Department of Oncology and Pathology
Karolinska Biomics Center
Karolinska Institutet, Stockholm, Sweden

PROTEOMIC ANALYSIS OF DNA DAMAGE INDUCED STRESS SIGNALING WITH FOCUS ON P53

S100A6 REGULATION, FUNCTION AND POTENTIAL AS BIOMARKER IN LUNG CANCER AND AS A NOVEL THERAPEUTIC TARGET

Lukas Orre

Karolinska Institutet

Stockholm 2008

Published by Karolinska Institutet. Printed by Larserics Digital Print AB, Sundbyberg

ISBN-978-91-7357-582-9

With endless love to Johanna, Ida and Amanda

(In order of appearance)

ABSTRACT

In this thesis powerful proteomics methods were used to reveal novel proteins involved in the cellular response to DNA damaging treatment. The goal was to find proteins with potential as biomarkers for prediction of cancer prognosis and response to cancer therapy, but also to find potential novel targets of cancer therapy. In addition, the impact of the tumor suppressor p53 on the cellular response to DNA damaging treatment was investigated.

When comparing two isogenic colon cancer cell lines (HCT116 p53wt and p53-/-) we were unable to detect p53 dependent differences in sensitivity to ionizing radiation (IR) or DNA damaging drugs. p53 did however alter the localization and reduce the abundance of Rad51, a key protein in homologous recombination repair, in response to IR. Our data thus indicate that p53 is involved in negative regulation of homologous recombination.

The same cell line pair was also used in a proteomics time course study to identify novel proteins involved in the cellular response to DNA damaging agents (IR). In this study we discovered that the small calcium binding protein S100A6 was upregulated in a p53 dependent manner in irradiated cells. In addition to the upregulation of S100A6 post irradiation we also discovered that the post translational modification pattern and the sub cellular localization were altered. The biological functions of S100A6 were not known at the time of this discovery, but several studies had implicated S100A6 in carcinogenesis as it was shown upregulated in several different types of cancer. We therefore decided to pursue this finding and study the biological role of S100A6 in detail.

The expression of S100A6 was investigated in stage I non-small cell lung cancer by immunohistochemistry using tissue microarrays. S100A6 was found overexpressed in cancer cells, and S100A6 expression correlated with wt p53 expression. S100A6 expression also correlated with patient survival in a subset of the cohort.

In order to elucidate the function of S100A6 we set out to identify novel S100A6 interacting proteins. Using immunoprecipitation and proteomics methods we identified Ubiquilin-1 as a novel S100A6 interacting protein. Ubiquilin-1 is involved in regulation of proteasome mediated degradation of ubiquitinated proteins such as p53 and IκBa. siRNA mediated silencing of S100A6 resulted in stabilisation of both p53 and IκBa. Mass spectrometry based proteomics further revealed that S100A6 silencing reduced proteasomal processing of NFκB2 p100. S100A6 has earlier been suggested involved also in degradation of β-catenin as S100A6 interacts with a component of the complex responsible for ubiquitination of β-catenin. Using S100A6 silencing we were able to show increased degradation of β-catenin. S100A6 silencing also increased the cellular sensitivity to ionizing radiation.

In conclusion our data indicates that overexpression of S100A6 in cancer cells would result in a survival benefit through inhibition of apoptosis via increased p53 degradation and stimulation of proliferation via increased NFκB and β-catenin signaling. Our data also indicates that S100A6 is upregulated at later timepoints post irradiation to inhibit apoptosis and cell cycle arrest, allowing cells with repaired DNA damage to re-enter the cell cycle. These findings suggest the potential of S100A6 as a novel target of cancer therapy.

LIST OF PUBLICATIONS

I. **Orre LM, Stenerlöw B, Dhar S, Larsson R, Lewensohn R, Lehtiö J.**
p53 is involved in clearance of ionizing radiation-induced RAD51 foci in a human colon cancer cell line.
Biochemical and Biophysical Research Communications. 2006. Apr 21;342(4):1211-7.

II. **Orre LM, Pernemalm M, Lengqvist J, Lewensohn R, Lehtiö J.**
Up-regulation, modification, and translocation of S100A6 induced by exposure to ionizing radiation revealed by proteomics profiling.
Molecular and Cellular Proteomics. 2007 Dec;6(12):2122-31.

III. **De Petris L, Orre LM, Kanter L, Pernemalm M, Koyi H, Lewensohn R, Lehtiö J.**
Tumor expression of S100A6 correlates with survival of patients with stage I non-small cell lung cancer.
(Submitted for publication)

IV. **Orre LM, Vernet E, Lengqvist J, Gräslund T, Lewensohn R, Lehtiö J.**
S100A6 interacts with Ubiquilin-1 and regulates degradation of p53, IκBα and NFκB2
(Submitted for publication)

Additional papers

Orre LM, Fält S, Szeles A, Lewensohn R, Wennborg A, Flygare J
Rad51-related changes in global gene expression
Biochemical and Biophysical Research Communications. 2006. 341(2):334-342.

Pernemalm M, Orre LM, Lengqvist J, Wikström P, Lewensohn R, Lehtiö J
Evaluation of three principally different intact protein pre-fractionation methods for plasma biomarker discovery
Journal of Proteome Research. 2008 (In press)

Eriksson H, Lengqvist J, Hedlund J, Orre LM, Persson B, Lehtiö J, Jakobsson P-J
Quantitative membrane proteomics analysis on human small cell lung cancer cells using narrow range peptide IPG-IEF
Proteomics. 2008 (In press)

CONTENTS

LIST OF ABBREVIATIONS

Apaf	apoptosis protease activating factor
ATM	ataxia telangiectasia mutated
ATP	adenosine tri-phosphate
ATR	ATM and Rad3-related
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2 associated X protein
Bcl	B-cell lymphoma
BER	base excision repair
Bid	BH3-interacting-domain death agonist
BRCA	breast cancer associated
CacyBP/SIP	calcyclin binding protein/Siah-1 interacting protein
CDK	cyclin dependent kinase
CDKI	cyclin dependent kinase inhibitor
DISC	death inducing signaling complex
DNA-PK	DNA-dependent protein kinase
DSB	DNA double strand break
ECM	extracellular matrix
EGF-R	epidermal growth factor receptor
ER	endoplasmatic reticulum
FANC	fanconi anemia complementation
FMCA	fluorimetric microculture cytotoxicity assay
HR	homologous recombination
IAP	inhibitor of apoptosis protein
IEF	isoelectric focusing
IHC	immunohistochemistry
IκB	inhibitor of kappa B
IP	immunoprecipitation
IPG	immobilized pH gradient
IR	ionizing radiation
iTRAQ	isobaric tag for relative and absolute quantification
LC	liquid chromathography
MALDI	matrix assisted laser desorption/ionization
Mdm-2	mouse double minute-2
MMP	matrix metallo proteinase
MMR	mismatch repair
MRN	Mre11/Rad50/Nbs1
MS	mass spectrometry
Nbs	nijmegen breakage syndrome
NER	nucleotide excision repair
NFκB	nuclear factor kappa B
NHEJ	non-homologous end joining
NSCLC	non-small cell lung carcinoma
PARP	poly (ADP-ribose) polymerase
PCD	programmed cell death
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrphoresis
pRB	retinoblastoma protein
RAGE	receptor for advanced glycation endproducts
SCLC	small cell lung carcinoma
SELDI	surface enhanced laser desorption/ionization
siRNA	small interfering RNA

TGF	transforming growth factor
TMA	tissue microarray
TNF	tumor necrosis factor
TOF	time of flight
TRAIL	TNF-related apoptosis inducing ligand
VEGF	vascular endothelial growth factor
XP	xeroderma pigmentosum
XRCC	X-ray cross complementation

1 BACKGROUND

1.1 CANCER

Hippocrates, the father of medicine, described a number of different cancer types about 400 years B.C. He used the Greek words oncos (swelling) for benign cancers and carcinos (crab) for malignant cancers and these are the roots of the modern words oncology and cancer. It is believed that the word carcinos was chosen as the cut surface of a solid tumor resembles a crab, with a round center and veins spreading out like legs on all sides. Today over 200 different types of cancer have been described (source: Cancer Research UK). In some cases cancer predisposition is genetically inherited (e.g. breast cancers caused by mutations in BRCA genes or colon cancer caused by mutations in the APC gene), but in most cases cancer is sporadic. Other causes of cancer are exposure to environmental factors such as UV-radiation (melanoma), radon (lung cancer) or chemical carcinogens. Certain infectious diseases have also been shown to induce cancer, most notably viral infections such as human papilloma virus (cervical cancer), but also bacterial infections such as *Helicobacter pylori* (gastric cancer). Life style factors such as tobacco smoking, diet, physical inactivity and excess body weight have also been shown to influence the risk of cancer. A striking variation in the risk of certain cancers is seen in different geographic areas, most likely reflecting variations in risk factors related to lifestyle or environment [1]. Most cancers are strongly correlated with age, reflecting that the development of cancer is dependent on the accumulation of genetic alterations and that this process takes time. Childhood cancers are rare and the predominating forms are leukemia and central nervous system cancers. Most cancers are diagnosed after initial recognition of symptoms (e.g. swelling or pain) or through screening programs (e.g. breast cancer and cervical cancer). For diagnostic purposes several types of medical tests such as blood tests, imaging and histological examination of biopsies are performed. Tumors are generally divided as being either benign (not invasive) or malignant (synonymous with cancer), the latter having the ability to spread through invasion of adjacent tissue and metastasis. Cancers are subdivided depending on their cellular origin into e.g. carcinoma (epithelial origin), sarcoma (connective tissue origin), leukemia and lymphoma (both deriving from blood cells). The progression or spread of the cancer is determined using a staging system (usually stage I-IV) where tumor size (T), spread to lymph nodes (N) and metastasis (M) are evaluated (TNM staging system). The tumor stage is then used for therapy guidance as for example a localized cancer can usually be cured through surgical resection while a metastatic cancer is associated with poor prognosis and usually treated with chemotherapy.

1.1.1 Cancer epidemiology

Worldwide it is estimated that 10.9 million persons are diagnosed with cancer, and that 6.7 million persons die from this disease every year. Globally the most commonly diagnosed types of cancer are lung cancer (1.35 million cases/year), breast cancer (1.15 million cases/year) and colorectal cancer (1 million cases/year) and the most common causes of cancer death are lung cancer (1.18 million deaths/year), stomach cancer (0.7 million deaths/year) and liver cancer (0.6 million deaths/year) [1]. In Sweden the

numbers of new cancer cases per year are 27000 and 24000 for males and females respectively. The most common types of cancer in Sweden are prostate cancer (36.5% of male cancer) and breast cancer (29.1% of female cancer) followed by cancers of the digestive system in both sexes. The most common causes of cancer death for both sexes are pancreatic cancer and bronchus/lung cancer. The cancer incidence in Sweden is slowly increasing, likely as a consequence of increasing age, improved diagnostics and screening programs, but this trend is likely also affected by alterations in environmental factors (e.g. UV exposure) and life style factors (e.g. smoking). (Source: the Swedish national board of health and welfare, "Cancer incidence in Sweden 2005", 2007).

1.1.2 Carcinogenesis

The development of cancer is a multistep process, where normal cells acquire a series of different tumor cell characteristics and ultimately break free from the restrains of normality and become tumor cells [2] (Figure 1). This process of transformation or carcinogenesis is not always the same; numerous different routes can be used. A driving force for transformation is genomic instability. Most of the tumor cell characteristics are consequences of genomic alterations such as mutations, deletions and insertions changing the functions of affected genes and gene products (proteins).

Tumor suppressor genes (e.g. *TP53* and *RB1* coding for p53 and retinoblastoma protein, pRB, respectively) are genes coding for proteins normally involved in cellular anti-cancer defence systems. These genes are commonly inactivated in cancer cells resulting in inability of the cell to hinder carcinogenesis. Proto-oncogenes (e.g. *MYC* or *RAS*) are genes coding for proteins with normal functions typically in regulation of cell growth. When over activated, e.g. through mutation or overexpression, these genes, now called oncogenes, become cancer inducing.

In analogy to Darwinian evolution, cells accumulate genetic alterations that results in a survival benefit. To avoid genetic alterations, cells are equipped with extremely efficient safety systems that monitor DNA and repair DNA damages as well as supervise delicate processes such as DNA synthesis and mitosis. Normally a cell is not allowed to divide unless DNA has been replicated without errors. It is unlikely that cancers would have the time to develop during the human lifespan if these safety systems were intact as carcinogenesis then would be a very slow process. However, key components of these systems such as the tumor suppressor p53 are often inactivated in tumor cells resulting in increased mutability and faster carcinogenesis. In fact, p53 is lost or functionally impaired in the vast majority of all human cancers [3].

Six different steps of carcinogenesis have been suggested in a seminal article by Douglas Hanahan and Robert A Weinberg [4]: Self-sufficiency in growth signals, insensitivity to antigrowth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. Each step represents a new acquired capability, and together these capabilities are shared by most if not all human tumors.

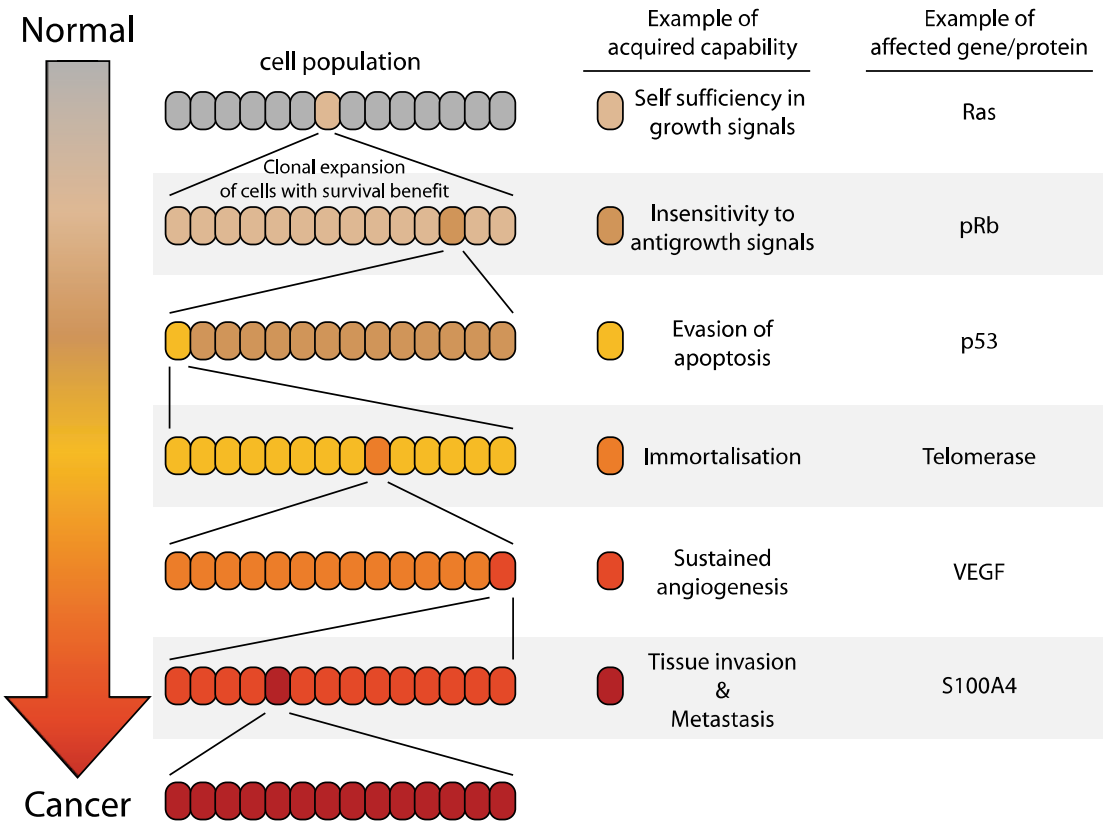

Figure 1. Carcinogenesis is a multi step process driven by clonal expansion of cells with a survival benefit. Each step in the figure represents the acquisition of a new tumor cell characteristic. The acquired capabilities described in this picture has been suggested to be hallmarks of cancer, shared by most if not all cancer cells [4]. The order in which these alterations occur may be other than suggested in this picture. Several alterations within the same functional area (e.g. apoptosis) are also common.

1.1.2.1 Growth signaling

Normal cells will not proliferate unless they receive a signal to do so. This signal can be transduced either through soluble growth factors that bind to cell surface receptors, or through interaction between cell surface molecules on one cell with other cells or components of the extracellular matrix (ECM). The signal received by these receptors is then transmitted to intracellular signaling molecules ultimately resulting in DNA replication and cell division. Three principle ways exist through which cancer cells achieve self sufficiency in growth signals: Alteration of extracellular signals, transcellular transducers or intracellular signaling pathways. Autocrine signaling i.e. when a cell produce the ligand for its own receptor, is an example of how cancer cells alter the extracellular signal and it has been shown for a number of cancer diseases such as lung cancers producing both ligand and receptor e.g. stem cell factor (SCF)/c-kit [5], transforming growth factor-α (TGF-α)/epidermal growth factor receptor (EGF-R) [6] or insulin-like growth factor (IGF)/IGF-R [7]. The transcellular transducers, e.g. growth factor receptors or integrins (ECM-receptors) are also frequently altered in cancer cells. One example of this is overexpression of receptors making the cell hyper reactive and responsive even to very low levels of ligand as is seen in the case of EGF-R in various

cancer types [8] and HER2/Neu in breast cancer [9]. Another example is mutation of the receptor gene resulting in expression of a truncated version of the receptor that signals constitutively even in the absence of ligand, e.g. in glioblastoma [10]. The intracellular signaling pathways that are activated by the receptors are very complex and can be altered at a multitude of different levels. A model example of altered intracellular signaling is activating mutations in Ras, a protein transmitting signals from growth factor receptors to the Raf-Mek-Erk signaling cascade resulting in increased proliferation [11]. Activating Ras mutations are found in 30% of human cancers.

1.1.2.2 Anti-growth signaling

A second layer of growth control is constituted of anti-growth signaling. In analogy to growth signaling, the cell senses its surrounding through receptors of growth inhibitors (e.g. TGF-β), cell surface molecules on other cells or components of the ECM. A normal cell receiving anti-growth signals would enter into temporary or permanent growth arrest. This is an obstacle cancer cells have to overcome in order to expand into macroscopic tumors. During the first gap phase (G1) of the cell cycle, the cell collects information from the surroundings to decide whether or not to allow entrance into the S phase during which the DNA is duplicated. A key component of this decision making is the Retinoblastoma protein (pRb). When hypophosphorylated, pRb complex with and inhibit the E2F family of transcription factors needed for induction of genes involved in S-phase entry. In the absence of anti-growth signals and presence of growth signals, cyclins are synthesized that bind to and activate cyclin dependent kinases (CDKs). These complexes phosphorylate pRb resulting in release of E2F transcription factors and entry into the S-phase. This signaling is further regulated by inhibitors of CDKs (CDKIs). It has been shown that one or several of the G1/S regulators are mutated in between 70-90% of all human tumors, resulting in insensitivity to growth inhibitory signals [12]. The frequent inactivation of G1/S regulators in cancer clearly demonstrates the importance of these systems in controlling proliferation.

1.1.2.3 Apoptosis

In normal tissue the total number of cells is kept at a constant level through the balance between cell proliferation and cell death. Carefully regulated cell death machinery is of great importance to the organism to ensure that cells with tumorigenic potential e.g. damaged DNA or deregulated growth signaling do not survive. Apoptosis or programmed cell death (PCD) is the major route of cell attrition present in higher organisms. The master regulator of apoptosis is the tumor suppressor protein p53 [3,13]. p53 can be activated by a variety of stimuli such as lack of nucleotides, exposure to radiation, oncogenic signaling, DNA damage, hypoxia or transcription blockage. The result of such activation is cell cycle arrest through upregulation of the CDKI p21. Cell cycle arrest allows the cell to repair DNA through several different DNA repair pathways also regulated in part by p53. If the cell is damaged beyond repair p53 will activate the apoptotic machinery through induction of proapoptotic factors (e.g. Bax) and repression of anti-apoptotic factors (e.g. Bcl-2) resulting in mitochondrial depolarization, release of cytochrome c and ultimately cleavage and activation of caspases, proteases that execute the death program through destruction of subcellular structures and organelles. Morphological changes associated with apoptosis are cell

shrinkage, chromatin condensation and nuclear fragmentation. The cell remnants are packed into small vesicles called apoptotic bodies that are engulfed by nearby phagocytes [14]. If the apoptotic machinery was intact in cancer cells it would be activated as cancer cells possess features that would normally result in PCD, e.g. genomic instability, oncogene activation and cell cycle checkpoint violation. As a consequence tumor cells have found ways to escape apoptosis. p53 is functionally impaired in most human cancers either through inactivating mutations or through other mechanisms such as overexpression of Mdm2, a ubiquitin ligase involved in degradation of p53 or deletion of the Mdm2 inhibitor p14arf [15,16]. Loss of proapoptotic proteins such as Bax or overexpression of anti-apoptotic proteins such as Bcl-2 are also common in cancer cells [17,18].

1.1.2.4 Immortalization

In order for a tumor to reach macroscopic, life-threatening size, the tumor must circumvent two additional proliferation-limiting safety systems existing in normal cells namely senescence and the telomerase/telomere system. Cells have an inherent limited number of allowed doublings (40-70), and when this number is reached the cell will enter into senescence or irreversible growth arrest [19]. Senescence is often avoided in tumors by the inactivation of tumor suppressors such as p53 and pRb (see above), allowing for additional population doublings (20-30). The telomeres (chromosome ends) consist of several thousands of repeats of a 6 base pair (bp) sequence element from which 50-200 bps are lost every replication round. This process would present a definitive limit of proliferation, as telomeres would eventually disappear resulting in loss of coding DNA for additional replication and finally chromosomal instability, crisis and cell death. Cancer cells steer clear of also this proliferation limitation through the expression of telomerase, an enzyme that adds hexanucleotide repeats onto the ends of telomeric DNA. Telomerase is expressed by 85-90% of malignant cells

1.1.2.5 Angiogenesis

In order to survive and proliferate all cells need access to the distribution system (capillary blood vessels) that delivers oxygen and nutrients and removes metabolic waste [20]. During normal development and organogenesis the simultaneous growth of blood vessels (angiogenesis) ensures that no cell is distanced more than 0.1 mm from vessels. Once a tissue is formed, angiogenesis is tightly regulated by positive and negative signaling. During the development of solid tumors formation of new blood vessels is essential and therefore stimulation of angiogenesis is a common feature of cancer growth. This deregulation could be either through overexpression of positive signaling from cancer cells through vascular endothelial growth factor (VEGF), acting on VEGF-receptors on endothelial cells, or through downregulation of angiogenesis inhibitors such as Trombospondin-1.

