 9623–9628 (2012).

247. Ali, A. M. *et al.* Disarming an Electrophilic Warhead: Retaining Potency in Tyrosine Kinase Inhibitor (TKI)-Resistant CML Lines while Circumventing Pharmacokinetic Liabilities. *ChemMedChem* **11,** 850–861 (2016).

248. Bendell, J. C. *et al.* Phase 1, open-label, dose-escalation, and pharmacokinetic study of STAT3 inhibitor OPB-31121 in subjects with advanced solid tumors. *Cancer Chemother. Pharmacol.* **74,** 125–130 (2014).

249. Oh, D.-Y. *et al.* Phase I Study of OPB-31121, an Oral STAT3 Inhibitor, in Patients with Advanced Solid Tumors. *Cancer Res. Treat.* **47,** 607–615 (2015).

250. Okusaka, T. *et al.* Phase 1 and pharmacological trial of OPB-31121, a signal transducer and activator of transcription-3 inhibitor, in patients with advanced

hepatocellular carcinoma. *Hepatol. Res.* **45,** 1283–1291 (2015).

251. Wong, A. L. *et al.* Phase I and biomarker study of OPB-51602, a novel signal transducer and activator of transcription (STAT) 3 inhibitor, in patients with refractory solid malignancies. *Ann. Oncol.* **26,** 998–1005 (2015).

252. Ogura, M. *et al.* Phase I study of OPB-51602, an oral inhibitor of signal transducer and activator of transcription 3, in patients with relapsed/refractory hematological malignancies. *Cancer Sci.* **106,** 896–901 (2015).

253. Yoo, C. *et al.* Phase I Dose-Finding Study of OPB-111077, a Novel STAT3 Inhibitor, in Patients with Advanced Hepatocellular Carcinoma. *Cancer Res. Treat.* 1–9 (2018). doi:10.4143/crt.2018.226

254. Tolcher, A. *et al.* A First-in-Human Phase I Study of OPB-111077, a Small-Molecule STAT3 and Oxidative Phosphorylation Inhibitor, in Patients with Advanced Cancers. *Oncologist* **23,** 658-e72 (2018).

255. Genini, D. *et al.* Mitochondrial dysfunction induced by a SH2 domain-targeting STAT3 inhibitor leads to metabolic synthetic lethality in cancer cells. *Proc. Natl. Acad. Sci.* **114,** E4924–E4933 (2017).

256. Hirpara, J. *et al.* Metabolic reprogramming of oncogene-addicted cancer cells to OXPHOS as a mechanism of drug resistance. *Redox Biol.* 101076 (2018). doi:10.1016/j.redox.2018.101076

257. Takakura, A. *et al.* Pyrimethamine inhibits adult polycystic kidney disease by modulating STAT signaling pathways. *Hum. Mol. Genet.* **20,** 4143–4154 (2011).

258. Khan, M. W. *et al.* The STAT3 inhibitor pyrimethamine displays anti-cancer and immune stimulatory effects in murine models of breast cancer. *Cancer Immunol. Immunother.* **67,** 13–23 (2018).

259. Ren, Z. *et al.* Crystal structure of unphosphorylated STAT3 core fragment. *Biochem. Biophys. Res. Commun.* **374,** 1–5 (2008).

260. Furtek, S. L., Matheson, C. J., Backos, D. S. & Reigan, P. Evaluation of quantitative assays for the identification of direct signal transducer and activator of transcription 3 (STAT3) inhibitors. *Oncotarget* **7,** (2016).

261. Molina, D. M. *et al.* Monitoring drug target engagement in cells and tissues using the cellular thermal shift assay. *Science (80-.).* **341,** 84–87 (2013).

262. Ishii, T. *et al.* CETSA quantitatively verifies in vivo target engagement of novel RIPK1 inhibitors in various biospecimens. *Sci. Rep.* **7,** 1–14 (2017).

263. Martinez, N. J. *et al.* A widely-applicable high-throughput cellular thermal shift assay (CETSA) using split Nano Luciferase. *Sci. Rep.* **8,** 1–16 (2018).

264. Mateus, A., Määttä, T. A. & Savitski, M. M. Thermal proteome profiling: Unbiased assessment of protein state through heat-induced stability changes. *Proteome Sci.* **15,** 1–7 (2017).

265. Peng, X. *et al.* APR-246/PRIMA-1MET inhibits thioredoxin reductase 1 and converts the enzyme to a dedicated NADPH oxidase. *Cell Death Dis.* **4,** 1–7 (2013).

266. Haffo, L. *et al.* Inhibition of the glutaredoxin and thioredoxin systems and

ribonucleotide reductase by mutant p53-targeting compound APR-246. *Sci. Rep.* **8,** 1–10 (2018).

267. Xiang, M. *et al.* Gene expression-based discovery of atovaquone as a STAT3 inhibitor and anticancer agent. *Blood* **128,** 1845–1853 (2016).