1.1.2.6 Invasion and metastasis

Tissue invasion, when the tumor breaches through the borders of the tissue of origin and invades the nearby stroma or through normal epithelial cell layers, and metastasis, where a subpopulation detaches from the original tumor, enters the blood or lymphatic vessels and give rise to a daughter tumor, can be regarded as the ultimate steps in

tumorigenesis. In fact, the cause of 90% of human cancer deaths is development of metastases [21]. Much is still unknown about the mechanisms driving tissue invasion and metastasis, and these areas have been described as the last great frontiers for exploratory cancer research. One key finding however is the deregulation of contact inhibition, where E-cadherin, a receptor that transmits antigrowth signals through β-catenin upon cell-to-cell contact, is functionally inactivated in many epithelial tumors. Another important finding is that cancer cells often overexpress extracellular proteases that degrade surrounding matrix and other obstacles that would otherwise hinder the invasion and metastasis. The ability of cancer cells to migrate, through for example cytoskeleton rearrangements is also often improved.

1.2 CANCER TREATMENT

1.2.1 Treatment options

1.2.1.1 Surgery, Radiotherapy or Drugs

Surgical removal of solid tumors is the oldest, and still most commonly used cancer treatment form. In fact surgery, alone or in combination with radiotherapy or chemotherapy, is the treatment that cures most cancer patients. The aim is to remove all cancer cells by resection of either a complete organ, or part of an organ. Surgery can also be used for diagnostic purposes or palliation. The current trend is to limit the amount of tissue removed, and to spare as much healthy tissue as possible [22]. The use of surgery in cancer treatment is limited by several different factors. In many cases spreading of the disease (metastasis) makes surgery inadequate as treatment, and the location of the tumor (e.g. vicinity to large blood vessels) can make surgery impossible. The status of the patient is also of importance as some patients may not endure surgery. Other common treatment options are radiotherapy and chemotherapy as will be discussed below. In certain cancers (e.g. breast and prostate) hormonal therapy has proved an efficient therapeutic option. Examples of hormonal therapy are to inhibit the proliferation stimulating effects of estrogen on breast cancer cells [23] or androgens on prostate cancer cells [24]. Immunotherapy is another novel treatment option where the goal is to induce the patients own immune system to target and kill tumor cells [25].

1.2.1.2 Radiotherapy

The first report of radiotherapy in treatment of cancer is from 1899, when the Swedish physicians Tage Sjögren and Tor Stenbeck cured patients with basal cell carcinoma using X-rays [26]. This was only four years after the discovery of X-rays by Wilhelm Röntgen, which rendered him the first Nobel price in physics 1901.

Radiotherapy is today a widely used cancer treatment, and roughly 50% of all cancer patients receive radiotherapy at some point. In about half of the cases radiotherapy is given with a curative intent and in the remaining cases it is given as palliative treatment. Radiotherapy is external, i.e. delivered from a source outside of the body, in the majority of cases, but radiotherapy could also be delivered as brachytherapy, i.e. a radioactive source is applied directly near or in the tumor. Radiotherapy can be either used as monotherapy, or in combination with surgery as pre or post operative radiotherapy (Source: the Swedish council on technology assessment in health care, “Radiotherapy in cancer”, 2003).

X-rays and γ-rays used in radiotherapy are two forms of electromagnetic radiation (photons). They do not differ in nature or properties, but are generated differently. X-rays are generated through acceleration of electrons in an electric device and when the accelerated electrons hit a solid target (e.g. gold or tungsten) the kinetic energy is converted into X-rays. γ-rays are emitted by radioactive isotopes that during decay give off excess energy in the form of electromagnetic radiation. Both forms of radiation are ionizing, i.e. their energy is sufficient to eject one or several orbital electrons from an atom or a molecule. The quantity of energy received is measured in Gray (Gy), representing the energy absorbed in joules/kg (1 Gy = 1 J/kg). When photons pass through tissue they will interact with molecules, and the consequence of this interaction is that energy is deposited to electrons (the Compton process). These electrons are

ejected from their orbits as an effect of the kinetic energy received from the photon, and the resulting fast moving electron may go on to take part in further interactions. The biological effects of radiation result principally from DNA damage. DNA can either be ionized directly by the incoming photons, resulting ultimately in DNA breaks or through interactions of DNA with fast moving electrons ejected from other molecular targets. As cells consist mainly of water (80%), water molecules are the most likely targets to interact with photons. If the water molecule is ionized, an H_2O^+ ion radical is produced. This ion radical is highly reactive and reacts with another water molecule to form a hydroxyl radical (OH·) which is also highly reactive. It is estimated that about two thirds of the DNA damage caused by X-rays and γ-rays is a consequence of reactions between hydroxyl radicals and DNA. Several different types of DNA damage are caused by ionizing radiation, e.g. base damage, single strand breaks and double strand breaks [27].

As with chemotherapy, side effects of radiotherapy limit the radiation dose that can be delivered. Side effects can be divided into acute (e.g. damage to skin and other epithelial surfaces, edema and infertility) and long term (e.g. fibrosis, hair loss and cancer). To minimize side effects of radiotherapy, fractionation is often used i.e. the radiation is delivered as smaller consecutive doses e.g. 64 Gy delivered as 2 Gy daily during several weeks. Tumors also differ in their sensitivity to irradiation and consequently radiotherapy is not always an option.

1.2.1.3 Cytotoxic drugs

Cytotoxic drugs, or chemotherapy, usually refers to anticancer agents that in some way target DNA and are more or less unspecific in the sense that both cancer cells and normal cells are affected by the drug. The use of cytotoxic drugs in cancer therapy has its origin in discoveries from world wars I and II, with the use of mustard-gas and chemical warfare [28]. After the sinking of a U.S ship containing mustard gas-bombs, the gas mixed with fuel oils and dispersed on the surface of the water. Men exposed to the mixture displayed decreased white blood cell counts and showed lymphotoxic symptoms. This discovery initiated research on the possible therapeutic potential of chemical warfare agents, and resulted in the use of a mustard gas analog, the nitrogen mustard Mustine in treatment of leukemia. It was also suggested that the toxic effects of nitrogen mustards was due to alkylation [29]. Since then several different groups of cytotoxic drugs have been developed targeting DNA or associated processes, and still today cytotoxic drugs are some of the most effective anticancer agents in clinical use [28].

Alkylating agents can be either monofunctional (one alkylating group) or bifunctional (two alkylating groups). Monofunctional alkylating agents cause single base modifications while bifunctional alkylating agents in addition has the possibility of crosslinking DNA with proteins or two DNA bases (intra strand or inter strand crosslinks). The interaction between the alkylating agent and DNA then gives rise to toxic lesions as the replication machinery tries to move through the modified DNA. Examples of alkylating agents in clinical use are cisplatin, mitomycin c and melphalan.

Antimetabolites (e.g. 5-fluorouracil and methotrexate) mimic nucleotides or their precursors and interfere with nucleotide metabolism or are incorporated into DNA.

Topoisomerase inhibitors act on topoisomerases, enzymes involved in relaxation of supercoiled DNA during DNA replication. During this process DNA is temporarily cleaved, and topoisomerase inhibitors stabilize this cleavage complex resulting in

replication dependent DNA breaks. Examples of topoisomerase inhibitors are topotecan (topoisomerase I inhibitor) and etoposide (topoisomerase II inhibitor) [30].

Antitumor antibiotics (e.g. Doxorubicin, Bleomycin) are cytotoxic compounds originally isolated from bacteria, in analogy to antibiotics used in treatment of infections. Doxorubicin seems to have several cytotoxic effects. Through intercalation (fitting between base pairs in DNA) doxorubicin inhibits replication, but it has also been shown that doxorubicin act as a topoisomerase II inhibitor [31]. Bleomycin is a radiomimetic drug that induces sequence specific cleavage of DNA [32].

Tubulin interacting agents act through alteration of microtubule polymerization and inhibition of mitosis [33], and in addition to blocking cell division, tubulin interacting agents induce apoptosis [34]. Examples of tubulin interacting agents are vinca alkaloids and taxanes.

An important limitation of chemotherapy in cancer treatment is the difficult side effects. As most cytotoxic agents act on DNA and associated processes, mainly affecting rapidly dividing cells, the pattern of side effects is mainly due to killing of rapidly dividing normal cells. Typical side effects are in the gastrointestinal region, related to the immune system, anemia, hair loss and pain.

Another limitation is that many tumors are resistant to specific cytotoxic agents (primary resistance), or develop resistance during treatment (secondary resistance). The reasons for this resistance can be several, e.g. increased drug efflux, decreased drug uptake, increased drug metabolism, alteration of the drug target or resistance to apoptosis.

Due to the side effects and the resistance, chemotherapy is commonly given as a combination of several cytotoxic agents. Combination therapy has the benefit of combining the cytotoxicity of several drugs with different side effects resulting in better tolerance to the treatment. Using a combination of drugs with different mechanisms of action also reduces the development of resistance.

1.2.2 Cellular effects of DNA damaging treatment

1.2.2.1 DNA damage sensors

As described above DNA damage is the primary cytotoxic effect of radiation therapy and most conventional cytotoxic drugs. The DNA damage is recognized by DNA surveillance proteins that initiate a cascade of events through phosphorylation of downstream targets. Three members of the phosphatidylinositol 3-kinase (PI-3) superfamily are considered to be central in this process: ATM (ataxia telangiectasia mutated), ATR (ATM- and Rad3-related) and DNA-PK (DNA-dependent protein kinase). Although partially redundant, these proteins play crucial roles in the DNA damage response.

ATM is a key protein in the cellular response to DNA double strand breaks (DSB) induced by ionizing radiation or certain cytotoxic drugs (e.g. Bleomycin, Cisplatin or Etoposide). DSBs are initially recognized by a complex of the three proteins Mre11/Rad50/Nbs1 (MRN) that binds to the broken DNA ends [35,36] (Figure 2A). It has been suggested that changes in DNA topology as a result of the DNA break could be important for detection of DSBs by the MRN complex [37].

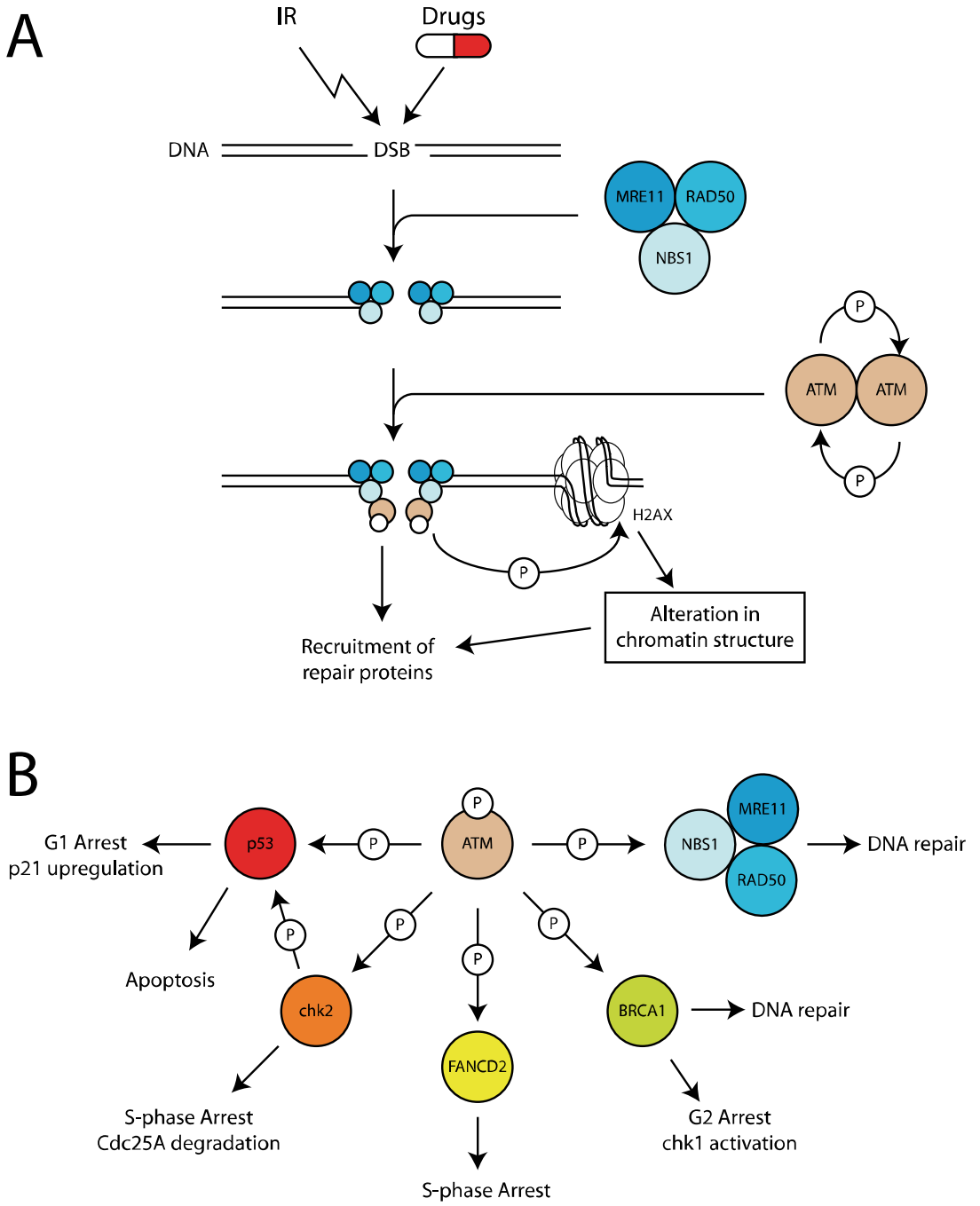

Figure 2. A. Within minutes after the formation of DNA double strand breaks (DSB), DNA damage sensors are recruited to the damaged DNA. A complex consisting of Mre11/Rad50/Nbs1 (MRN) binds to the DNA ends of the DSB and recruits ATM homodimers that are activated through autophosphorylation resulting in dimer dissociation. ATM then phosphorylate histone H2AX resulting in alteration in chromatin structure and recruitment of additional proteins involved in e.g. DNA repair to the protein complex bound to the broken DNA end. B. Activated ATM has the capability of activating a number of different downstream processes through phosphorylation of target proteins. p53 phosphorylation results in induction of apoptosis and p21 upregulation leading to cell cycle arrest. Cell cycle arrest is also induced through phosphorylation of Chk2, FANCD2 and BRCA1. ATM dependent phosphorylation of Nbs1 in the MRN complex is important for DNA repair, as is BRCA1 phosphorylation.

The MRN complex then recruits the inactive ATM dimere to the DSB [36], resulting in autophosphorylation of ATM and dissociation of the dimere into active ATM monomers [38]. The activation of ATM induce a chain reaction where a large portion of the nuclear ATM pool is activated, and in addition ATM phosphorylates components of the histone complexes (H2AX) [39] leading to additional changes in chromatin structure which facilitate the recruitment of a number of proteins (e.g. repair proteins), and the formation of these proteins into foci. A large number of ATM kinase substrates have been reported [40] and a few of them will be discussed below (Figure 2B). Activated ATM phosphorylates p53 on S15 [41,42] resulting in transcription of p53 target genes such as the CDKI p21 resulting in G1/S checkpoint activation. Alternatively, activated p53 can induce apoptosis through transcription of proapoptotic genes. ATM is also involved in p53 stabilisation through phosphorylation and inhibition of ubiquitin ligases (Mdm2 [43] and COP1 [44]) responsible for p53 ubiquitination leading to proteasomal degradation. Chk2 is another target of ATM, and once activated Chk2 phosphorylates p53 on S20 for further activation, and Cdc25A resulting in its degradation and intra-S phase checkpoint activation [45]. FANCD2 [46] and BRCA1 [47] are additional targets of ATM suggested to be involved in intra-S phase checkpoint. BRCA1 is also implicated in regulation of the G2/M transition through activation of Chk1, and regulation of DNA repair. The MRN complex, responsible in part for ATM activation, is itself a substrate of ATM as ATM phosphorylates Nbs1 [48].

ATR is the main sensor of stalled replication forks, a consequence of hinders in the way of the replication machinery. Treatment with DNA damaging agents such as alkylating agents or topoisomerase inhibitors, or exposure to UV-radiation cause lesions in DNA resulting in stalled replication forks that potentially leads to DNA breaks. In order to cope with such replication stress, cell cycle checkpoints are activated to allow repair of the DNA lesion and restart of replication. When DNA polymerases stall, MCM helicases continue to unwind the DNA generating a long stretch of single strand (ss) DNA [49]. The ssDNA binding protein RPA then coats ssDNA and ATR and ATR interacting protein (ATRIP) are recruited to the stalled fork [50]. Several other proteins are also recruited to ssDNA/RPA such as Rad17, the 911 complex (Rad9/Rad1/Hus1) [51] and TopBP [52]. ATR then phosphorylates Rad17, 911 and TopBP, resulting ultimately in further activation of ATR. Once activated ATR phosphorylates Chk1, which in turn phosphorylate Cdc25A and C resulting in intra-S and G2 checkpoint activation [53,54].

DNA-PK is composed of the catalytic subunit DNA-PKcs and the regulatory subunits Ku70 and Ku80. Like ATM and ATR, DNA-PK is suggested to be a molecular sensor of DNA damage, activating downstream targets through phosphorylation, however its main function in the DNA damage response pathway is in repair of DSBs as will be described below. The Ku70/80 heterodimer recognizes and binds to DNA breaks and recruits DNA-PKcs resulting in the formation of the DNA-PK complex [55]. DNA-PKcs is then autophosphorylated [56], increasing its binding to Ku70/80 heterodimer and downstream target phosphorylation. p53 is both activated and stabilized by DNA-PK activation through phosphorylation of p53 and Mdm2 respectively [57,58]. It has been suggested that p53 activation by DNA-PK selectively regulates apoptosis [59]. DNA-PK has also been shown to phosphorylate H2AX [60], and may act as a scaffold protein to recruit DNA repair proteins to sites of DNA damage.

There is a substantial amount of redundancy and crosstalk in the cellular DNA damage response. For example exposure to ionizing radiation activates not only ATM

but also ATR [61], and ATM deficiency results in impaired repair also of UV-induced DNA damage [62]. Further, p53 is a downstream target of ATM, ATR and DNA-PK, and Chk1 and Chk2 can be phosphorylated by both ATM and ATR, indicating that multiple common downstream targets exist.

1.2.2.2 p53 as the guardian of the genome

As described above, the tumor suppressor protein p53 is affected in virtually all situations where DNA integrity is threatened. p53 activity is basically regulated by two different mechanisms in response to stress signaling. First the stability of p53 is increased through disruption of its interaction with several different ubiquitin ligases (Mdm2, Cop1 [63] and Pirh2 [64]), that normally tags p53 for proteasomal degradation. Second, the ability of p53 to bind to, and promote transcription of target genes is increased. The stability and activation of p53 is regulated by post translational modifications as for example ATM, ATR and DNA-PK dependent phosphorylation of S15 on p53 inhibits interaction with ubiquitin ligases. Human p53 has 23 different phosphorylation sites and a number of lysines that are modified by ubiquitinylation, acetylation, sumoylation, neddylation and methylation, and it is believed that the pattern of these modifications determines target gene activation [65]. Through its target genes p53 then regulates the cells fate through involvement in cell cycle control, DNA repair and apoptosis as will be described below. The nuclear level of p53 is under strict control through several autoregulatory negative feedback loops (e.g. p53 dependent transcription of Mdm2, Cop1 and Pirh2). This is important as excessive p53 signaling could result in premature cell death, and there is also a need to downregulate p53 activity during stress relief to allow cells with repaired DNA damage to re-enter the cell cycle [66].

1.2.2.3 Cell cycle checkpoints

Cell cycle progression is controlled by the sequential activation and degradation of cyclins and activation of their partners the cyclin dependent kinases (CDKs). During early G1-phase of the cell cycle (the first gap phase), in response to mitogenic signaling (e.g. through the Wnt/β-catenin, Ras/Raf/Mek/Erk or NFκB signaling pathways), D-type cyclins are synthesized and complex together with CDK4 or 6. The CyclinD-CDK4/6 complexes initiate the phosphorylation of pRb which is completed by CyclinE-CDK2 as cells move through the restriction point. Once passed the restriction point cells are no longer sensitive to the withdrawal of serum or mitogens, and hyperphosphorylated pRb dissociates from E2F transcription factors resulting in transcription of S-phase proteins and entry into the S-phase of the cell cycle (synthesis of DNA). During early S-phase CyclinE is replaced by CyclinA in the complex with CDK2 and at late S-phase/early G2 phase CDK2 is replaced by CDC2 in the complex with CyclinA. During late G2 phase and entrance into mitosis, or M-phase, CyclinA is finally replaced by CyclinB to form the complex CyclinB/CDC2. Once the cell has completed mitosis, the two daughter cells are ready to receive new mitogenic signals and re-enter the cell cycle. Progression through the cell cycle is tightly regulated by CDK inhibitors (CDKIs), interacting with CDKs to inhibit their association with Cyclins. CDKs are also regulated by inhibitory phosphorylation, and dephosphorylation by Cdc25A and C activates CDK2 and CDC2 respectively. CDKIs of the INK4 family (inhibiting CyclinD-CDK4/6), p27 and p21 (widely acting CDKIs) can be activated by

anti-growth signaling through the TGF-β pathway [67]. In response to cellular stress a number of different cell cycle checkpoints are activated (Figure 3). First, activated ATM/ATR and DNA-PK phosphorylate p53 resulting in transcription of the CDKI p21 (waf/cip). P21 is a widely acting CDKI, interacting with both CDK2 and CDC2 to primarily inhibit the transition from G1 to S phase. A second checkpoint (intra-S) is activated as ATM (and ATR [68]) phosphorylate Chk2 which in turn phosphorylate Cdc25A, tagging it for degradation. Cdc25A is a phosphatase responsible for CDK2 activation through removal of inhibitory phosphorylations. BRCA1 has also been implicated in the intra-S checkpoint as ATM dependent phosphorylation of S1387 in BRCA1 is important for this checkpoint [69]. Similarly, ATM dependent phosphorylation of FANCD2 is also required for the intra-S checkpoint [46]. The G2/M checkpoint controls the transition into mitosis and this transition is inhibited through ATR (and ATM [70]) dependent phosphorylation of Chk1. Chk1 then phosphorylates Cdc25C which causes interaction with 14-3-3 proteins and sequestration of Cdc25C in the cytoplasm where it can no longer activate CDC2 through dephosphorylation [54]. BRCA1 is implicated also in control of the G2/M transition as ATM and ATR dependent phosphorylation of BRCA1 on S1423 is important for G2/M checkpoint activation [71].

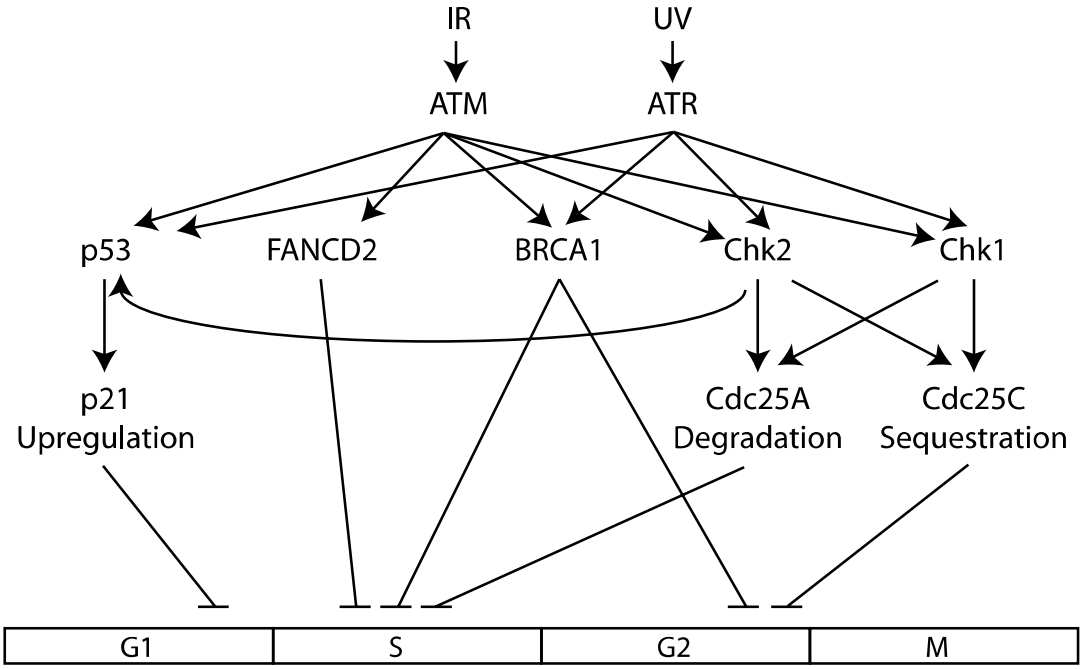

Figure 3. In response to DNA damaging treatment, checkpoints at several different phases of the cell cycle are activated. ATM and ATR control these checkpoints through phosphorylation of target proteins. See text for furter details.

1.2.2.4 DNA repair

If the DNA is damaged the cell will try to restore this lesion with as few genetic alterations as possible to avoid loss of coding material and to preserve DNA integrity. To do this cells are equipped with a variety of specialized DNA repair systems, roughly divided into five different pathways namely mismatch repair (MMR), base excision repair (BER), nucleotide excision repair (NER), non-homologous end-joining (NHEJ)

and homologous recombination (HR). Each repair pathway recognizes specific types of DNA lesions and their mechanism of repair will be described briefly below.

MMR is primarily involved in correction of base-base mismatches and insertion/deletion mispairs introduced during DNA replication and recombination [72]. These lesions are recognized by heterodimers consisting of MSH2 and either MSH6 or MSH3 and a second heterodimer, consisting of MLH1 and either PMS1 or 2, is recruited. PCNA and the exonuclease EXO1 then associate with the complex to excise mismatched bases after which DNA polymerase δ re-synthesizes DNA and DNA ligase I ligates the nick. MMR is not essential for repair of DNA damage caused by DNA damaging agents, rather MMR has been shown required for the cytotoxic effects of several anticancer drugs through recruitment of DNA damage sensors resulting in induction of apoptosis [73].

BER eliminates bases that are either damaged (e.g. by reactive oxygen species (ROS) or DNA damaging drugs such as alkylating agents) or inappropriate (e.g. introduced through treatment with antimetabolites) [74]. The first step of BER is base excision, where a DNA glycosylase cleaves the damaged base, creating an apurinic or apyrimidinic (AP) site. Next, an AP endonuclease (APE1) process the AP site generating nicked DNA, followed by excision of the lesion, DNA polymerase β dependent gap filling and sealing of the remaining nick by the XRCC1-DNA ligase 3 complex. BER can also be activated by single strand breaks (SSB), caused e.g. by irradiation. In this case the SSB is recognized by a complex consisting of XRCC1 and PARP1, and these two proteins then help recruit other proteins involved in later stages of BER.

NER is responsible for repairing thymidine dimers formed by exposure to UV irradiation and other bulky DNA lesions induced e.g. by crosslinking agents [75]. Many of the genes encoding proteins involved in NER (XPA-XPG) are mutated in the different subgroups of the autosomal recessive disease Xeroderma Pigmentosum, manifested by inability to repair UV-induced DNA damage and predisposition to cancer. It is generally accepted that the initial damage recognition is through the proteins XPA, RPA and the XPC-Rad23B complex. XPC-Rad23B then recruits the transcription factor IIH complex (TFIIH) consisting of nine subunits. Two of the subunits, XPB and XPD with helicase activity then unwind the DNA surrounding the damage, creating a "DNA bubble" around the lesion. XPG and XPF-ERCC1 are then employed to make incisions on each side of the lesion, and a 24-32 nucleotides long oligomer containing the DNA lesion is excised. Using the undamaged DNA as template, DNA polymerase δ or ε then synthesize a new strand of DNA to replace the damaged one and a DNA ligase then seals the nicks. Another branch of NER, transcription-coupled NER, is employed when DNA polymerase II stalls at sites of DNA damage, and differs from the one described above (global genomic-NER) only in the steps involving recognition of the DNA lesion.

NHEJ is one of the two major pathways for repairing DNA double strand breaks (DSBs), introduced e.g. through treatment with ionizing radiation or radiomimetics. NHEJ is active predominantly in G1 and S phases of the cell cycle and provides a fast, error prone repair by ligating the DNA ends together [76]. This repair pathway can result in loss of genetic material due to processing of DNA ends before ligation. The initiation of NHEJ is through recognition of both ends of the DSB by the Ku70/80 heterodimer. Ku70/80 then recruits the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), forming the DNA-PK complex. The DNA ends are then brought together by the

bound DNA-PK complexes, and ssDNA overhangs are removed either through the endonuclease activity of ARTEMIS or through DNA synthesis. In the last step of NHEJ, DNA ends are joined together by DNA ligase IV/XRCC4 complex.

HR is the other main repair pathway for DSBs and in addition HR is involved in repair of replication-associated DSBs e.g. induced by topoisomerase inhibitors. In late S and G2 phases of the cell cycle, sister chromatides give the possibility of recombination by the slower HR with no loss of genetic material at the DNA lesion as a result. The first step of HR after DSB recognition is believed to be resection of the DNA to yield 3' single-strand overhangs. This process is thought to be carried out by the MRN complex consisting of Mre11, Rad50 and Nbs1. Mre11 has ssDNA exonuclease activity [77], Rad50 is thought to facilitate DNA unwinding through its ATPase activity [78] and Nbs1 is believed to be involved in activation of the complex as Nbs1 is a direct substrate for ATM phosphorylation [48]. After initial resection of the DNA, ssDNA is coated with RPA and Rad51 is recruited to form nucleoprotein filaments on the ssDNA. Homologous DNA pairing and strand exchange is then performed, a process promoted by Rad52, Rad54 and Rad51 paralogs. An emerging number of additional proteins are also suggested to be involved in the control of HRR based on protein interaction studies and gene silencing e.g. BRCA2 [79], BRCA1 [80], BLM [81], WRN [82] and p53 [83].

p53 has been shown involved in regulation of virtually all DNA repair pathways [84]. This function of p53 is mediated either through regulation of transcription of DNA repair genes, or through direct interaction with components of the repair machinery. Interestingly, p53 seems to stimulate certain repair pathways like MMR or NER, while negatively regulating HR. It is likely that HR needs to be tightly controlled as excessive HR could potentially result in hyper recombination and genomic instability.

1.2.2.5 Apoptosis

If the damage induced by cytotoxic agents or irradiation is overwhelming or irrepairable, the damaged cells are removed by induction of apoptosis (programmed cell death, PCD). Through apoptosis multicellular organisms can dispose of unwanted cells in a controlled manner, minimizing damage to neighbouring cells and avoiding the release of immunostimulatory molecules. This is accomplished through the activation of a family of cystein proteases, caspases, responsible for cleavage of several hundred target proteins [85]. Characteristic apoptotic features include cell shrinkage, chromatin condensation, DNA fragmentation, membrane blebbing and finally engulfment of the cell corpse by phagocytes [14]. Apoptosis is generally subdivided into the intrinsic and extrinsic pathways [86] (Figure 4).

The intrinsic pathway is initiated through mitochondrial permeabilization, a process regulated by the Bcl-2 family of pro- and anti-apoptotic proteins [18]. If the balance of pro- and anti-apoptotic Bcl-2 proteins is tilted in favour of apoptosis, the proapoptotic Bcl-2 proteins Bak and Bax will oligomerize within the mitochondrial outer membrane. These oligomers create pores in the outer membrane, permitting efflux of intermembrane space proteins such as cytochrome c. Cytochrome c induces the formation of the apoptosome, a complex consisting of Apaf-1, pro-caspase 9, dATP and cytochrome c [87]. Apoptosome complexes then aggregate into heptamers in which caspase 9 is cleaved and activated, leading to the cleavage and activation of executioner caspases 3 and 7. Mitochondrial permeabilization can also be caused by calcium release

from the endoplasmatic reticulum (ER) in response to ER stress induced e.g. by oxidative stress [88]. A rise in intracellular calcium levels triggers the opening of channels located in the mitochondrial membrane (e.g. PT pores) and release of cytochrome c.

The extrinsic pathway is stimulated through binding of extracellular apoptosis inducing ligands such as Fas ligand, tumor necrosis factor-α (TNF-α) or TRAIL to members of the TNF-receptor family containing an intracellular death domain (e.g. Fas-R/CD95, TNF-R and TRAIL-R). Ligand receptor interaction results in receptor trimerisation and recruitment of intracellular adapter proteins FADD (Fas associated death domain) or TRADD (TNF-R associated death domain), necessary for recruitment of initiator pro-caspases 8 and 10. The assembled complex consisting of receptor/adaptor/pro-caspase is called the death inducing signaling complex (DISC) [89], and within this complex pro-caspases 8 and 10 are activated through autoproteolytic cleavage. Activated initiator caspases are then released and can either directly cleave and activate executioner caspases 3 and 7 (type I cells) or engage the mitochondrial pathway through cleavage of the Bcl-2 family member Bid [90]. Truncated Bid (tBid) promotes oligomerization of Bax like proteins resulting in mitochondrial outer membrane permeabilization, cytochrome c release and apoptosome formation (type II cells) [91].

Apoptosis is further regulated by additional layers of pro- and anti-apoptotic proteins. Inhibitor of apoptosis proteins (IAPs) is a family of important negative regulators of caspases, including e.g. XIAP, c-IAPs and survivin. IAPs either bind directly to caspases such as caspase 9, 3 and 7 and suppress their catalytic activity, or act as ubiquitin ligases to target caspases for degradation [92]. IAPs are in turn inhibited through caspase dependent cleavage [93] and by the actions of Smac/Diablo [94] and Omi/Htra2 [95]. Apoptosis is also regulated by the stress-activated protein kinases (SAPK) JNK (c-Jun N-terminal protein kinase) and p38. Targets of JNK phosphorylation are e.g. p53 and members of the Bcl-2 family [96], while p38 has been suggested to induced Bax translocation to mitochondria and cytochrome c release [97]. The exact role of SAPK signaling in apoptosis is however complex as e.g. JNK has been shown to have pro- or antiapoptotic functions, depending on cell type, nature of the death stimulus and activity of other signaling pathways [96].

p53 is one of the main regulators of apoptosis. In response to DNA damaging treatment, p53 accumulates in the nucleus and promotes transcription of a number of pro-apoptotic genes such as the pro-apoptotic Bcl-2 family members Bax, Noxa, Puma and Bid, as well as Apaf-1 and death receptors Fas-R and TRAIL-R [98]. p53 has also been shown to inhibit transcription of several anti-apoptotic genes e.g. Bcl-2 [99] and survivin [100]. In addition, a transcription independent role of p53 in promotion of apoptosis has been suggested through p53 accumulation in the cytoplasm, inhibition of anti-apoptotic Bcl-2 family members and activation of Bax and Bak [101].

Apoptosis is not the only route to cell death in response to DNA damaging treatment. Other possible outcomes include necrosis, mitotic catastrophe or senescence [102]. Necrosis is an energy independent form of cell death characterized by vacuolation of the cytoplasm, breakdown of the plasma membrane and induction of inflammation around the dying cell as a consequence of released immunostimulatory molecules [103]. In energy deprived cells apoptosis is no longer an option as this is an energy dependent process, and in these cases the mode of cell death could be switched to necrosis. Mitotic catastrophe is caused by failure to activate cell cycle checkpoints in response to DNA damage resulting in aberrant mitosis [104]. Mitotic catastrophe is associated with

multinucleate, giant cells containing uncondensed chromosomes. Senescence is a form of permanent growth arrest triggered by DNA damage, oncogenic signaling and telomere shortening [105]. p53 and pRb has been identified as two of the principal regulators of senescence.

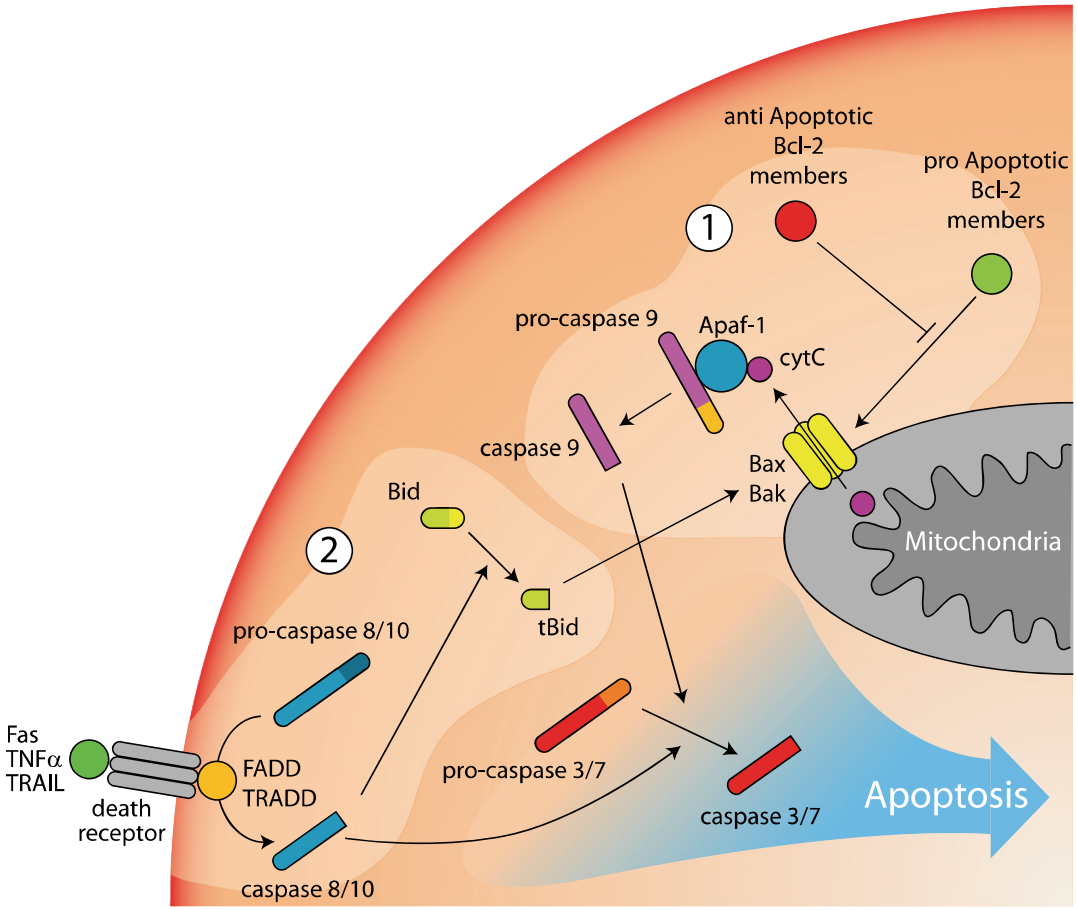

Figure 4. Apoptosis is generally subdivided into the intrinsic and the extrinsic pathway. In the intrinsic pathway (1), pro- and antiapoptotic members of the Bcl-2 protein family controls permeabilization of mitochondria. In response to proapoptotic signaling e.g. through p53, the proapoptotic Bcl-2 family members Bax and Bak oligomerize in mitochondrial outer membrane permitting for efflux of cytochrome c. Cytochrome c associates with Apaf-1 and pro-caspase 9, forming the apoptosome complex in which pro-caspase 9 is cleaved to active caspase 9. Caspase 9 then cleave executioner pro-caspases 3 and 7 resulting in caspase 3 and 7 dependent cleavage of target proteins and apoptosis. The extrinsic pathway (2) is activated as proapoptotic extracellular ligands such as Fas, TNF-α or TRAIL bind to cellular death receptors. Upon ligand-receptor interaction, intracellular adapter proteins FADD or TRADD associate with the intracellular domains of death receptors and pro-caspase 8 or 10 is recruited to form the Death inducing signalling complex (DISC). In this complex pro-caspase 8/10 is cleaved and activated caspase 8/10 then cleave executioner caspases 3 and 7 (type I cells). Alternatively, caspase 8/10 cleaves Bid, and truncated Bid (tBid) engage the intrinsic pathway through promoting Bax and Bak oligomerization (type II) cells.

1.3 TARGETED CANCER THERAPY

1.3.1 Critical targets of cancer therapy

The aim of modern anticancer treatment is targeted therapy where specific features of tumor cells are used as starting points for rational therapy design. One could argue that also traditional cytotoxic drugs are targeted as they act preferentially on rapidly dividing cells. However the usability of these traditional drugs is often limited by the difficult side effects they also possess on endogenous normal rapidly dividing cells. Truly targeted therapies should be able to discriminate between normal cells and cancer cells. The hallmarks of cancer postulated by Douglas Hanahan and Robert Weinberg [4], described above, will be used as a base for the discussion below on current and future efforts to improve anticancer treatment.

Targeting growth signaling. As discussed above cancer cell proliferation is driven by deregulated growth signaling. This knowledge has been used in the development of targeted anti cancer therapy such as imatinib (Gleevec) for treatment of chronic myeloid leukemia (CML) [106]. Imatinib binds to the Bcr-Abl fusion tyrosine kinase and inhibits its constitutive signaling, thereby inhibiting the progrowth signaling. The first example ever of targeted therapy however, is maybe the use of Tamoxifen for treatment of breast cancer. Tamoxifen inhibits proliferative signaling through inhibition of the estrogen receptor (ER). Other examples of growth factor receptors targeted by drugs are EGF-R (gefitinib, Iressa) and HER2/neu (trastuzumab, Herceptin) [107]. There are drugs in clinical trials aiming for inhibition also of intracellular growth signaling components such as Ras (tipifarnib) [108].

Targeting evasion of apoptosis. Restoration of the apoptotic machinery constitutes an attractive way of selectively targeting cancer cells. It is believed that many cancer cells are primed to enter into apoptosis triggered e.g. by oncogenic activation and deregulated growth signaling, however due to abrogation in the apoptotic machinery the execution state is never reached. Reactivation of apoptotic signaling could therefore be a way to allow for execution of apoptosis selectively in cancer cells. Mechanisms of action for some of the drugs in clinical trials are through reactivation of mutated p53 [109], inhibition of p53 degradation [110], inhibition of antiapoptotic Bcl-2 [111] and inhibition of the apoptosis inhibitors XIAP [112] and survivin [113].

Targeting limitless replication. Telomerase is expressed by 85-90% of malignant cells and inhibition of this enzyme would be detrimental to these cells, as the telomeres would be lost resulting in permanent growth arrest or cell death. Two approaches for targeting telomerase/telomere systems reaching clinical trials in the near future are molecules interfering with the interaction between telomerase and telomeres, or molecules acting directly on the telomeres leading to telomere degradation [114].

Targeting angiogenesis. Interfering with angiogenesis, potentially leading to starvation and death of the tumor has long been a potential therapeutic niche that researchers have put high hope in. With the introduction of bevacizumab (Avastin), an antibody targeting vascular endothelial growth factor (VEGF), on the market new fuel is put into research on drugs targeting angiogenesis [115].

Targeting tissue invasion and metastasis. The first targets of cancer therapy within this field were matrix metallo proteinases (MMP), but inhibitors of MMPs did not meat the expectations [116]. New generations of drugs aiming to inhibit tissue invasion and metastasis are now entering clinical trials with Src-kinase inhibitors (modulation of

adhesion complex turnover) and urokinase (protease) inhibitors leading the way, followed by ROCK (kinase linked to cell motility) inhibitors [117].

Targeting DNA repair. Components of DNA repair pathways are often altered in cancer cells. These alterations can present themselves either as increased activity in certain repair pathways, resulting in resistance to DNA damaging agents, or as defects in specific pathways that can be exploited pharmacologically [118]. Combination therapies with cytotoxic agents and inhibitors of proteins involved in DNA repair (e.g. PARP, Chk1/2, ATM and DNA-PK) are currently in pre-clinical and clinical trials [118]. Interestingly, certain subsets of tumors with defects in proteins involved in DNA repair (e.g. BRCA1 and BRCA2) have been shown hypersensitive to PARP inhibition [119]. PARP inhibitors cause single strand breaks and stalled replication forks ultimately resulting in DNA double strand breaks if not resolved properly. Tumors deficient in components of the homologous recombination repair pathway fail in repairing these lesions and are consequently sensitive to monotherapy with PARP inhibitors, currently tested in clinical trials.

1.3.2 Knowing what to target-Predictive biomarkers

With the development of new molecularly targeted anticancer agents it is also equally important to develop methods to characterize the tumors [120] - if the target is not there, targeted therapy will not cure the patient. Predictive markers can be defined as factors that indicates sensitivity or resistance to a specific treatment, and such markers would be extremely important when deciding what targeted drugs to use. Only a few predictive markers are used within the clinic today, examples are estrogen and progesterone receptor positivity in breast cancer indicating that the patient will benefit from hormone therapy and HER2/neu positivity also in breast cancer indicating that the therapeutic antibody trastuzumab (Herceptin) would be effective in killing the tumor [121].

Different methods for developing predictive markers could be considered. The most direct way would be to sample the tumor and measure the presence of the specific target. This could be done directly through the use of affinity based assays or cyto/histochemistry to measure the presence or level of a protein, or through genetic analysis where mutations and other aberrations (deletions, translocations or amplifications), altering the activity of a protein could be assessed. It is not certain however that the increased level of a protein or the presence of a specific mutation can answer the question of whether a patient will benefit from a treatment or not. Other tumor characteristics could modify the treatment response, even if the specific target is present. In order to address this question, cohort studies could be designed where differences in molecular patterns between responders and non-responders to a specific drug could be studied.

The possibility to study molecular patterns in cancer cells has improved dramatically with the new omics methods, but there is still a need for high-throughput multiplex assays that can be used as diagnostic tools. Due to a growing catalogue of potential drugs, it will be increasingly important to know which drugs to choose. One of the most important factors deciding treatment outcome is that the right drug is delivered as soon as possible. Most patients do not have the time to randomly try the effect of several different treatments, as the tumor will keep growing until it is properly treated. Also the

cost of these treatments is often very high, further underscoring the importance to give the right drug to the right patient.

1.3.3 Targeted therapy conclusions

Cancer is the common name for a large family of diseases affecting a broad range of organs within the body. Different cancer diseases do share common features as described above, but that does not mean that they all have the same genomic alterations. Cancer cells are heterogeneous and the complexity of the molecular alterations in these cells is overwhelming. It is of great importance to understand what the causes and consequences of these molecular alterations are. This basic knowledge can then be used in rational design of targeted cancer therapies. Future cancer therapy will most probably be based on the combination of molecularly targeted anticancer agents, each being the result of thorough research and knowledge within a specific part of the cancer biology field. Conventional chemotherapy and irradiation will likely keep their positions as cornerstones in cancer treatment, but in combination with targeted therapy [122]. Each tumor should also be systematically characterized with respect to a large panel of features, or biomarkers, in order to direct the treating physician to the right combination of drugs. Combining anti-cancer drugs has long been used in clinical practice, but up to date primarily to minimize the development of resistance and too severe side effects. Since most traditional anti cancer drugs has a common target, namely DNA, the combination of drugs for synergistic effects has limited value. With the development of targeted anti cancer drugs, aiming for a broad range of specific features, a new possibility of using rational combinations to kill cancer cells more effectively has emerged. New technologies enabling high throughput characterization of tumors will lead to the discovery of new molecular targets of anti cancer therapy. One of the important tasks for the future will be to determine the most promising combinations of targeted drugs, and to evaluate these combinations clinically [107].

1.4 S100A6

1.4.1 The S100 protein family

The S100 family of proteins is a large family of calcium binding proteins with more than 20 members identified today [123]. In 1965, the first S100 protein was isolated from bovine brain by Moore and coworkers as part of a systematic attempt to isolate proteins specific to the nervous system and it was named S-100 because of its solubility in saturated ammonium sulphate at pH 7 [124,125]. Subsequent studies revealed that this fraction contained predominantly two proteins, S100B and S100A1 with molecular weights of approximately 10 kDa. All of the S100 proteins are low molecular weight proteins ranging from 9 to 13 kDa with 25-65% identity at the amino acid level. The majority of S100 genes are clustered on chromosome 1q21 and these S100 proteins are named S100A followed by consecutive Arabic numbers i.e. S100A1, S100A2 and so on. S100 genes from other chromosomal regions are named S100 followed by a single letter i.e. S100B or S100P [126]. The S100 protein family seems phylogenetically young, so far S100 proteins are found only in vertebrates [126].

A combining feature of S100 proteins is the presence of two EF-hand calcium binding domains; one "S100 specific" or "pseudo EF-hand" located at the N-terminal portion of the protein, and one classical EF-hand located at the C-terminal part of the protein. The classical calcium binding EF-hand motif was first described by Kretsinger and Nockolds, and it consists of two α-helices with an intervening 12-residue calcium binding loop [127]. The pseudo EF-hand specific for S100 proteins has 14- amino acids in the loop and lower affinity to calcium than the classical EF-hand [128]. Separating the two EF-hand domains is an intermediate region known as the hinge. This region and the C-terminal region flanking the classical EF-hand domain are the most variable regions between the different S100 proteins and they have been suggested to give the specific biological activity of the individual proteins. Intracellular calcium levels are tightly controlled and range from resting levels of about 100 nM to signalling levels of close to 1 μM. Calcium binding to the classical EF-hand on S100 proteins ($K_d \approx$ 10-50 μM) suggests that the affinity between S100 proteins and calcium is to weak to be physiologically relevant, however this affinity is improved upon target protein binding and certain post translational modifications [129]. The calcium affinity to the pseudo EF-hand is even weaker ($K_d \approx$ 200-500 μM), disputing the *in vivo* relevance of calcium binding to this site [130]. Binding of calcium to the classical EF-hand of S100 proteins induce a dramatic change in conformation, resulting in a more open structure and exposure of the hinge region involved in target protein interaction (Figure 5).

It is generally suggested that within cells most S100 proteins exist as homodimers held together by non-covalent bonds [131], but in several cases formation of heterodimeres has also been shown e.g. S100A8/S100A9 [132]. Post translational modifications of S100 proteins have also been shown, e.g. through phosphorylation of S100A8 and S100A9 [133] or redox modifications of cystein residues in S100A1 and S100B [134,135], adding another level of S100 activity regulation. S100 proteins are non ubiquitous proteins showing distinct tissue and cell type specificity [136] indicating that different S100 proteins have distinct cellular functions in spite of the structural similarities. Both intracellular and extracellular roles have been suggested for S100 proteins as reviewed by Donato [131].

S100 proteins have no known enzymatic activity, so the biological functions are generally believed to be through interaction with other proteins and regulation of these

target proteins functions. Much effort has been put into finding the interaction partners of S100 proteins, and today more than 100 target proteins have been suggested [123]. Most of these interactions are calcium dependent and the suggested functions of these interactions can be divided into five major groups: (i) regulation of phosphorylation mediated by protein kinases, (ii) modulation of enzymatic activity, (iii) maintenance of cell shape and motility, (iv) influence of some signal-transduction pathways, and (v) promotion of calcium homoeostasis. A large number of target proteins have been suggested for S100 proteins however the physiological relevance and the biological consequence of these interactions, in many cases, remain to be determined.

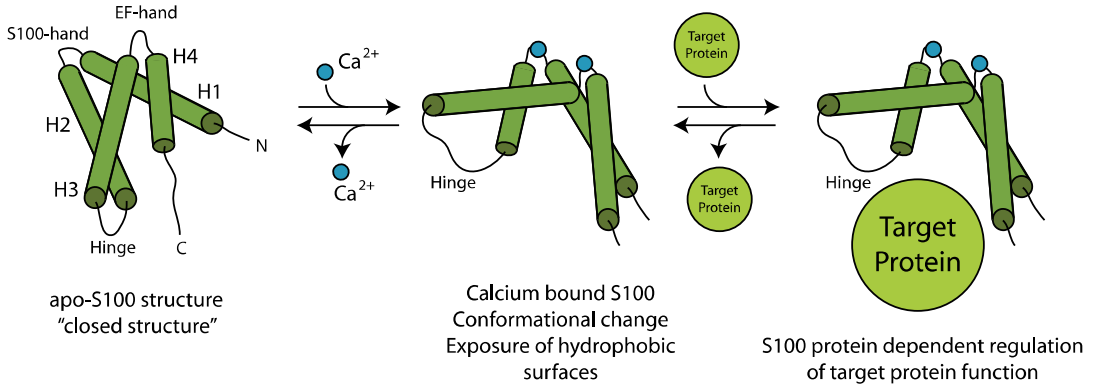

Figure 5. Calcium interaction of S100 proteins induces a dramatic conformational change, exposing hydrophobic binding surfaces on the S100 protein. Most S100 protein interactions are dependent on this conformational change. S100 proteins excert their biological functions through regulating the activities of the bound target proteins.

1.4.2 S100 proteins in disease

In the past few years S100 protein family members have been shown to have critical connections to major diseases such as inflammatory diseases, neurodegradation, cardiomyopathies and cancer.

Three members of the S100 family, S100A8, S100A9 and S100A12, predominantly expressed in neutrophils and macrophages, are strongly associated with pro-inflammatory functions. S100A8 and S100A9 form a heterodimer that is released from activated neutrofils and has a chemotactic effect in the local microenvironment through interaction with several different cell surface receptors, e.g. the receptor for advanced glycation end products (RAGE) [137]. S100A8 and S100A9 have been shown upregulated in several inflammatory disorders such as rheumatoid arthritis and cystic fibrosis [138]. S100A12 directly activates endothelial cells, mononuclear phagocytes and lymphocytes through interaction with RAGE [139], resulting in secretion of proinflammatory mediators. The above mentioned three S100 proteins, together with S100A7 have all been shown upregulated in psoriatic lesions, suggesting a role in the development of psoriasis [140,141].

S100B, one of the first S100 proteins discovered in bovine brain, has been shown upregulated in response to traumatic brain injury in peripheral blood and cerebrospinal fluid [142]. S100B has also been shown upregulated in the post mortem brain tissue in Alzheimer's disease and Down's syndrome. Produced primarily by astrocytes, S100B

exerts autocrine and paracrine effects on glia, neurons, and microglia [143]. The biological effect of secreted S100B depends on concentration. At low levels S100B stimulate neurite outgrowth and act as a neurotrophic factor [144], while higher concentrations of S100B induce inflammation and apoptosis [145,146]. Also S100A6 has been implicated in neurodegenerative disorders in a limited number of publications. In Alzheimer's disease it was shown, both in human post mortem brain and in mouse models, that S100A6 protein was up-regulated in astrocytes surrounding the amyloid deposits of senile plaques [147]. Also in amyotrophic lateral sclerosis (ALS) overexpression of S100A6 has been shown in astrocytes located near affected motoneurons [148,149]. So far no biological function of S100A6 in the development of neurodegenerative diseases has been shown.

S100A1 is highly expressed in mammalian myocardium, where it modulates contractile performance [150]. S100A1 has been shown upregulated in right ventricular hypertrophy [151], and downregulated at end stage heart failure [152]. At the molecular level, S100A1 has been shown to interact with components of the calcium homeostasis system regulating cardiac muscle contraction/relaxation such as the cardiac ryanodine receptor, the SR Ca_2_-ATPase 2a (SERCA2a)-phospholamban (PLB) complex, cardiac titin, and mitochondrial F_1-ATPase [153].

1.4.3 S100 proteins in cancer

Several S100 proteins have been shown deregulated in cancer as will be described below.

S100A1 is overexpressed in renal cell carcinoma but not in normal tissue [154], and further S100A1 has been validated as a marker for differential diagnostics and prognosis determination in renal cancer [155,156]. S100A1 has also been suggested to inhibit the pro-metastatic activities of S100A4 through heterodimerization [157].

S100A2 has been suggested to be a tumor suppressor as downregulation of S100A2 expression has been shown during progression of cancer. Such correlations have been shown in non small cell lung cancer (NSCLC) [158,159], breast cancer [160], prostate cancer [161], oral cancer [162,163], bladder cancer [164] and melanoma [165]. Hypermethylation of the S100A2 promoter is thought to be the mechanism for this loss of S100A2 expression [160]. However other groups have reported upregulation of S100A2 in NSCLC and a correlation with lymphatic invasion [166-168] and in pancreatic cancer S100A2 has been correlated to poor prognosis [169]. It has been shown that S100A2 interacts with the tumor suppressor p53 and increase its transcriptional activities [170], but further studies are needed to establish the biological consequences of S100A2 downregulation.

S100A4 has been intensively studied in human cancer and the data supporting its role in tumor progression is overwhelming. Upregulation of S100A4 has been shown in cancer of the bladder [164,171], endometrium [172], pancreas [173,174], colorectum [175-178], lung [167,179], thyroidea [180], breast [181-184], oral cavity [185], stomach [186], prostate [161], gallbladder [187] and esophagus [188]. In most cases the upregulation of S100A4 has been correlated to poor prognosis, and in many cases to the development of metastasis. In analogy to S100A2, methylation of the S100A4 promoter has been suggested as a regulatory mechanism for S100A4 expression and hypomethylation has been correlated with high expression [172,174]. The biological role of S100A4 in tumor progression in general and metastasis in particular has also been studied in many laboratories. It has been shown that S100A4 interacts with p53 and that this interaction inhibits phosphorylation of p53

and modulate its transcriptional activity [189]. The prometastatic functions of S100A4 has been studied in rodents, most widely in breast cancer models, and it has been shown that overexpression of S100A4 itself is not tumorigenic but that it correlates with the development of metastasis [190-192]. Further supporting the causal role of S100A4 in the process of metastasis, it has been shown that downregulation of S100A4 suppress the formation of metastasis in animal models of lungcarcinoma and osteosarcoma [193,194]. On the molecular level S100A4 interacts with nonmuscle myosin II and has been shown involved in the regulation of cellular motility [195,196]. S100A4 is also secreted, and extracellular S100A4 stimulates matrix metallo protease activity [197,198]. These findings support the role of S100A4 in metastasis as remodelling of the extracellular matrix through proteolysis and cell migration are important in initiation of the metastatic cascade.

S100A6 expression has been directly correlated with the neoplastic phenotype, being more expressed in cancer than in normal tissues. Upregulation of S100A6 has been shown in pancreas [169,199-202], where S100A6 expression was reported to be a negative prognostic factor [202] and in colorectal [203-207] cancers where it was more expressed in invading fronts and liver metastasis then in primary tumors. Increased expression of S100A6 has also been shown in thyroid cancer [208,209], gastric cancer [210], breast cancer [211] and in some cutaneous tumors [212]. In opposite of the above findings increased S100A6 levels in osteosarcoma [213] have been coupled with improved survival and lower risk of metastasis. Moreover, loss of S100A6 in tumors compared to benign proliferative lesions has been observed for prostate cancer [214] and melanoma [215]. At the mRNA level however it has been shown that higher expression of S100A6 mRNA in malignant melanoma correlates with a shorter survival time [165]. As in the case of S100A2 and S100A4 promoter hypermethylation is suggested to regulate S100A6 expression during cancer progression [216,217]. The biological functions of S100A6 will be discussed below.

S100A7 has been studied primarily in breast cancer, where its expression has been correlated to more aggressive tumors [218-220]. S100A7 was shown to interact with Jab-1 (c-Jun activation domain-binding protein 1) leading to increased activity of the NFκB pro survival pathway [218,221,222]. The level of S100A7 has also been studied and shown upregulated in bladder cancer [223], skin cancer [224] and lung cancer [225,226].

S100A8/S100A9 is mostly associated with inflammatory conditions as described above, but in several cases deregulation of the two components of this heterodimer has been observed in cancer. Upregulation of S100A8 has been shown in prostate cancer [227], bladder cancer [228] and endometrial cancer [229] and association of S100A9 to tumor differentiation has been shown in liver, lung, thyroid and breast cancer [230-233]. Upregulation of both proteins has been shown in colon cancer [234] while downregulation of both proteins has been shown in head and neck cancer [235] and esophagus cancer [236]. The involvement of S100A8/S100A9 in both inflammation and cancer suggests that this heterodimer may play a key role in inflammation-associated cancer.

S100A11 downregulation was shown to correlate with bladder cancer progression and also loss of S100A11 expression was associated with poor survival suggesting a tumor suppressor function of S100A11 [237]. A possible role of S100A11 could be through inhibition of cell cycle progression as overexpression of S100A11 results in upregulation of p21 [238]. However, in lung cancer, colon cancer and prostate cancer S100A11 increased with higher tumor stage and in lung cancer high level of S100A11 correlated with positive lymph node status [239-241].

S100B is closely associated with the development of malignant melanoma and increased serum levels of S100B correlates with increasing melanoma grade [242-244]. An increased serum level of S100B has also been shown to correlate with metastasis and decreased survival [245,246]. The correlation between melanoma tumor load and S100B serum levels makes S100B a useful diagnostic marker for melanoma and a potential marker for treatment response assessment [247,248]. S100B has been shown by several groups to interact with p53 [249,250]. It has also been shown that this interaction inhibits p53 oligomerization and transcriptional activity and reduces the abundance of p53 [249,251]. Conversely inhibition of S100B using antisense resulted in increased p53 levels [252]. In addition it was shown in the same paper that p53 is a transcription factor for S100B, suggesting a negative feed back loop for p53 through S100B.

1.4.4 S100A6

S100A6 was originally discovered in the mid 80ies by several different groups. In 1986 Calabretta and coworkers cloned a gene called 2A9 (S100A6) after a differential screening of genes to find sequences inducible by serum [253,254]. The gene was not expressed in quiescent cells, but was induced by stimulation with serum, platelet derived growth factor and epidermal growth factor. Exponentially growing cells also expressed the 2A9 gene and expression was shown increased in leukemia [255,256]. The full gene of 2A9 was sequenced the following year, and named Calcyclin (Calcium binding, cell cycle related) [257]. The Calcyclin gene was shown to have three exons and was localized to the long arm of chromosome 1. In 1987 Kuznicki and coworkers isolated a novel calcium binding protein named CaBP (Calcium Binding Protein (S100A6)) from Ehrlich-ascites-tumor cells [258]. The size of the protein was estimated to be 10.5 kDa and two calcium binding sites with different affinity were suggested. Calcium binding of CaBP altered UV absorbance, fluorescence intensity and affinity to phenyl-Sepharose, all indications of a calcium induced conformational change. The S100A6 gene was also independently cloned by Murpy and coworkers in 1988 and named PRA (Prolactin Receptor Associated) as the protein encoded by the gene co-purified with the prolactin receptor [259]. The clustered organization of S100 genes in chromosome 1q21 (S100A1-S100A18) was later used to introduce a new logical nomenclature, based on the physical arrangement of S100 genes on the chromosome, and so the name S100A6 was suggested to be used instead of 2A9, Calcyclin, CaBP and PRA [260].

The three dimensional structure of S100A6 was initially determined in solution in the apo state by NMR spectroscopy [261]. It was suggested that apo S100A6, like most other S100 proteins, formed symmetric homodimers, and that each subunit consists of two helix-binding loop-helix (EF-hand) motifs packed in a parallel fashion, joined by a linker loop (hinge). The authors further suggested that the apo S100A6 dimer was held together by hydrophobic interactions and that the two subunits were packed in an antiparallel fashion. The same group later showed, using NMR, that calcium binding of S100A6 only induced small changes in conformation and they concluded that calcium binding would not be sufficient to trigger fundamental changes in protein–target interactions [262]. In stark contrast to these findings it was later shown by X-ray crystallography that calcium binding of S100A6 results in a dramatic change in the global shape and charge distribution of the S100A6 dimer, leading to the exposure of two hydrophobic target binding sites [263]. The largest conformational changes seen

upon calcium binding were reorientation of helix III (86°) and marked changes in the position of the hinge region and the C-terminal part of the protein. As pointed out earlier these regions are the most variable between different S100 proteins, and exposure of these regions would enable specific interactions for different S100 proteins. It has been suggested that S100A6 forms heterodimers with S100B, but the functional consequences of this interaction has not been determined [264,265].

The calcium affinity of S100A6 has been studied by several different groups [258,266,267]. As for other S100 proteins the calcium affinity for the C-terminal EF-hand of S100A6 (K_d ≈20mM) would allow for calcium binding during calcium influx, while the N-terminal pseudo EF-hand affinity is likely too weak to bind calcium at physiological concentrations. The affinity of S100A6 and other S100 proteins to calcium is modulated by target protein interaction and also by the oxidative state of cystein residues in the protein, indicating that in its proper environment the affinity between S100A6 and calcium is much higher [134,266]. It has also been shown that S100A6 binds zinc, and that the affinity of this binding is 2-300 times higher than the affinity to calcium [266]. However, due to extremely low intracellular levels of zink the physiological relevance of this interaction in regulation of intracellular S100A6 functions is questionable.

The tissue expression of S100A6 has been studied using northern blot, where it was shown that S100A6 mRNA expression in normal human tissue was high in lung, colon, kidney, placenta, ovary and mammary gland [165]. The highest mRNA level was seen in lung, which was also seen for mRNA levels of S100A4 and S100A2. In mouse tissue S100A6 mRNA expression was found in greatest amount in organs containing proliferating cells, e.g. epidermis, skin, stomach, uterus of pregnant mouse, placenta, and decidua [268]. Using immunohistochemistry it was shown that only epithelial cells and fibroblasts showed positive S100A6 staining in all human tissues examined (breast, heart, intestine, kidney, liver, ovary, placenta, stomach, thymus, and uterus), further suggesting involvement in proliferation [269]. Immunohistochemistry has also been used to study S100A6 distribution in brain, where positive staining has been shown in neurons [270] and astrocytes [271].

Several transcription factors involved in promotion of S100A6 mRNA expression has been found. Using a series of deletion mutants it was shown that the 164-base pair fragment just upstream of the S100A6 gene contained the region responsive to growth factor regulation [272]. This region was later shown to bind an AP-1 like transcription factor responsible for S100A6 upregulation in neuroblastoma cell lines in response to treatment with retinoic acid [273]. Further, two E-box motifs has been identified in the S100A6 gene promoter (-593/-588 and -283/-278), both binding upstream stimulatory factor (USF) transcription factors [274,275]. These motifs were also shown important for activation of the S100A6 promoter in response to treatment with the fatty acid palmitate. Moreover, S100A6 gene expression has been shown upregulated in response to agents evoking oxidative stress ($CdCl_2$, curcumin or H_2O_2) through the binding of Nrf2 transcription factor to an antioxidant response element (-290/-281) [276]. In addition, treatment of an hepatoblastoma cell line with TNFα resulted in upregulation of S100A6 transcription through the binding of NFκB/p65 to the S100A6 promoter (-460/-451) [277]. Overexpression of NFκB/p65 was shown to increase promoter activity while overexpression of the NFκB/p65 inhibitor IκBα decreased the activity.

Epigenetic mechanisms have also been shown affecting the transcription of the S100A6 gene as for example hypermethylation of the promoter region is associated

with loss of transcription in medulloblastoma [217] and prostate cancer [216]. Modification of histone H3 bound to the S100A6 promoter region is an additional epigenetic mechanism suggested to be involved in regulation of S100A6 transcription [278].

1.4.5 Biological functions of S100A6

The biological functions of S100A6 are mainly thought to be through interaction with and regulation of other proteins as S100A6 have no shown enzymatic activity of its own. Research aiming to explain the biological role of S100A6 has therefore been focused on finding S100A6 interacting proteins.

Several different groups have reported interactions between S100A6 and different members of the annexin protein family (Annexin A2 [279,280], Annexin A6 [280] and Annexin A11 [281-284]), although with weak affinity and with no suggested biological function.

Two groups have shown interaction between S100A6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), however the interaction did not seem to alter the enzymatic activity of GAPDH [279,280].

The chicken isoform of S100A6 interacts with caldesmon, an actin binding protein regulating assembly and stabilization of microfilaments. S100A6 was shown to inhibit the interaction between caldesmon and actin, thereby reducing caldesmons inhibitory effect on acto-myosin ATPase activity [285,286]. These results were later disputed as the affinity of the interaction between S100A6 and caldesmon was to low to modulate ATPase activity [287]. Tropomyosin is another actin binding protein involved in regulation of the cytoskeleton, and like caldesmon it has been shown to interact with the chicken isoform of S100A6 [288]. In S100A6 antisense studies it was later shown that downregulation of S100A6 resulted in disruption of tropomyosin labeled microfilaments and a flattened, spread cell morphology [289]. However no actual co-localisation between S100A6 and Tropomyosin was shown as S100A6 localized to the nucleus. In addition chicken S100A6 has been shown to interact with Calponin [290] and Lysozyme, but without affecting enzymatic activity [291].

The most well established S100A6 interacting protein is CacyBP (Calcyclin (S100A6) Binding Protein). This protein was first identified as a protein interacting with S100A6 with higher affinity then previously described proteins such as Annexin 2 or GAPDH [292]. When the gene was cloned, nucleotide sequence analysis confirmed that this was a novel protein expressed predominantly in mouse brain, and it was named CacyBP [293]. The interaction was studied in detail and it was shown that S100A6 interacted with the C-terminal region of CacyBP [294]. The human homologue of CacyBP was identified in a yeast two-hybrid screen to find proteins interacting with Siah-1, an E3-ligase involved in phosphorylation independent ubiquitination of β-catenin, and it was named SIP (Siah-1 Interacting Protein) [295]. Further it was shown that CacyBP/SIP interacted with another component of the ubiquitination machinery, Skp1, and it was concluded that CacyBP/SIP was important for proper formation of the ubiquitination complex. Mapping of the interacting domains showed that the N-terminal part of CacyBP/SIP was sufficient for interaction with Siah-1, and that the C-terminal part of CacyBP/SIP was required for interaction with Skp1. Supporting these results it has been shown that forced overexpression of CacyBP/SIP in gastric cells results in increased degradation of β-catenin, decreased activity of the β-catenin co-transcription factor Tcf/LEF, inhibition of cell proliferation, suppressed tumorigenesis in vitro and

prolonged survival time in tumor bearing nude mice [296]. Down regulation of CacyBP/SIP using siRNA resulted in opposite effects. Similar results has been shown in human renal cancer cells and it has also been shown in clinical tissue samples that CacyBP/SIP was downregulated in renal cell carcinoma compared to matched non-tumorous tissue [297]. Several other S100 proteins has been shown to interact with CacyBP/SIP (S100A1, S100A12, S100B and S100P) [298] and together with S100A6 these proteins have also been shown to interact with sgt1, a protein with some homology to CacyBP/SIP also potentially involved in protein ubiquitination [299].

A biological role of S100A6 in regulation of proliferation, initially suggested by Calabretta and coworkers [253] has been further supported by S100A6 silencing experiments. Using S100A6 antisense expressing pulmonary fibroblasts it was shown that downregulation of S100A6 inhibited cell proliferation [289]. Similar results were obtained for S100A6 siRNA treated pancreatic cells where it was also shown that S100A6 downregulation inhibited the invasive potential [200].

S100A6 have also been suggested to be involved in secretion processes. S100A6 positivity was shown in rabbit pancreatic islets cells using immunohistochemistry and S100A6 exposure of permeabilized islets was shown to stimulate insulin release [300,301]. High levels of S100A6 has been shown in placenta and decidua (see above), and exposure of cultured trophoblast cells with S100A6 resulted in increased secretion of mouse placental lactogen II [302,303]. A role of S100A6 in secretion is also supported by in situ hybridization data showing elevated S100A6 mRNA level in mouse tissues involved in exocytosis [304].

Extracellular functions have been shown for several different S100 proteins and S100A6 has been detected in the medium of cultured breast cancer [305] and glioblastoma cell lines [306]. An extracellular function of S100A6 was further suggested as S100A6 was shown to interact *in vitro* with the receptor of advanced glycation end products (RAGE) as has been shown for several other S100 proteins [139,307,308]. Treatment of neuroblastoma cell lines with high concentrations of recombinant S100A6 resulted in increased intracellular ROS levels and apoptosis in a RAGE dependent manner [306]. Implication of S100A6 in the regulation of apoptosis has also been suggested as S100A6 was shown to enhance the sensitivity to apoptosis induced by a calcium ionophore in hepatocellular carcinoma cell lines [309]. The mechanism for apoptosis induction by S100A6 was suggested to be through modulation of transcriptional regulation of caspase-3. In breast cancer cell lines an additional link between S100A6 and caspases has been suggested as Trail and etoposide treatment of cells resulted in a caspase dependent decrease in S100A6 protein level [310]. In contrast increased expression of S100A6 has been shown in gastric cancer cell lines in response to treatment with the DNA damaging drugs doxorubicin or 5-fluorouracil [210].

2 THE PRESENT STUDY

2.1 AIMS

The general aim of this thesis was to use omics methods to find novel therapy related changes in the cellular proteome. The aim was further to investigate the potential of affected proteins as biomarkers for prediction of prognosis and response to therapy, but also as novel targets of cancer therapy. In addition the impact of p53 on DNA damage induced stress signaling was investigated. The tumor suppressor p53 is mutated in approximately 50% of human cancers and most likely its functions are impaired through other mechanisms in a large portion of remaining tumors, underlining the importance of this protein in carcinogenesis. Intense research since the discovery of p53 30 years ago have resulted in vast knowledge about the regulation of p53 and its involvement in various cellular processes. However, this research has also highlighted the complexity surrounding p53 and many questions remain to be answered. One aim of the research presented in this thesis was to further elucidate the cellular functions of p53 and processes related to this tumor suppressor. When S100A6 was revealed as part of the cellular response to DNA damaging treatment (paper II), our aim was to pursue this discovery and elucidate the previously unknown functions of this protein.

The specific aims were the following:

To investigate p53 dependent differences in cellular sensitivity to DNA damaging agents and p53 dependent differences in DSB repair.

To identify novel proteins involved in p53 dependent signaling in response to DNA damaging treatment.

To investigate the role of S100A6 in the cellular response to DNA damaging treatment.

To investigate the function of S100A6 in normal physiology and in carcinogenesis.

To evaluate the expression of S100A6, S100A4 and p53 in NSCLC and their possible prognostic value in stage I NSCLC.

2.2 MATERIAL AND METHODS

Materials and methods used in **papers I-IV** are described in detail in each paper. A brief description and comments are presented below.

2.2.1 Cells and treatments

2.2.1.1 Cell lines

All cell lines used in **papers I-IV** are of human origin and established from cancerous tissue. Cell lines were cultured in humidified air in incubators under controlled conditions with a temperature of 37°C and at 5% CO_2. All culture media were supplemented with 10% heat inactivated fetal bovine serum, 100 IU/ml penicillin and 100µg/ml streptomycin. The human colon cancer cell line HCT116 and its isogenic subcell line HCT116 p53-/- [311] (**papers I and II**), kindly provided by Professor Bert Vogelstein, were cultured in McCoy´s 5A medium. Lung cancer cell lines U1810 (NSCLC), H69 (SCLC), H23 (NSCLC) (used in **paper II**) and A549 (NSCLC) (used in **papers II-IV**) were cultured in Dulbecco's modified Eagle medium. Cells, cultured in exponential phase, were trypzinated and re-seeded 2-3 times weekly. Viability was routinely monitored by exclusion of trypan blue. All cell lines were tested negative for mycoplasma. All treatments and measurements were performed on sub-confluent cell cultures.

2.2.1.2 Irradiation

All irradiations (γ-rays) were performed using a ^{60}Co (cobolt) source at a dose rate of 1.5 Gy/min (**papers I, II and IV**). Cells were seeded 24h before exposure to ionizing radiation after which cells were returned to the incubator. The doses used for irradiation ranged from 8-12 Gy as specified in individual papers. All control cells in irradiation experiments were mock treated, i.e. they were handled in parallel with irradiated cells except they were not exposed to IR.

2.2.1.3 Cytotoxic drugs

A panel of clinically used cytotoxic drugs was used in **paper I** for measurements of p53s impact on cellular sensitivity to DNA damaging treatment. The drugs used were Doxorubicin (topoisomerase II inhibitor/intercalating agent), Etoposide (topoisomerase II inhibitor), Cisplatin (DNA-crosslinker), Mitomycin (DNA alkylator), Topotecan (topoisomerase I inhibitor) and Fluorouracil (antimetabolite). Five different doses were used for each drug, ranging from 10 ng/ml to 100 µg/ml, and sensitivity was measured using FMCA (see below). In **paper IV** Doxorubicin was used to assess the impact of this drug on S100A6 protein level.

2.2.2 Quantification of specific biomolecules

2.2.2.1 Western blot

Western blotting was routinely used to measure protein abundance in **papers I, II and IV**. Cells cultured as described above were washed once in phosphate buffered saline (PBS) and harvested using trypsin. After trypsination, cells were washed once in culture medium, twice in PBS and snap frozen in liquid nitrogen. Soluble proteins were extracted using repeated freeze-thaw cycles in a pH 7.5 buffer containing the mild

detergent Triton X-100, CHAPS and a cocktail of protease inhibitors. After centrifugation the total protein content in the resulting cell lysate was measured spectrophotometrically using a protein assay. A normalized amount of protein (typically 50 μg) was then size resolved using gel electrophoresis, and blotted onto a nitrocellulose membrane using tank blot equipment. Remaining proteins in the gel were visualized using comassie staining to control for errors e.g. uneven loading or transfer. The membrane was blocked in milk and incubated over night at 4°C with a primary antibody directed against the protein of interest. The membrane was then washed repeatedly and incubated 1 hour at room temperature in horseradish peroxidase coupled secondary antibody, recognizing the primary antibody. After additional washing the enzymatic activity of bound secondary antibody was used to activate a chemoluminiscence substrate and the resulting light emission was captured on film. The relative intensity of western blot bands was then used for comparisons of protein abundance and visualization in individual papers. For normalization purposes a control antibody was used to detect the protein level of a housekeeping protein showing stable expression (anti-Tubulin or anti-Fodrin). In **paper IV** the band intensity was measured densitometrically using software (Quantity One, BioRad, USA) and reported as a mean of three independent experiments.

2.2.2.2 Quantitative real-time PCR

Quantitative real-time PCR was used in **paper IV** for quantification of mRNA expression of S100A6, p53, IκBα, β-catenin and NFκB2. Polymerase chain reaction (PCR) is a method used for quantification of mRNA through enzymatic amplification of a specific sequence of DNA. Cells were cultured as described above and total cellular mRNA was isolated using RNeasy kit. The next step is reverse transcription where mRNA is translated into cDNA by the enzyme reverse transcriptase. Enzymatic amplification of the sequence of interest is then achieved by using specific oligonucleotide primers for guidance of the DNA polymerase enzyme. The amplification is driven by a series of repeated temperature cycles (typically 25-35) consisting of three steps; Denaturation (95°C) where double stranded DNA dissociates through disruption of hydrogen bonds, Annealing (58°C) during which the primers base pair with the template DNA and Elongation (72°C) when the DNA polymerase synthesize a new DNA strand. In quantitative real-time PCR the progression of the amplification is measured after each completed cycle through the use of a specific dye, SYBR green. This dye recognizes and binds to double stranded DNA and when bound, SYBR green emits light upon excitation. The number of cycles needed to overcome a preset threshold for the light signal is dependent of the amount of mRNA in the original sample and can therefore be used for relative comparison. A housekeeping mRNA (β-actin) was also measured for normalization purposes.

2.2.3 Measurements of sensitivity to DNA damaging agents

2.2.3.1 Clonogenic survival assay

Clonogenic survival assay is used to measure the capacity of cells to proliferate and form new clones. It is typically used to determine the fraction of cells that not only survive a specific treatment, but also continue to divide. This assay was used in **paper I** to determine the p53 dependency in radiosensitivity. A specific number of cells were

plated in cell culture dishes, allowed to adhere to the surface and then irradiated with different doses (1-4 Gy). After 10 days cells were washed and fixed and colonies containing at least 50 cells were counted after staining with Giemsa (DNA stain). The surviving fraction (SF) is then calculated as the ratio between the number of colonies in irradiated cells and the number of colonies in untreated cells.

2.2.3.2 Fluorometric microculture cytotoxicity assay (FMCA)

For high throughput purposes short term cytotoxicity assays are preferred over the more time consuming and laborious clonogenic survival assay. In **paper I** such an assay, FMCA, was used to estimate p53s impact on cellular sensitivity to DNA damaging drugs. FMCA is based on the measurement of fluorescence generated by hydrolysis of FDA (fluorescein diacetate) to fluorescent fluorescein by cells with intact plasma membrane. The assay was run in 384 well plates and 72 h after treatment with DNA damaging drugs the fraction of surviving cells were calculated through comparing the fluorescence signal in treated cells to the signal in untreated cells. Note that this assay does not determine the ability of cells to divide and form new colonies, but only the presence of intact cells.

2.2.4 DNA repair assay

In **paper I** the ability of cells to rejoin DNA double strand breaks (DSB) induced by high dose irradiation (40Gy from a ^{137}Cs (cesium) source) was assayed as a measurement of DSB repair. ^{14}C labeled irradiated cells, given a limited time to repair DSBs, were trypsinated, mixed with agarose and molded into plugs. Cells inside the plugs were then lysed to prepare naked chromosomal DNA and the plugs were loaded into an agarose gel. DNA was then size resolved using pulsed-field gel electrophoresis (PFGE). DNA fragments smaller than 5.7 Mbp were quantified by liquid scintillation measuring the amount of ^{14}C, and used as an estimation of remaining DSB. Note that this assay does not measure correct DNA repair but only the remaining amount of small DNA fragments.

2.2.5 Sub cellular localization of proteins

In **papers I and II** immuno fluorescence microscopy was used to study the subcellular localization of proteins. Cells were fixed on glass slides using formaldehyde and permeabilized using the detergent Triton X-100. The cells were then incubated with a specific antibody recognizing the protein of interest, washed and subsequently incubated with a fluorophore conjugated secondary antibody recognizing the primary antibody. The specific fluorescence of the fluorophore was then detected using a fluorescence microscope to determine the subcellular localization of the protein of interest. DAPI DNA-staining was used in all fluorescence experiments to visualize nuclei.

2.2.6 RNA interference

RNA interference, used in **papers II and IV,** is a method used to reduce the cellular level of a specific mRNA sequence. This is done through the introduction of a short sequence (21-23 nucleotides) of double stranded RNA known as short interfering RNA or siRNA. The siRNA sequence matches the sequence of the target mRNA in the cell.

siRNA is delivered into the cell either through the exposure of cells to chemically synthesized siRNAs as in **paper II**, or using an expression system that produce short hairpin RNA (shRNA) that is cleaved by the cellular machinery into siRNA (used in **paper IV**). The siRNAs is then incorporated into the cellular RNA-induced silencing complex (RISC). RISC then processes the siRNA and binds to the target mRNA complementary to the siRNA resulting in cleavage of the mRNA and thereby silencing of gene expression.

2.2.7 Immunoprecipitation

Immunoprecipitation or immunocapture is a method used to identify protein interactions used in **paper IV**. The principle is that a specific antibody is coupled to a support (e.g. agarose, beads or column), either covalently, or through interaction between the antibody and other molecules coupled to the support e.g. protein G or A. A protein mixture, typically a cell lysate, is then exposed to the support coupled antibody. The antibody will then bind to its target protein, retaining this protein and interacting proteins to the support. After washing, the target protein and interacting proteins are eluted (immunoprecipitate). The immunoprecipitate can then be analysed for the presence of specific proteins using western blot or used for discovery of novel interactions via mass spectrometry based peptide sequencing. In all immunoprecipitation experiments relevant control antibodies were included to distinguish specific interactions (i.e. with the target protein) from unspecific interactions (i.e. with the antibody or the support).

2.2.8 Proteomics methods

It is estimated that from the 20-25000 genes present in the human genome more than 500000 different variants and isoforms of proteins are derived. The increased complexity at the protein level is a result of processes such as alternative splicing, post translational protein modifications and proteolytic processing. The proteome is the protein content of a specific biological system at a specific time. Proteomics are methods to study the proteome. Since we don't have the methods to measure all proteins in a complex sample at the same time, today's proteomics methods sample a fraction of for example the cellular proteome. At best proteomics methods approach coverage similar to what is seen in current microarray platforms [312]. The first step in most proteomics methods is to fractionate the proteins in a complex sample to simplify the detection and quantification. This can be achieved e.g. through electrophoresis, chromatography or isoelectric focusing. Proteins are then detected e.g. through staining methods or using a mass spectrometer. Mass spectrometry itself adds an additional separation step of proteins or peptides. Proteomics methods can be subdivided into top-down proteomics, where detection and quantification is performed at the protein level, and bottom-up proteomics, starting with digestion of the proteins into peptides used for detection, quantification and identification of proteins. The abundance of individual proteins can be estimated using staining or labelling techniques, or through label free quantification where protein abundance is estimated from mass spectrometry data. In many cases the complexity needs to be additionally reduced e.g. for detection of low abundant proteins, and for this purpose various additional prefractionation techniques has been developed.

2.2.8.1 SELDI-TOF-MS

SELDI-TOF-MS (surface enhanced laser desorption/ionization-time of flight-mass spectrometry) is a top-down (detection of intact proteins) mass spectrometry based proteomics method used in **paper II and III**. In this method aluminium based chips coated with chromatographic surfaces are used to enrich for a certain subset of the proteome, thereby reducing the complexity and increasing the possibility of detecting low abundant proteins. Chips with different surface coatings can then be used to analyze the same sample to increase the proteome coverage. Four different surfaces were used in **paper II**, H50 (hydrophobic interaction, used also in **paper III**), CM10 (cation exchange), Q10 (anion exchange) and IMAC Cu (metal (copper) ion binding). Cell lysates with normalized total protein concentration were incubated on spots on the SELDI ProteinChip array to allow for interaction between proteins and chip surfaces. After washing and drying, a matrix solution (sinapinic acid) was added to each spot for efficient ionization in the mass spectrometer. ProteinChip arrays were then loaded into the mass spectrometer inside which a laser is used to ionize the protein/matrix mixture. Ionized proteins are then accelerated through an electric potential, travel through the free flight tube and reach the detector. The time needed for each ionized protein to travel from the ProteinChip surface to the detector is proportional to the mass/charge ratio of the ionized protein. The output from the mass spectrometer is a mass spectrum showing a number of peaks spread out over a mass/charge range corresponding to proteins with specific mass/charge ratios. The intensity of each peak corresponds with the number of proteins reaching the detector at the same time i.e. having the same mass/charge. Relative quantification is given through the comparison of specific peaks intensity between different samples. Different proteins are more or less difficult to ionize, why comparison of different peaks (i.e. different proteins) in the same spectrum can not be used for relative quantification. In general larger proteins are more difficult to ionize presenting a limitation for top down proteomics with today's mass spectrometers. SELDI-TOF-MS will not give any direct information of the identity of the protein peaks. The protein of interest therefore has to be isolated and identified using additional steps. We used a two step chromatographic method consisting of size fractionation and reversed-phase fractionation. The protein of interest (S100A6) was located using screening of the chromatographic fractions with SELDI-TOF-MS. After the reversed-phase fractionation the protein was judged sufficiently pure for identification using LC-MS/MS.

2.2.8.2 LC-MS/MS

In **paper II** LC-MS/MS was used for identification of S100A6. The reversed phase fraction containing our protein of interest from SELDI experiments was digested using trypsin. The resulting peptides were then loaded into a CapLC-Q-TOF (Capillary liquid chromatography-quadrupole-time of flight). Briefly peptides were separated using chromatography, ionized through electrospray ionization and fragmented in the quadrupole. The peptide fragments are then accelerated through the flight tube and detected at the end of the tube. The resulting fragment spectrum is then used for database searches of matching peptides.

2.2.8.3 Fractionation

An alternative to online LC-MS/MS as described above is to do the peptide fractionation in separate steps before mass spectrometry. In **paper IV**, fractionation of peptides was done using nano-LC chromatography and the resulting fractions were spotted automatically onto MALDI target plates together with matrix solution (CHCA) using a spotting robot. In the siRNA experiment an additional fractionation step, peptide isoelectric focusing (IEF), was included before chromatography to increase the coverage. Immobilized pH gradient (IPG) strips (pH-gradient 3.7-4.9) were used to separate peptides based on their isoelectric point, strips were then cut, peptides were eluted and a subset of the IEF fractions were analysed using LC-MALDI-MS/MS. Only a subset of the peptides will focus in this pH-range, but in theory the majority of all proteins have at least one peptide in this pH-range (Eriksson et al. Proteomics 2008, in press). Therefore peptide IEF reduce the complexity of the sample allowing for better proteome coverage.

2.2.8.4 MALDI-MS/MS

MALDI-MS/MS (matrix assisted laser desorption/ionization) was used in **paper IV** for identification and relative quantification of peptides (bottom-up proteomics). The MALDI target plate with peptides fractionated and spotted together with matrix solution was loaded into a MALDI TOF/TOF (time of flight/time of flight) instrument. When operated in MS mode this instrument quantifies and determines mass/charge ratios of peptides resulting in a mass spectrum as in SELDI-TOF. In MS/MS mode peptides are separated in the flight tube, and a peptide with a specific mass/charge is selected and directed into a CID (collision induced dissociation) chamber where the peptide is fragmented as it collide with gas molecules. The mass/charge ratios of the resulting fragments are then determined by the time needed to reach the detector. The fragment spectrum is subsequently used to determine the aminoacid sequence of the peptide with the aid of database information. Databases are then further used to search for proteins containing peptides with matching aminoacid sequences.

2.2.8.5 iTRAQ

iTRAQ (isobaric tag for relative and absolute quantification) are tags used for mass spectrometry based quantification of proteins or peptides. Isobaric tags consist of a reporter group ranging from 114-117 Da (4-plex version) and a balancing group making the tags identical in mass and chemistry. In **paper IV** a 4-plex version of iTRAQ labels were used in both proteomics experiments (Figure 6). Soluable proteins or immunoprecipitates were individually digested with trypsin and labelled with four different isobaric tags (iTRAQ labels). Labelled peptides were then pooled, fractionated using HPLC (nano-LC) alone or in combination with peptide IEF and spotted onto a MALDI target using a spotting robot. MALDI-MS/MS was then used to identify the proteins by peptide sequencing (1) and the relative quantity of the peptides in the four different samples was estimated by relative peak intensities of the four different reporter ions (2).

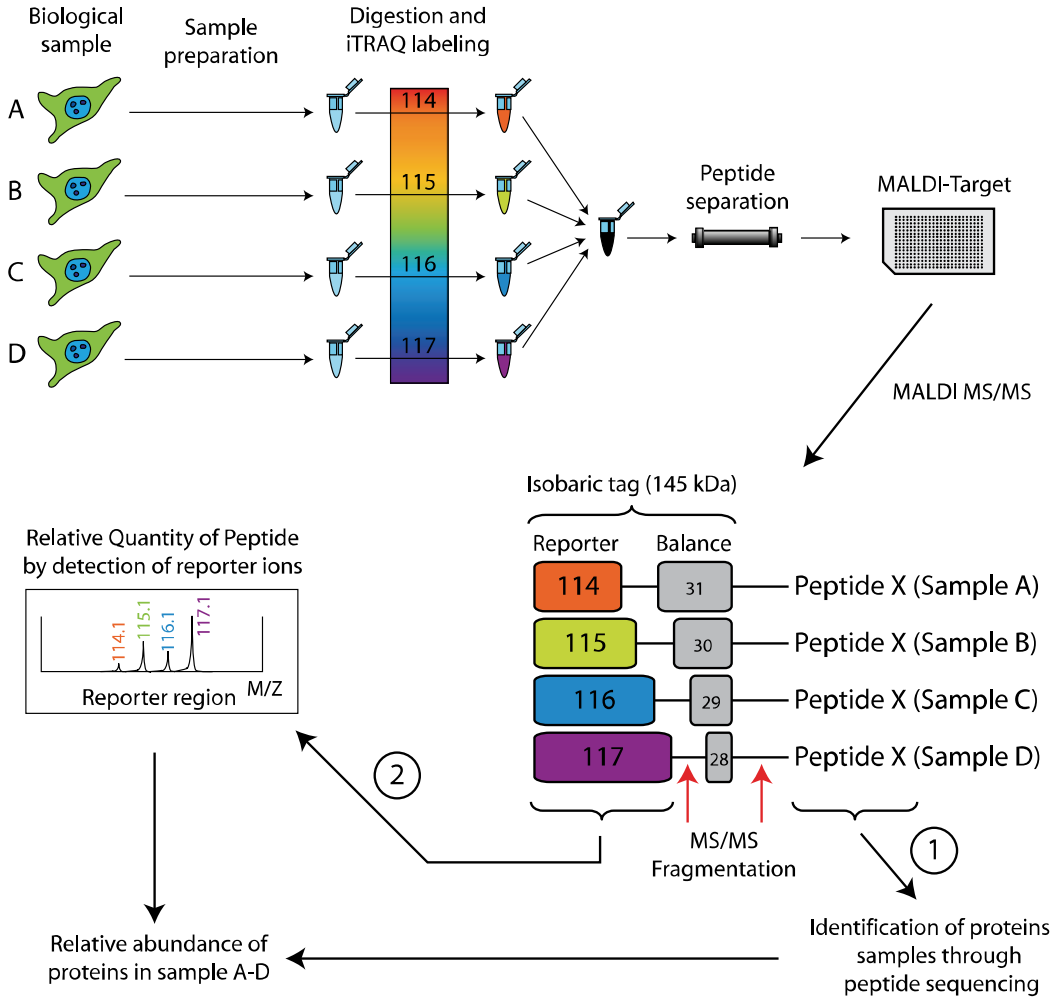

Figure 6. iTRAQ labelling for relative quantification of peptides and proteins. Samples were individually digested and labelled with four different isobaric tags. The labelled peptides were then pooled and separated using peptide IEF and/or nano-LC and automatically spotted on a MALDI target plate. MALDI-MS/MS was then used to identify the proteins in the sample through peptide sequencing (1), and the relative quantity of the peptides was estimated by relative peak intensities of the four different reporter ions (2).

2.2.9 Tissue microarray

2.2.9.1 Biobank material

In **paper III**, two separate patient cohorts with early stage non-small-cell lung cancer (NSCLC) were included. The samples in these cohorts were taken from biobanks at Karolinska University Hospital and the study was approved by the Institutional Review Boards at Karolinska Institutet and at Stockholm's Count Council. Follow-up data were obtained from the Swedish Cancer registry and from single patient files. In cohort I tumor cell suspensions from tumors of patients receiving surgery with a curative intent at Karolinska Hospital Solna between 1980 and 1992 was used for SELDI-TOF-MS

analysis. In cohort II formalin-fixed and paraffin-embedded tumor tissue from patients with stage I NSCLC receiving surgery between 1987 and 1992 was used for construction of Tissue microarrays.

2.2.9.2 Tissue microarray

Tissue microarray (TMA) is a method used for the simultaneous measurement of specific proteins in a large number of tissue samples using immunohistochemistry (IHC). A hollow needle is used to remove tissue cores from paraffin embedded tissue samples. Multiple cores from different samples are then inserted into a recipient block of paraffin in a precisely spaced, array pattern. Sections from the recipient block is then cut and adhered to microscope slides (TMA slides). For IHC, the TMA slides were then deparaffinized, rehydrated, washed, blocked and incubated with primary antibody directed against the protein of interest. After washing the TMA slide was incubated with a biotinylated secondary antibody directed against the primary antibody followed by washing and incubation with streptavidin coupled peroxidase reagent. 3.3-diaminobenzidin was then used for detection of peroxidase activity and visualization of protein abundance and localisation. Nuclear counterstaining was performed using Mayer's hematoxylin. Evaluation of IHC staining and scoring was done by an experienced pathologist in concordance with previously published scoring systems for each protein.

2.3 RESULTS AND DISCUSSION

2.3.1 Paper I

The entrance point to the studies resulting in **paper I** was previous work on the functions of Rad51 [313] and reports of p53 dependent regulation of Rad51 [83,314,315]. In order to further study the role of p53 in the cellular response to DNA damaging agents we used the p53 wt colon cancer cell line HCT116 and its isogenic p53-/- sub cell line [311]. Although without detecting p53 dependent differences in cellular sensitivity to DNA damaging drugs or irradiation, we were able to show differences in abundance and localization of Rad51 in response to irradiation. Rad51 foci formation was indistinguishable between p53 wt and -/- cells after exposure to ionizing radiation (IR), but resolution of foci was seen only in wt cells. Resolution of foci also correlated in time with the development of larger Rad51 formations residing in nucleoli and a decrease in Rad51 protein level.

Based on these findings we suggested that p53 was involved in disruption of Rad51 nucleoprotein filaments after completion of DNA repair. Other situations when a shutdown of HR should be beneficial is in response to excessive recombination to prevent genomic instability [316], or during the switch from DNA repair to apoptosis [317]. Using real time measurements of nucleation, growth and dissociation of Rad51-DNA nucleoprotein filaments it has recently been shown that Rad51 depolymerizes slowly from dsDNA, highlighting the importance of partner proteins to facilitate Rad51 depolymerization [318]. p53s interaction with Rad51 presents a possibility of p53 to regulate Rad51 polymerization [83]. In addition to p53s role in transactivation independent suppression of HR, p53 has also been shown to inhibit Rad51 transcription [319], presenting an additional level of regulation.

The decrease in Rad51 protein level detected in p53 wt cells post irradiation could be an effect of p53 dependent suppression of Rad51 transcription, increased Rad51 degradation or both. Our data indicated that nucleolar localization of Rad51 is coupled to Rad51 degradation. Rad51 has been suggested as a target of sumoylation [320,321] and ubiquitination [322], both examples of modifications regulating protein localization and degradation. The exact mechanism of Rad51 degradation and the potential role of nucleoli remain to be discovered.

We did not detect any differences in DNA repair as measured by PFGE, indicating that whatever functions p53 may have in regulation of HR or other repair pathways, p53 has no marked impact on the rejoining of DNA double strand breaks (DSBs) in response to ionizing radiation. The reason for this could be that the alternative route to DSB repair, NHEJ, is dominating in repair of IR induced DSBs [323], and that NHEJ is independent on p53 status.

Studying the literature, no clear correlation can be made between p53 status and cellular sensitivity to DNA damaging agents as shown for IR [324,325] and DNA damaging drugs [326]. This is somewhat surprising given the central role of p53 in regulation of apoptosis, cell cycle and DNA repair. One possible explanation for this is redundancy in cellular signaling, where other proteins (e.g. p73 [327]) cover up for loss of p53 in induction of apoptosis [328]. Another explanation could be that p53 negative cells die from mitotic catastrophe due to defective cell cycle checkpoints instead of apoptosis [329,330].

2.3.2 Paper II

The evolution of our labs and research focus towards proteomics opened new and exciting possibilities of unbiased exploratory research. Once again we turned to the cell line pair used in **paper I**, this time to search for novel p53 dependent changes in the cellular proteome in response to IR. HCT116 p53 wt and -/- cells were irradiated and sampled at several timepoints after treatment, and the resulting cell lysates were profiled using top-down proteomics. Using mass spectrometry based proteomics (SELDI-TOF-MS) we were able to monitor up to 500 protein peaks in the sub 30 kDa mass area. The most striking change in protein profile was an irradiation and p53 dependent increase of a 10.2 kDa protein. Chromatographic isolation of the protein, followed by trypsination and LC-MS/MS based peptide sequencing resulted in the identification of the protein as S100A6. A detailed analysis of the proteomics data revealed that in addition to S100A6 upregulation in response to irradiation, the post translational modification pattern of S100A6 was altered in a p53 dependent manner. In untreated p53 wt cells unmodified and glutathionylated S100A6 were the predominating forms, whereas in irradiated cells glutathionylated S100A6 decreased and cysteinylated S100A6 increased. This change in modification pattern was not seen in p53-/- cells. The p53 dependence in S100A6 expression and modification pattern in response to IR was confirmed using a lung cancer (LC) cell line panel. In the LC cell lines we discovered that another S100 protein, S100A4, also was expressed in a p53 dependent manner, and that S100A4 showed similar changes as S100A6 in post translational modification pattern post irradiation. The p53 dependency in expression of S100A6 and S100A4 was further confirmed through p53 silencing in the p53 wt lung cancer cell line A549. Since earlier reports described calcium dependent translocation of S100A6 [331,332], we decided to study the effect of irradiation on S100A6 subcellular localization. Using immunofluorescence microscopy we could show that S100A6 translocated from the nucleus to the cytoplasm in A549 cells post irradiation, and that S100A6 colocalized in the cytoplasm with tropomyosin, a component of the cytoskeleton previously shown to interact with S100A6 [288].

Both S100A6 and S100A4 seem to be connected with development of cancer as both proteins have been shown upregulated in a number of different malignancies (discussed above). While the functions of S100A4 have been suggested to be regulation of p53 and cellular processes coupled to metastasis [333], the biological functions of S100A6 have remained largely unknown. Our studies reported in **paper II** were the first ever to implicate S100A6 in the cellular response to DNA damaging agents. This finding in it self did not assign S100A6 a specific biological function as multiple major cellular systems are affected by irradiation such as proliferation, DNA repair and apoptosis. The connection with p53 suggested that S100A6 may be involved in processes related to apoptosis, however the timing of the upregulation indicated that S100A6 was at least not part of initiation of apoptosis. Increase in S100A6 protein level as well as S100A6 subcellular localization and post-translational modification in response to IR were all late events most pronounced at the 48h timepoint and later. Also no evidence exist of p53 being a transcription factor for S100A6 indicating the involvement of other transcription factors which in turn are affected by p53.

It is generally accepted that the functions of S100 proteins are dependent on interactions with other proteins as S100 proteins themselves have no catalytic activities. A literature search on S100A6 interacting proteins and our findings of cytoplasmic

S100A6 localisation post irradiation directed our interest to tropomyosin, a component of the cytoskeleton involved in the formation of stress fibers and cytoskeleton rearrangements. As we were able to show colocalisation of S100A6 and tropomyosin in irradiated cells we suggested that S100A6 is involved in regulation of actin-tropomyosin dependent cytoskeleton organization.

The interaction between S100 proteins and their targets are in most cases calcium dependent [123]. Calcium binding to EF-hands on S100 proteins induces conformational changes resulting in the exposure of hydrophobic binding surfaces suggested to be the primary interaction sites of other proteins. The activities of S100 proteins are also believed to be dependent of homo and heterodimerization between different S100 family members. *In vitro* studies have suggested an additional level of regulation as the calcium affinity of S100 proteins was shown affected by redox modifications of conserved cysteine residues located in N- or C- terminal extensions of S100 proteins [134,266]. Our findings of cysteinylation and glutathionylation of S100A6 and S100A4, together with the radiation dependency in the pattern of these post translational modifications, indicate that redox modification is a relevant regulatory mechanism for S100 proteins *in vivo*. Treatment of cells with DNA damaging agents results in release of calcium from intracellular depots, changes in redox milieu and alteration of cytoplasmic glutathione and cystein content. These variations in the intracellular environment provide means for regulation of interaction between S100 proteins and their targets.

2.3.3 Paper III

As we in **paper II** detected large differences in S100A6 and S100A4 protein levels between lung cancer cell lines, and also a p53 dependency in the expression pattern of S100A6 and S100A4 we set out to explore the situation in clinical non small cell lung cancer (NSCLC) and reported the results in **paper III**. The expression and prognostic role of S100A6 in NSCLC have previously not been assessed. The study is based primarily on two separate cohorts. In cohort 1, a biobank material consisting of enriched tumor cell lysates from 39 patients with resected NSCLC was analyzed using a mass spectrometry based proteomics method (SELDI-TOF-MS) for detection of S100A6. Cohort 2, containing 103 surgically resected stage I NSCLC cases and 14 normal lung parenchyma specimens arrayed in tissue microarrays (TMA), was analyzed using immunohistochemistry for detection of S100A6, S100A4 and p53 expression.

Using SELDI-TOF-MS we were able to detect the same S100A6 post translational modifications (i.e. cysteinylation and glutathionylation) in clinical material that were detected in cell lines in **paper II**. As these modifications are redox sensitive and therefore possibly affected by e.g. long time storage, we assessed the presence of modifications also in freshly prepared NSCLC samples confirming the pattern in cohort 1. We were unable to correlate the S100A6 modification pattern to tumor type or patient survival, and concluded that cysteinylated S100A6 was the predominating form detected in cohort 1. High S100A6 peak intensity (cysteinylated form) was associated with longer median survival time (35 vs 18 months for high and low peak intensity, respectively), but without reaching statistical significance. We were also able to detect S100A6 in plasma and pleural effusion samples for the first time indicating a potential extracellular role of S100A6.

As cohort 1 indicated a potential correlation between S100A6 expression and patient survival, we decided to investigate this further in a larger cohort. In addition **paper II** showed p53 dependence in S100A6 expression, and suggested that S100A4 expression was also correlated with S100A6 levels. Therefore the expression of both p53 and S100A4 was assessed as well. TMA slides with duplicate samples from each tumor were stained with appropriate antibodies and scored by an experienced pathologist, blind to clinical data. Each antibody was used to stain two TMA slides, and each case was scored by combining the evaluation from the resulting 4 samples. On the basis of previous reports, immunoreactivity was scored as either positive or negative for S100A6 S100A4 and p53, being positive in 26/103, 22/102 and 31/103 cases respectively. S100A6 positivity correlated directly with S100A4 positivity and inversely with p53 positivity. All normal specimens were negative in staining of S100A6 and S100A4. The inverse correlation with p53 is in agreement with the results in **paper II** showing a correlation between wt p53 and S100A6, as immunohistochemical staining of p53 is a sign of a missense mutation leading to accumulation of p53, while wt p53 expression is typically below the detection limit [334]. In the overall population a trend was detected (p=0.07) for longer survival time in S100A6 positive cases than in negative cases with a median survival time (MST) of 92.6 and 61.5 months respectively. This trend reached statistic significance (p=0.02) when selecting only p53 negative cases (72 pts) showing MST for S100A6 positive and negative cases of 112 and 61.5 months respectively. A significant difference in MST was also detected when examining prognostic impact of S100A6 in S100A4 positive

cases. Double positive cases showed improved survival compared to cases positive for only S100A4 (MST 132 and 63 months respectively). Expression and prognostic value of S100A6 have been studied in other tumor forms with conflicting results. Most reports show increased expression of S100A6 in cancer cells compared to normal, but in some cases increased expression of S100A6 correlated with poor prognosis (e.g. pancreatic cancer) while in others correlating with improved survival (osteosarcoma). These differences could be due to tissue specific functions of S100A6 or an effect of stage distribution and sample size differences between studies. Our results also indicate that expression of other proteins affects the prognostic impact of S100A6. As discussed in **paper II**, and will be discussed below (**paper IV**), S100A6 is regulated by, and is involved in regulation of several different cancer related signaling pathways. To fully evaluate the prognostic value of S100A6 in subgroups of stage I NSCLC a larger cohort should be used.

S100A4 positivity has been correlated with bad prognosis, tumor stage, invasion and development of metastasis in a number of different tumor forms. We could not demonstrate a prognostic value of S100A4 expression in stage I NSCLC. Earlier reports have shown a correlation between S100A4 expression and poor survival in NSCLC [167,179]. However, these studies consisted mainly of adenocarcinomas and did not examine stage I tumors exclusively, suggesting that S100A4 is involved in the metastatic phenotype at later stages or histology specifically.

p53 expression could also not be correlated with prognosis in cohort 2, supporting some previous reports [335,336], while not supporting others [337,338]. Studying p53 in cancer with immunohistochemistry has its limitations. p53 is mutated in more than 50% of NSCLC and in many, but not all cases p53 mutations results in nuclear accumulation of p53 that can be detected with immunohistochemistry. The subgroup with negative p53 staining is therefore a heterogeneous group consisting of both p53 wt and p53 mutated cases. To fully validate the impact of wt p53 on survival, mutation analysis should be done.

2.3.4 Paper IV

After our findings in **papers II and III**, we were more curious then ever to elucidate the biological functions of S100A6 in normal physiology, stress response and carcinogenesis. As discussed earlier, the functions of S100 proteins are mainly thought to be through interaction and regulation of other proteins since S100 proteins have no shown catalytic activity of their own. We therefore decided to search for novel S100A6 interacting proteins using our well suited in-house proteomics methods and A549 lung cancer cells. S100A6 interacting proteins were retrieved using an immunoprecipitation protocol where the antibody was covalently coupled to the gel support allowing elution of bound proteins without antibody (Ab) contamination. Eluted immunoprecipitates (α-control Ab (untreated), α-control Ab (IR), α-S100A6 Ab (untreated) and α-S100A6 Ab (IR)) were then digested and labeled using four different isobaric tags. The labeled peptides were pooled, fractionated using HPLC and automatically spotted onto a MALDI-target. MALDI-MS/MS was then used to identify the proteins in the precipitate by peptide sequencing and the relative quantity of the peptide in the four different precipitates was given by relative quantification of the four different reporter ions. Out of 177 identified proteins in the precipitates, where the vast majority were not S100A6 specific, ubiquilin-1 was the highest ranking S100A6 interacting protein. The interaction between S100A6 and ubiquilin-1 was validated using western blotting and additional immunoprecipitation with α-ubiquilin antibody.

Ubiquilins (1 and 2, also called PLIC-1 and -2) are proteins suggested to be involved in regulation of proteasomal degradation of ubiquitinated proteins. Initially it was suggested that ubiquilin acted as a shuttle to transport ubiquitinated proteins to proteasomes for destruction, as ubiquilin interacts with both polyubiquitinated proteins and subunits of the proteasome [339,340]. Surprisingly it was shown that ubiquilin overexpression resulted in stabilization of ubiquitinated proteins normally degraded by proteasomes (p53 and IκBα). It has also been shown that the Kaposi´s sarcoma associated herpesvirus (KSHV) K7 protein inhibits apoptosis and stimulates proliferation through an interaction with ubiquilin-1, resulting in disruption of the interaction between ubiquilin-1 and polyubiquitinated proteins and ultimately in rapid degradation of p53 and IκBα [341]. An alternative to the shuttle theory, where ubiquilin-1 promotes degradation of ubiquitinated proteins, is therefore another theory where ubiquilin-1 protects ubiquitinated proteins from degradation through interaction with the polyubiquitin tail, possibly inhibiting a direct interaction between polyubiquitinated proteins and proteasomes.

To test the effect of S100A6 expression on degradation of proteins known to be affected by ubiquilin we constructed stable S100A6 siRNA expressing A549 cells. Measuring both mRNA and protein levels of p53 and IκBα we were able to conclude that S100A6 silencing resulted in accumulation of both p53 and IκBα protein without notably altering mRNA expression. Our results indicate that S100A6 can inhibit the role of ubiquilin-1 in stabilization of p53 and IκBα in analogy to KSHV K7.

IκBα is an inhibitor of NFκB transcription factors, acting through interaction with and sequestration of NFκB transcription factors in the cytoplasm. In response to a wide variety of signals IκBα is phosphorylated and ubiquitinated resulting in its degradation and the release of NFκB transcription factors that enters the nucleus and promote transcription of target genes. General effects of NFκB transcription factors are inhibition of apoptosis (through transcription of e.g. Bcl-2 and IAPs) and stimulation of

proliferation (through transcription of Myc and Cyclin D). NFκBα has also been shown to promote transcription of S100A6 [277]. Studying the time course pattern of protein expression in A549 cells post irradiation in **paper IV**, degradation of IκBα precedes S100A6 upregulation suggesting that NFκBα activation is the cause of S100A6 upregulation in response to IR. As S100A6 silencing resulted in stabilization of IκBα, our data further indicate that S100A6 could be part of a stimulatory feedback loop for NFκB signaling through promotion of IκBα degradation.

Our results in **papers II and III** show a p53 dependency in the expression of S100A6. p53 is not a known transcription factor of S100A6, but it has been shown that p53 induces NFκBα activity [342,343] which in turn could explain the p53 dependent S100A6 expression. Silencing of S100A6 in **paper IV** resulted in stabilisation of p53. In addition, upregulation of S100A6 in A549 cells post irradiation preceded a decrease in p53 protein level. Our data indicate that in response to p53 upregulation S100A6 is upregulated (through activation of NFκBα) and promotes degradation of p53. S100A6 seems therefore to be part of a negative feedback loop for p53, although not as direct as in the case of ubiquitin ligases Mdm-2 and Cop-1. A negative feedback loop for p53 has earlier been described involving another S100 family member, namely S100B [252]. Downregulation of p53 signaling has been suggested an important part of a post stress recovery period at later timepoints post treatment with DNA damaging agents [66,344]. During this period a relief in proapoptotic and cell cycle arrest signaling allows cells with repaired DNA to re-enter the cell cycle. Both Mdm-2 and Cop-1 are strong candidates in such post stress recovery through driving p53 degradation, and our data suggest that S100A6 is an additional component through inhibition of ubiquilin dependent p53 stabilisation.

Earlier studies have suggested that S100A6 is involved in regulation of β-catenin degradation as S100A6 interacts with CacyBP/SIP, a component of the protein complex responsible for ubiquitination of β-catenin. Overexpression of CacyBP/SIP has been shown to promote β-catenin degradation [296], but the role of S100A6 has not earlier been described. Using our stable S100A6 siRNA cells we were able to conclude that S100A6 inhibits β-catenin degradation, possibly through inhibition of CacyBP/SIP. This presents an additional role of S100A6 in stimulation of proliferation through β-catenin stabilization.

To find additional proteins affected by S100A6 we performed a second proteomics experiment. This time we used A549 S100A6 siRNA and control cell lysates from irradiated and untreated cells. Digestion, labelling and pooling was performed as in the first experiment and in addition peptide isoelectric focusing was added as a first line fractionation step to increase the number of identified proteins. Over 2500 proteins were identified with relative quantification. Interestingly, the highest ranking S100A6 dependent change was an increase of NFκB2 p100 in S100A6 siRNA cells. NFκB2 p100 is activated through phosphorylation and ubiquitination dependent partial degradation in proteasomes, resulting in the active transcription factor NFκB2 p52. Measuring both mRNA and protein level of NFκB2 p100 we were able to conclude that S100A6 silencing resulted in stabilization of the full length transcriptionally inactive NFκB2 protein. These data indicate that in addition to stimulating NFκB signaling through promoting IκBα degradation, S100A6 stimulate this pathway through increasing the processing of NFκB2 p100 into NFκB2 p52. A proapoptotic transcription independent role of NFκB2 p100 through activation of caspase-8 has also been described [345], presenting another anti-apoptotic function of S100A6.

As described above our data suggests that S100A6 stimulates proliferation and inhibits apoptosis through regulation of degradation of key proteins in central cellular pathways. In concordance with this role of S100A6 we could also show that S100A6 silencing resulted in increased sensitivity to ionizing radiation. Although we did detect increased p21 in S100A6 siRNA cells compared to control cells, we did not detect differences in cell growth. Further studies should be performed including analysis of cell cycle distribution in response to S100A6 silencing in order to fully understand the impact of S100A6 on proliferation and cell cycle progression.

Our data reported in **paper IV** indicate that overexpression of S100A6 in cancer would be beneficial for tumor growth through stimulation of proliferation and inhibition of apoptosis. We also suggest that S100A6 is a potential target of cancer therapy, and that inhibition of the cellular functions of S100A6 could stimulate apoptosis and inhibit proliferation, thereby inhibiting tumor growth.

2.3.5 General conclusions

The general aim of this thesis was to use omics methods to find novel therapy related changes in the cellular proteome. The aim was further to investigate the potential of affected proteins as biomarkers for prediction of prognosis and response to therapy, and also as novel targets of cancer therapy. In addition, our aim was to identify p53 dependent effects of DNA damaging treatment. Both general effects, such as sensitivity to treatment and DNA repair, and specific effects, such as p53 dependent alteration of specific protein abundance were studied. S100A6 was identified as a protein upregulated in a p53 dependent manner post irradiation. The connection of S100A6 with cancer and the lack of knowledge on the cellular functions of S100A6 directed our research towards this protein. The main findings were the following:

- p53 is involved in the resolution of Rad51 foci 48h and later post exposure to IR. The resolution of foci coincided with a decrease in Rad51 protein level only detected in p53 wt cells. These findings support a role of p53 in negative regulation of homologous recombination.

- S100A6 is upregulated after treatment with DNA damaging agents such as IR and doxorubicin, and also by agents evoking oxidative stress (H_2O_2). IR also alters the post translational modification pattern of S100A6 and the subcellular localization. S100A6 upregulation, modification and translocation are late events, most prominent 48h after irradiation and later. S100A6 silencing results in increased IR induced cell death.

- S100A6 expression is p53 dependent. The protein level of S100A6 was higher in p53 wt HCT116 colon cancer cells compared to p53 -/- HCT116 cells. S100A6 level was also higher in A549 (p53 wt) lung cancer cells compared to lung cancer cell lines with mutated p53, and p53 silencing in A549 cells resulted in reduced S100A6 protein level. In stage I NSCLC S100A6 expression correlated with negative p53 staining, indicating wt p53.

- The expression of S100A6 also correlated with S100A4 expression in HCT116 wt/p53-/- cells, lung cancer cell lines and stage I NSCLC.

- S100A6 interacts with Ubiquilin-1, a protein involved in degradation of ubiquitinated proteins such as p53 and IκBα. S100A6 silencing resulted in reduced degradation of p53, IκBα and NFκB2/p100.

- S100A6 silencing increased the degradation of β-catenin.

- S100A6 is upregulated in stage I NSCLC and correlates with the differentiation grade. S100A6 is a positive prognostic factor in p53 negative and also in S100A4 positive stage I NSCLC.

In normal physiology S100A6 is expressed in proliferating cells as has been described earlier [253]. This expression is likely at least in part a consequence of NFκB activation in response to pro-growth signaling, as NFκB has been shown to promote transcription of

S100A6 [277]. Our data indicate that S100A6 act to further stimulate proliferation through increasing IκBα degradation and decreasing β-catenin degradation.

We have also shown that cellular exposure to DNA damaging agents such as IR and doxorubicin results in increased expression of S100A6. Based on the timing of this increased expression and our data showing anti apoptotic and proliferation stimulating activities of S100A6, we suggest that S100A6 is involved in post stress recovery. It has been suggested that a relief in apoptotic and cell cycle arrest signaling (post stress recovery) is important to allow cells with repaired DNA damage to re-enter the cell cycle and escape apoptosis [66,344].

S100A6 has been shown overexpressed in a number of different cancer types, and our data indicate that such overexpression is stimulating cancer progression through inhibition of apoptosis and stimulation of proliferation. In fact, high S100A6 expression has been correlated with poor prognosis in cancer types such as pancreatic cancer and melanoma [165,202]. However, we and others have shown opposite correlation where S100A6 expression is correlated with improved prognosis. This contradiction could be a result of tissue specific functions of S100A6, but it may also be due to the expression of other proteins. As an example we have shown that S100A6 expression correlates with the expression of wt p53, possibly masking specific effects of S100A6.

Our data also show a correlation between the expression of S100A6 and S100A4. Heterodimerization between different members of the S100 family has been shown in several papers [123], and such interactions may modulate the biological functions of the individual proteins. As an example it was shown that S100A1 through heterodimerization with S100A4 could inhibit S100A4 induced cellular motility *in vitro* and S100A4 induced formation of metastases *in vivo* [157]. If S100A6 and S100A4 forms heterodimers, and the consequences of such an interaction remain to be determined.

Finally, our data suggests that inhibition of the cellular functions of S100A6 through pharmacological intervention would sensitise cancer cells to apoptosis inducing treatment. It is also possible that cancers overexpressing S100A6 are dependent of the apoptosis protection and proliferation stimulation that is induced by S100A6. Thus our data indicate the potential of S100A6 as a novel target for cancer therapy.

3 ACKNOWLEDGEMENTS

This research was supported by grants from the Swedish Cancer Society, the Cancer Society in Stockholm, Stockholm County Council and the European Commission (6th FP CHEMORES-project and 7th FP APOSYS-project).

Karolinska Institutet, Department of Oncology and Pathology and Karolinska Biomics Center are acknowledged for providing a stimulating research platform.

I would like to thank the following persons for help, support, and for making my PhD period memorable and joyful:

First of all I would like to acknowledge my supervisor and friend Janne Lehtiö. His appearance in the group and as my supervisor improved the research in general and my projects in particular. By critically listening to my endless research theories he helped me sort out the good ones from the bad. In addition I have really enjoyed our work unrelated discussions both at "neuro-fiket" and elsewhere. I would especially like to thank him for allowing me to explore my own ideas.

Professor Rolf Lewensohn for introducing me into cancer research, for having grand visions and for always having faith in me, and Marianne Langéen for teaching me about cell culturing and for being patient with my lack of administrative capacity.

My initial supervisors Anders Nilsson and Jenny Flygare for laying the foundation of my research career, I hope you didn't leave because of me ☺.

Maria Pernemalm, my closest fellow PhD student and room mate, for always being cheerful, for being razor sharp in research discussions, and for being a wizard on all kinds of complicated machinery.
The proteomics PhD gang: Luigi De Petris (with family) for teaching me about clinical research, Italian Sunday lunch culture and for bringing me cheese, Hanna Eriksson for spreading sunshine in the labs and offices and Sara Ståhl for having a refreshingly prestige-free relation to research and all three of you for being good friends.
The proteomics Post doc crew: Johan Lengqvist for patiently explaining mass spectrometry related issues to me over and over again, and for making it all work in the labs. I look forward to digging into our new projects. Anders Nordström (with family) for meatballs, dinosaurs and discussions about post PhD decisions. I have high hopes for our future joint meatballsomics (or was it metabolomics) projects. Jenny Forshed for education in the statistics field, boating discussions and for sharing the knowledge of the amount of chaos produced by children.
All the other great people in the proteomics group and the other groups at KBC for contributing to the excellent atmosphere at KBC: Lina Hultin-Rosenberg, Birgitta Sundelin, Susanne Becker, Helena Bäckvall, Flavia Melotti, Per-Johan Jakobsson, Barbro Linderholm, Leif Stenke, Kristinas Viktorsson and her group, Serhiy Souchelnytskyi and his group (good luck with your thesis Nimesh) and many more.

My collaborators and coautors: Bo Stenerlöw, Sumeer Dhar, Rolf Larsson, Lena Kanter, Hirsh Koyi, Erik Vernet and Torbjörn Gräslund. It's been a pleasure working with you all and I'm looking forward to working with you again.

Jag vill speciellt tacka min fantastiska familj som alltid har stått bakom mig. Mamma och Martin, jag lovar att jag ska hålla en begriplig föreläsning för er som beskriver vad det är jag egentligen håller på med. Pappa och Karin, jag hoppas att du kan sova lite bättre igen snart pappa, när du inte längre behöver oroa dig för hur det ska gå med min disputation ☺.
Min kära syster Hanna och hennes familj Tobbe, Olle, Ebba och lille Viggo vill jag tacka för att ni alltid är så snälla och hjälpsamma. Tack Tobbe för att du hjälpte mig med bilderna.

Min extra familj, Jansson-Sjörs. Tack Kerstin och Rolf för att ni är så kärleksfulla och gästfria. Tack Hugo och Katrin, hoppas vi hinner träffas lite mer framöver. Tack Malin, Jörgen, Elsa, Alva och Maja. Nu har vi också årskort på Parken Zoo så det kommer hängas en hel del i E-tuna i sommar.

Familjen Sandahl, Johan, Lina, Signe och en till när som helst. Tack för allt roligt umgänge och för er vänskap. Hoppas allt går bra med tillökningen, jag ser fram emot att lära känna ytterligare en Sandahl.

Grabbgänget: Micke, Kalle, Gustav, Per, Stoffe och Christer. Tack för att ni är de bästa vänner man kan önska sig. Om det bara fanns tid skulle jag gärna ha grabbhelg 52 gånger per år. Tack också till era fantastiska familjer.

Rönnbygänget: Lina och Jon, Viktoria och Lars-Åke, Charlotta och Jon, Robert och Mirja, Staffan och Clara, Danne och Anna, Koppe och Sofie, Tompa och Linda. Tack för alla skärgårdshelger, middagar och fester. Ni är verkligen underbara.

Tack Olle och Mårten för att ni har delat apotekarutbildningen, forskarskolan och doktorandlivet med mig. Det har varit ett nöje. Jag vill speciellt tacka dig Olle för gemensamma resminnen, träningsprogram och fotbollsmatcher. Jag hoppas att du bestämmer dig för att flytta tillbaka hem till Solna till slut.

Mest av alla vill jag tacka min älskade hustru Johanna och våra fantastiska små flickor Ida och Amanda. Tack Johanna för att du alltid finns där för mig, för att du förstår mig och för att du täcker upp för mig när jag inte räcker till. Tack Ida och Amanda för att ni får mig att förstå vad som är viktigt i livet.

4 REFERENCES

1. Parkin, D. M., Bray, F., Ferlay, J. & Pisani, P. Global cancer statistics, 2002. *CA Cancer J Clin* **55**, 74-108 (2005).
2. Kinzler, K. W. & Vogelstein, B. Lessons from hereditary colorectal cancer. *Cell* **87**, 159-170 (1996).
3. Levine, A. J. p53, the cellular gatekeeper for growth and division. *Cell* **88**, 323-331 (1997).
4. Hanahan, D. & Weinberg, R. A. The hallmarks of cancer. *Cell* **100**, 57-70 (2000).
5. Krystal, G. W., Hines, S. J. & Organ, C. P. Autocrine growth of small cell lung cancer mediated by coexpression of c-kit and stem cell factor. *Cancer Res* **56**, 370-376 (1996).
6. Putnam, E. A. *et al.* Autocrine growth stimulation by transforming growth factor-alpha in human non-small cell lung cancer. *Surg Oncol* **1**, 49-60 (1992).
7. Macaulay, V. M. *et al.* Autocrine function for insulin-like growth factor I in human small cell lung cancer cell lines and fresh tumor cells. *Cancer Res* **50**, 2511-2517 (1990).
8. Salomon, D. S., Brandt, R., Ciardiello, F. & Normanno, N. Epidermal growth factor-related peptides and their receptors in human malignancies. *Crit Rev Oncol Hematol* **19**, 183-232 (1995).
9. Kurebayashi, J. Biological and clinical significance of HER2 overexpression in breast cancer. *Breast Cancer* **8**, 45-51 (2001).
10. Heimberger, A. B., Suki, D., Yang, D., Shi, W. & Aldape, K. The natural history of EGFR and EGFRvIII in glioblastoma patients. *J Transl Med* **3**, 38 (2005).
11. Schubbert, S., Shannon, K. & Bollag, G. Hyperactive Ras in developmental disorders and cancer. *Nat Rev Cancer* **7**, 295-308 (2007).
12. Malumbres, M. & Barbacid, M. To cycle or not to cycle: a critical decision in cancer. *Nat Rev Cancer* **1**, 222-231 (2001).
13. Oren, M. Decision making by p53: life, death and cancer. *Cell Death Differ* **10**, 431-442 (2003).
14. Taylor, R. C., Cullen, S. P. & Martin, S. J. Apoptosis: controlled demolition at the cellular level. *Nat Rev Mol Cell Biol* **9**, 231-241 (2008).
15. Eymin, B., Gazzeri, S., Brambilla, C. & Brambilla, E. Mdm2 overexpression and p14(ARF) inactivation are two mutually exclusive events in primary human lung tumors. *Oncogene* **21**, 2750-2761 (2002).
16. Momand, J., Jung, D., Wilczynski, S. & Niland, J. The MDM2 gene amplification database. *Nucleic Acids Res* **26**, 3453-3459 (1998).
17. Adams, J. M. & Cory, S. The Bcl-2 apoptotic switch in cancer development and therapy. *Oncogene* **26**, 1324-1337 (2007).
18. Youle, R. J. & Strasser, A. The BCL-2 protein family: opposing activities that mediate cell death. *Nat Rev Mol Cell Biol* **9**, 47-59 (2008).
19. Newbold, R. F. The significance of telomerase activation and cellular immortalization in human cancer. *Mutagenesis* **17**, 539-550 (2002).
20. Tonini, T., Rossi, F. & Claudio, P. P. Molecular basis of angiogenesis and cancer. *Oncogene* **22**, 6549-6556 (2003).
21. Yilmaz, M., Christofori, G. & Lehembre, F. Distinct mechanisms of tumor invasion and metastasis. *Trends Mol Med* **13**, 535-541 (2007).
22. Mintzer, D. The changing role of surgery in the diagnosis and treatment of cancer. *Am J Med* **106**, 81-89 (1999).
23. Bush, N. J. Advances in hormonal therapy for breast cancer. *Semin Oncol Nurs* **23**, 46-54 (2007).
24. Daskivich, T. J. & Oh, W. K. Recent progress in hormonal therapy for advanced prostate cancer. *Curr Opin Urol* **16**, 173-178 (2006).
25. Stagg, J., Johnstone, R. W. & Smyth, M. J. From cancer immunosurveillance to cancer immunotherapy. *Immunol Rev* **220**, 82-101 (2007).

26. Birkenhake, S. & Sauer, R. Historical essentials influencing the development of radiooncology in the past 100 years. *Experientia* **51**, 681-685 (1995).
27. Hall, E.J., in *Radiobiology for the radiologist*, edited by J.R. John (Lippinkott Williams & Wilkins, Philadelphia, 2000), pp. 5-16.
28. Hurley, L. H. DNA and its associated processes as targets for cancer therapy. *Nat Rev Cancer* **2**, 188-200 (2002).
29. Gilman, A. & Philips, F. S. The Biological Actions and Therapeutic Applications of the B-Chloroethyl Amines and Sulfides. *Science* **103**, 409-436 (1946).
30. Pommier, Y. Topoisomerase I inhibitors: camptothecins and beyond. *Nat Rev Cancer* **6**, 789-802 (2006).
31. Tewey, K. M., Rowe, T. C., Yang, L., Halligan, B. D. & Liu, L. F. Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II. *Science* **226**, 466-468 (1984).
32. Hecht, S. M. Bleomycin: new perspectives on the mechanism of action. *J Nat Prod* **63**, 158-168 (2000).
33. Sengupta, S. & Thomas, S. A. Drug target interaction of tubulin-binding drugs in cancer therapy. *Expert Rev Anticancer Ther* **6**, 1433-1447 (2006).
34. Wang, T. H., Wang, H. S. & Soong, Y. K. Paclitaxel-induced cell death: where the cell cycle and apoptosis come together. *Cancer* **88**, 2619-2628 (2000).
35. Lee, J. H. & Paull, T. T. Direct activation of the ATM protein kinase by the Mre11/Rad50/Nbs1 complex. *Science* **304**, 93-96 (2004).
36. Lee, J. H. & Paull, T. T. ATM activation by DNA double-strand breaks through the Mre11-Rad50-Nbs1 complex. *Science* **308**, 551-554 (2005).
37. Shroff, R. *et al.* Distribution and dynamics of chromatin modification induced by a defined DNA double-strand break. *Curr Biol* **14**, 1703-1711 (2004).
38. Bakkenist, C. J. & Kastan, M. B. DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature* **421**, 499-506 (2003).
39. Burma, S., Chen, B. P., Murphy, M., Kurimasa, A. & Chen, D. J. ATM phosphorylates histone H2AX in response to DNA double-strand breaks. *J Biol Chem* **276**, 42462-42467 (2001).
40. Lavin, M. F. & Kozlov, S. ATM activation and DNA damage response. *Cell Cycle* **6**, 931-942 (2007).
41. Banin, S. *et al.* Enhanced phosphorylation of p53 by ATM in response to DNA damage. *Science* **281**, 1674-1677 (1998).
42. Canman, C. E. *et al.* Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. *Science* **281**, 1677-1679 (1998).
43. Khosravi, R. *et al.* Rapid ATM-dependent phosphorylation of MDM2 precedes p53 accumulation in response to DNA damage. *Proc Natl Acad Sci U S A* **96**, 14973-14977 (1999).
44. Dornan, D. *et al.* ATM engages autodegradation of the E3 ubiquitin ligase COP1 after DNA damage. *Science* **313**, 1122-1126 (2006).
45. Matsuoka, S. *et al.* Ataxia telangiectasia-mutated phosphorylates Chk2 in vivo and in vitro. *Proc Natl Acad Sci U S A* **97**, 10389-10394 (2000).
46. Taniguchi, T. *et al.* Convergence of the fanconi anemia and ataxia telangiectasia signaling pathways. *Cell* **109**, 459-472 (2002).
47. Cortez, D., Wang, Y., Qin, J. & Elledge, S. J. Requirement of ATM-dependent phosphorylation of brca1 in the DNA damage response to double-strand breaks. *Science* **286**, 1162-1166 (1999).
48. Lim, D. S. *et al.* ATM phosphorylates p95/nbs1 in an S-phase checkpoint pathway. *Nature* **404**, 613-617 (2000).
49. Byun, T. S., Pacek, M., Yee, M. C., Walter, J. C. & Cimprich, K. A. Functional uncoupling of MCM helicase and DNA polymerase activities activates the ATR-dependent checkpoint. *Genes Dev* **19**, 1040-1052 (2005).
50. Zou, L. & Elledge, S. J. Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. *Science* **300**, 1542-1548 (2003).
51. Zou, L., Liu, D. & Elledge, S. J. Replication protein A-mediated recruitment and activation of Rad17 complexes. *Proc Natl Acad Sci U S A* **100**, 13827-13832 (2003).

52. Kumagai, A., Lee, J., Yoo, H. Y. & Dunphy, W. G. TopBP1 activates the ATR-ATRIP complex. *Cell* **124**, 943-955 (2006).
53. Busino, L. *et al.* Degradation of Cdc25A by beta-TrCP during S phase and in response to DNA damage. *Nature* **426**, 87-91 (2003).
54. Peng, C. Y. *et al.* Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216. *Science* **277**, 1501-1505 (1997).
55. Featherstone, C. & Jackson, S. P. Ku, a DNA repair protein with multiple cellular functions? *Mutat Res* **434**, 3-15 (1999).
56. Chan, D. W. *et al.* Autophosphorylation of the DNA-dependent protein kinase catalytic subunit is required for rejoining of DNA double-strand breaks. *Genes Dev* **16**, 2333-2338 (2002).
57. Mayo, L. D., Turchi, J. J. & Berberich, S. J. Mdm-2 phosphorylation by DNA-dependent protein kinase prevents interaction with p53. *Cancer Res* **57**, 5013-5016 (1997).
58. Shieh, S. Y., Ikeda, M., Taya, Y. & Prives, C. DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. *Cell* **91**, 325-334 (1997).
59. Wang, S. *et al.* The catalytic subunit of DNA-dependent protein kinase selectively regulates p53-dependent apoptosis but not cell-cycle arrest. *Proc Natl Acad Sci U S A* **97**, 1584-1588 (2000).
60. Park, E. J., Chan, D. W., Park, J. H., Oettinger, M. A. & Kwon, J. DNA-PK is activated by nucleosomes and phosphorylates H2AX within the nucleosomes in an acetylation-dependent manner. *Nucleic Acids Res* **31**, 6819-6827 (2003).
61. Myers, J. S. & Cortez, D. Rapid activation of ATR by ionizing radiation requires ATM and Mre11. *J Biol Chem* **281**, 9346-9350 (2006).
62. Hannan, M. A. *et al.* Deficiency in the repair of UV-induced DNA damage in human skin fibroblasts compromised for the ATM gene. *Carcinogenesis* **23**, 1617-1624 (2002).
63. Dornan, D. *et al.* The ubiquitin ligase COP1 is a critical negative regulator of p53. *Nature* **429**, 86-92 (2004).
64. Leng, R. P. *et al.* Pirh2, a p53-induced ubiquitin-protein ligase, promotes p53 degradation. *Cell* **112**, 779-791 (2003).
65. Toledo, F. & Wahl, G. M. Regulating the p53 pathway: in vitro hypotheses, in vivo veritas. *Nat Rev Cancer* **6**, 909-923 (2006).
66. Brooks, C. L. & Gu, W. p53 ubiquitination: Mdm2 and beyond. *Mol Cell* **21**, 307-315 (2006).
67. Reynisdottir, I., Polyak, K., Iavarone, A. & Massague, J. Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-beta. *Genes Dev* **9**, 1831-1845 (1995).
68. Wang, X. Q., Redpath, J. L., Fan, S. T. & Stanbridge, E. J. ATR dependent activation of Chk2. *J Cell Physiol* **208**, 613-619 (2006).
69. Xu, B., O'Donnell, A. H., Kim, S. T. & Kastan, M. B. Phosphorylation of serine 1387 in Brca1 is specifically required for the Atm-mediated S-phase checkpoint after ionizing irradiation. *Cancer Res* **62**, 4588-4591 (2002).
70. Gatei, M. *et al.* Ataxia-telangiectasia-mutated (ATM) and NBS1-dependent phosphorylation of Chk1 on Ser-317 in response to ionizing radiation. *J Biol Chem* **278**, 14806-14811 (2003).
71. Xu, B., Kim, St & Kastan, M. B. Involvement of Brca1 in S-phase and G(2)-phase checkpoints after ionizing irradiation. *Mol Cell Biol* **21**, 3445-3450 (2001).
72. Li, G. M. Mechanisms and functions of DNA mismatch repair. *Cell Res* **18**, 85-98 (2008).
73. Li, G. M. The role of mismatch repair in DNA damage-induced apoptosis. *Oncol Res* **11**, 393-400 (1999).
74. Hegde, M. L., Hazra, T. K. & Mitra, S. Early steps in the DNA base excision/single-strand interruption repair pathway in mammalian cells. *Cell Res* **18**, 27-47 (2008).

75. Shuck, S. C., Short, E. A. & Turchi, J. J. Eukaryotic nucleotide excision repair: from understanding mechanisms to influencing biology. *Cell Res* **18**, 64-72 (2008).
76. Weterings, E. & Chen, D. J. The endless tale of non-homologous end-joining. *Cell Res* **18**, 114-124 (2008).
77. Paull, T. T. & Gellert, M. The 3' to 5' exonuclease activity of Mre 11 facilitates repair of DNA double-strand breaks. *Mol Cell* **1**, 969-979 (1998).
78. Paull, T. T. & Gellert, M. Nbs1 potentiates ATP-driven DNA unwinding and endonuclease cleavage by the Mre11/Rad50 complex. *Genes Dev* **13**, 1276-1288 (1999).
79. Sharan, S. K. *et al.* Embryonic lethality and radiation hypersensitivity mediated by Rad51 in mice lacking Brca2. *Nature* **386**, 804-810 (1997).
80. Scully, R. *et al.* Association of BRCA1 with Rad51 in mitotic and meiotic cells. *Cell* **88**, 265-275 (1997).
81. Wu, L., Davies, S. L., Levitt, N. C. & Hickson, I. D. Potential role for the BLM helicase in recombinational repair via a conserved interaction with RAD51. *J Biol Chem* **276**, 19375-19381 (2001).
82. Baynton, K. *et al.* WRN interacts physically and functionally with the recombination mediator protein RAD52. *J Biol Chem* **278**, 36476-36486 (2003).
83. Sturzbecher, H. W., Donzelmann, B., Henning, W., Knippschild, U. & Buchhop, S. p53 is linked directly to homologous recombination processes via RAD51/RecA protein interaction. *Embo J* **15**, 1992-2002 (1996).
84. Helton, E. S. & Chen, X. p53 modulation of the DNA damage response. *J Cell Biochem* **100**, 883-896 (2007).
85. Luthi, A. U. & Martin, S. J. The CASBAH: a searchable database of caspase substrates. *Cell Death Differ* **14**, 641-650 (2007).
86. Jin, Z. & El-Deiry, W. S. Overview of cell death signaling pathways. *Cancer Biol Ther* **4**, 139-163 (2005).
87. Li, P. *et al.* Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* **91**, 479-489 (1997).
88. Grimm, S. & Brdiczka, D. The permeability transition pore in cell death. *Apoptosis* **12**, 841-855 (2007).
89. Kischkel, F. C. *et al.* Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. *Embo J* **14**, 5579-5588 (1995).
90. Scaffidi, C. *et al.* Two CD95 (APO-1/Fas) signaling pathways. *Embo J* **17**, 1675-1687 (1998).
91. Li, H., Zhu, H., Xu, C. J. & Yuan, J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* **94**, 491-501 (1998).
92. Vaux, D. L. & Silke, J. IAPs, RINGs and ubiquitylation. *Nat Rev Mol Cell Biol* **6**, 287-297 (2005).
93. Deveraux, Q. L. *et al.* Cleavage of human inhibitor of apoptosis protein XIAP results in fragments with distinct specificities for caspases. *Embo J* **18**, 5242-5251 (1999).
94. Du, C., Fang, M., Li, Y., Li, L. & Wang, X. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell* **102**, 33-42 (2000).
95. Suzuki, Y. *et al.* A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. *Mol Cell* **8**, 613-621 (2001).
96. Liu, J. & Lin, A. Role of JNK activation in apoptosis: a double-edged sword. *Cell Res* **15**, 36-42 (2005).
97. Van Laethem, A. *et al.* Activation of p38 MAPK is required for Bax translocation to mitochondria, cytochrome c release and apoptosis induced by UVB irradiation in human keratinocytes. *Faseb J* **18**, 1946-1948 (2004).
98. Vousden, K. H. & Lu, X. Live or let die: the cell's response to p53. *Nat Rev Cancer* **2**, 594-604 (2002).

99. Miyashita, T., Harigai, M., Hanada, M. & Reed, J. C. Identification of a p53-dependent negative response element in the bcl-2 gene. *Cancer Res* **54**, 3131-3135 (1994).
100. Hoffman, W. H., Biade, S., Zilfou, J. T., Chen, J. & Murphy, M. Transcriptional repression of the anti-apoptotic survivin gene by wild type p53. *J Biol Chem* **277**, 3247-3257 (2002).
101. Schuler, M. & Green, D. R. Transcription, apoptosis and p53: catch-22. *Trends Genet* **21**, 182-187 (2005).
102. Okada, H. & Mak, T. W. Pathways of apoptotic and non-apoptotic death in tumour cells. *Nat Rev Cancer* **4**, 592-603 (2004).
103. Golstein, P. & Kroemer, G. Cell death by necrosis: towards a molecular definition. *Trends Biochem Sci* **32**, 37-43 (2007).
104. Takai, H. *et al.* Aberrant cell cycle checkpoint function and early embryonic death in Chk1(-/-) mice. *Genes Dev* **14**, 1439-1447 (2000).
105. Dimri, G. P. What has senescence got to do with cancer? *Cancer Cell* **7**, 505-512 (2005).
106. Hehlmann, R., Hochhaus, A. & Baccarani, M. Chronic myeloid leukaemia. *Lancet* **370**, 342-350 (2007).
107. Dancey, J. E. & Chen, H. X. Strategies for optimizing combinations of molecularly targeted anticancer agents. *Nat Rev Drug Discov* **5**, 649-659 (2006).
108. Mesa, R. A. Tipifarnib: farnesyl transferase inhibition at a crossroads. *Expert Rev Anticancer Ther* **6**, 313-319 (2006).
109. Bykov, V. J. *et al.* Restoration of the tumor suppressor function to mutant p53 by a low-molecular-weight compound. *Nat Med* **8**, 282-288 (2002).
110. Issaeva, N. *et al.* Small molecule RITA binds to p53, blocks p53-HDM-2 interaction and activates p53 function in tumors. *Nat Med* **10**, 1321-1328 (2004).
111. Li, J., Viallet, J. & Haura, E. B. A small molecule pan-Bcl-2 family inhibitor, GX15-070, induces apoptosis and enhances cisplatin-induced apoptosis in non-small cell lung cancer cells. *Cancer Chemother Pharmacol*, (2007).
112. Cummings, J. *et al.* Validation of pharmacodynamic assays to evaluate the clinical efficacy of an antisense compound (AEG 35156) targeted to the X-linked inhibitor of apoptosis protein XIAP. *Br J Cancer* **92**, 532-538 (2005).
113. Wang, T. T., Qian, X. P. & Liu, B. R. Survivin: potential role in diagnosis, prognosis and targeted therapy of gastric cancer. *World J Gastroenterol* **13**, 2784-2790 (2007).
114. Kelland, L. Targeting the limitless replicative potential of cancer: the telomerase/telomere pathway. *Clin Cancer Res* **13**, 4960-4963 (2007).
115. Zakarija, A. & Soff, G. Update on angiogenesis inhibitors. *Curr Opin Oncol* **17**, 578-583 (2005).
116. Coussens, L. M., Fingleton, B. & Matrisian, L. M. Matrix metalloproteinase inhibitors and cancer: trials and tribulations. *Science* **295**, 2387-2392 (2002).
117. Elvin, P. & Garner, A. P. Tumour invasion and metastasis: challenges facing drug discovery. *Curr Opin Pharmacol* **5**, 374-381 (2005).
118. Helleday, T., Petermann, E., Lundin, C., Hodgson, B. & Sharma, R. A. DNA repair pathways as targets for cancer therapy. *Nat Rev Cancer* **8**, 193-204 (2008).
119. Bryant, H. E. *et al.* Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature* **434**, 913-917 (2005).
120. Roberts, T. G., Jr. & Chabner, B. A. Beyond fast track for drug approvals. *N Engl J Med* **351**, 501-505 (2004).
121. Duffy, M. J. Predictive markers in breast and other cancers: a review. *Clin Chem* **51**, 494-503 (2005).
122. Chabner, B. A. & Roberts, T. G., Jr. Timeline: Chemotherapy and the war on cancer. *Nat Rev Cancer* **5**, 65-72 (2005).
123. Santamaria-Kisiel, L., Rintala-Dempsey, A. C. & Shaw, G. S. Calcium-dependent and -independent interactions of the S100 protein family. *Biochem J* **396**, 201-214 (2006).

124. Moore, B. W. A soluble protein characteristic of the nervous system. *Biochem Biophys Res Commun* **19**, 739-744 (1965).
125. Moore, B. W. & McGregor, D. Chromatographic and Electrophoretic Fractionation of Soluble Proteins of Brain and Liver. *J Biol Chem* **240**, 1647-1653 (1965).
126. Marenholz, I., Heizmann, C. W. & Fritz, G. S100 proteins in mouse and man: from evolution to function and pathology (including an update of the nomenclature). *Biochem Biophys Res Commun* **322**, 1111-1122 (2004).
127. Kretsinger, R. H. & Nockolds, C. E. Carp muscle calcium-binding protein. II. Structure determination and general description. *J Biol Chem* **248**, 3313-3326 (1973).
128. Donato, R. S100: a multigenic family of calcium-modulated proteins of the EF-hand type with intracellular and extracellular functional roles. *Int J Biochem Cell Biol* **33**, 637-668 (2001).
129. Zimmer, D. B., Wright Sadosky, P. & Weber, D. J. Molecular mechanisms of S100-target protein interactions. *Microsc Res Tech* **60**, 552-559 (2003).
130. Donato, R. S-100 proteins. *Cell Calcium* **7**, 123-145 (1986).
131. Donato, R. Intracellular and extracellular roles of S100 proteins. *Microsc Res Tech* **60**, 540-551 (2003).
132. Hunter, M. J. & Chazin, W. J. High level expression and dimer characterization of the S100 EF-hand proteins, migration inhibitory factor-related proteins 8 and 14. *J Biol Chem* **273**, 12427-12435 (1998).
133. Guignard, F., Mauel, J. & Markert, M. Phosphorylation of myeloid-related proteins MRP-14 and MRP-8 during human neutrophil activation. *Eur J Biochem* **241**, 265-271 (1996).
134. Goch, G., Vdovenko, S., Kozlowska, H. & Bierzynski, A. Affinity of S100A1 protein for calcium increases dramatically upon glutathionylation. *Febs J* **272**, 2557-2565 (2005).
135. Zhukova, L., Zhukov, I., Bal, W. & Wyslouch-Cieszynska, A. Redox modifications of the C-terminal cysteine residue cause structural changes in S100A1 and S100B proteins. *Biochim Biophys Acta* **1742**, 191-201 (2004).
136. Zimmer, D. B., Cornwall, E. H., Landar, A. & Song, W. The S100 protein family: history, function, and expression. *Brain Res Bull* **37**, 417-429 (1995).
137. Hermani, A., De Servi, B., Medunjanin, S., Tessier, P. A. & Mayer, D. S100A8 and S100A9 activate MAP kinase and NF-kappaB signaling pathways and trigger translocation of RAGE in human prostate cancer cells. *Exp Cell Res* **312**, 184-197 (2006).
138. Yui, S., Nakatani, Y. & Mikami, M. Calprotectin (S100A8/S100A9), an inflammatory protein complex from neutrophils with a broad apoptosis-inducing activity. *Biol Pharm Bull* **26**, 753-760 (2003).
139. Hofmann, M. A. *et al.* RAGE mediates a novel proinflammatory axis: a central cell surface receptor for S100/calgranulin polypeptides. *Cell* **97**, 889-901 (1999).
140. Broome, A. M., Ryan, D. & Eckert, R. L. S100 protein subcellular localization during epidermal differentiation and psoriasis. *J Histochem Cytochem* **51**, 675-685 (2003).
141. Semprini, S. *et al.* Evidence for differential S100 gene over-expression in psoriatic patients from genetically heterogeneous pedigrees. *Hum Genet* **111**, 310-313 (2002).
142. Rothermundt, M., Peters, M., Prehn, J. H. & Arolt, V. S100B in brain damage and neurodegeneration. *Microsc Res Tech* **60**, 614-632 (2003).
143. Adami, C. *et al.* S100B expression in and effects on microglia. *Glia* **33**, 131-142 (2001).
144. Van Eldik, L. J., Christie-Pope, B., Bolin, L. M., Shooter, E. M. & Whetsell, W. O., Jr. Neurotrophic activity of S-100 beta in cultures of dorsal root ganglia from embryonic chick and fetal rat. *Brain Res* **542**, 280-285 (1991).
145. Fulle, S. *et al.* Calcium and fos involvement in brain-derived Ca(2+)-binding protein (S100)-dependent apoptosis in rat phaeochromocytoma cells. *Exp Physiol* **85**, 243-253 (2000).

146. Li, Y., Barger, S. W., Liu, L., Mrak, R. E. & Griffin, W. S. S100beta induction of the proinflammatory cytokine interleukin-6 in neurons. *J Neurochem* **74**, 143-150 (2000).
147. Boom, A. *et al.* Astrocytic calcium/zinc binding protein S100A6 over expression in Alzheimer's disease and in PS1/APP transgenic mice models. *Biochim Biophys Acta* **1742**, 161-168 (2004).
148. Hoyaux, D. *et al.* S100A6, a calcium- and zinc-binding protein, is overexpressed in SOD1 mutant mice, a model for amyotrophic lateral sclerosis. *Biochim Biophys Acta* **1498**, 264-272 (2000).
149. Hoyaux, D. *et al.* S100A6 overexpression within astrocytes associated with impaired axons from both ALS mouse model and human patients. *J Neuropathol Exp Neurol* **61**, 736-744 (2002).
150. Most, P. *et al.* Cardiac S100A1 protein levels determine contractile performance and propensity toward heart failure after myocardial infarction. *Circulation* **114**, 1258-1268 (2006).
151. Ehlermann, P. *et al.* Right ventricular upregulation of the Ca(2+) binding protein S100A1 in chronic pulmonary hypertension. *Biochim Biophys Acta* **1500**, 249-255 (2000).
152. Remppis, A. *et al.* Altered expression of the Ca(2+)-binding protein S100A1 in human cardiomyopathy. *Biochim Biophys Acta* **1313**, 253-257 (1996).
153. Most, P., Remppis, A., Pleger, S. T., Katus, H. A. & Koch, W. J. S100A1: a novel inotropic regulator of cardiac performance. Transition from molecular physiology to pathophysiological relevance. *Am J Physiol Regul Integr Comp Physiol* **293**, R568-577 (2007).
154. Teratani, T. *et al.* Induced transcriptional expression of calcium-binding protein S100A1 and S100A10 genes in human renal cell carcinoma. *Cancer Lett* **175**, 71-77 (2002).
155. Li, G. *et al.* S100A1: a powerful marker to differentiate chromophobe renal cell carcinoma from renal oncocytoma. *Histopathology* **50**, 642-647 (2007).
156. Rocca, P. C. *et al.* Diagnostic utility of S100A1 expression in renal cell neoplasms: an immunohistochemical and quantitative RT-PCR study. *Mod Pathol* **20**, 722-728 (2007).
157. Wang, G. *et al.* Mutually antagonistic actions of S100A4 and S100A1 on normal and metastatic phenotypes. *Oncogene* **24**, 1445-1454 (2005).
158. Feng, G., Xu, X., Youssef, E. M. & Lotan, R. Diminished expression of S100A2, a putative tumor suppressor, at early stage of human lung carcinogenesis. *Cancer Res* **61**, 7999-8004 (2001).
159. Wang, H. *et al.* Overexpression of S100A2 protein as a prognostic marker for patients with stage I non small cell lung cancer. *Int J Cancer* **116**, 285-290 (2005).
160. Wicki, R., Franz, C., Scholl, F. A., Heizmann, C. W. & Schafer, B. W. Repression of the candidate tumor suppressor gene S100A2 in breast cancer is mediated by site-specific hypermethylation. *Cell Calcium* **22**, 243-254 (1997).
161. Gupta, S. *et al.* Differential expression of S100A2 and S100A4 during progression of human prostate adenocarcinoma. *J Clin Oncol* **21**, 106-112 (2003).
162. Suzuki, F. *et al.* S100A2 expression as a predictive marker for late cervical metastasis in stage I and II invasive squamous cell carcinoma of the oral cavity. *Oncol Rep* **14**, 1493-1498 (2005).
163. Tsai, S. T. *et al.* S100A2, a potential marker for early recurrence in early-stage oral cancer. *Oral Oncol* **41**, 349-357 (2005).
164. Matsumoto, K. *et al.* Expression of S100A2 and S100A4 predicts for disease progression and patient survival in bladder cancer. *Urology* **70**, 602-607 (2007).
165. Maelandsmo, G. M. *et al.* Differential expression patterns of S100A2, S100A4 and S100A6 during progression of human malignant melanoma. *Int J Cancer* **74**, 464-469 (1997).
166. Bartling, B. *et al.* S100A2-S100P expression profile and diagnosis of non-small cell lung carcinoma: impairment by advanced tumour stages and neoadjuvant chemotherapy. *Eur J Cancer* **43**, 1935-1943 (2007).

167. Matsubara, D. *et al.* Differential expression of S100A2 and S100A4 in lung adenocarcinomas: clinicopathological significance, relationship to p53 and identification of their target genes. *Cancer Sci* **96**, 844-857 (2005).
168. Smith, S. L. *et al.* S100A2 is strongly expressed in airway basal cells, preneoplastic bronchial lesions and primary non-small cell lung carcinomas. *Br J Cancer* **91**, 1515-1524 (2004).
169. Ohuchida, K. *et al.* Over-expression of S100A2 in pancreatic cancer correlates with progression and poor prognosis. *J Pathol* **213**, 275-282 (2007).
170. Mueller, A. *et al.* The calcium-binding protein S100A2 interacts with p53 and modulates its transcriptional activity. *J Biol Chem* **280**, 29186-29193 (2005).
171. Davies, B. R. *et al.* Expression of S100A4 protein is associated with metastasis and reduced survival in human bladder cancer. *J Pathol* **196**, 292-299 (2002).
172. Xie, R. *et al.* Hypomethylation-induced expression of S100A4 in endometrial carcinoma. *Mod Pathol* **20**, 1045-1054 (2007).
173. Oida, Y. *et al.* Increased S100A4 expression combined with decreased E-cadherin expression predicts a poor outcome of patients with pancreatic cancer. *Oncol Rep* **16**, 457-463 (2006).
174. Rosty, C. *et al.* Overexpression of S100A4 in pancreatic ductal adenocarcinomas is associated with poor differentiation and DNA hypomethylation. *Am J Pathol* **160**, 45-50 (2002).
175. Hemandas, A. K., Salto-Tellez, M., Maricar, S. H., Leong, A. F. & Leow, C. K. Metastasis-associated protein S100A4--a potential prognostic marker for colorectal cancer. *J Surg Oncol* **93**, 498-503 (2006).
176. Cho, Y. G. *et al.* Overexpression of S100A4 is closely associated with progression of colorectal cancer. *World J Gastroenterol* **11**, 4852-4856 (2005).
177. Flatmark, K. *et al.* Nuclear localization of the metastasis-related protein S100A4 correlates with tumour stage in colorectal cancer. *J Pathol* **200**, 589-595 (2003).
178. Gongoll, S. *et al.* Prognostic significance of calcium-binding protein S100A4 in colorectal cancer. *Gastroenterology* **123**, 1478-1484 (2002).
179. Kimura, K. *et al.* Clinical significance of S100A4 and E-cadherin-related adhesion molecules in non-small cell lung cancer. *Int J Oncol* **16**, 1125-1131 (2000).
180. Zou, M., Al-Baradie, R. S., Al-Hindi, H., Farid, N. R. & Shi, Y. S100A4 (Mts1) gene overexpression is associated with invasion and metastasis of papillary thyroid carcinoma. *Br J Cancer* **93**, 1277-1284 (2005).
181. Lee, W. Y. *et al.* Expression of S100A4 and Met: potential predictors for metastasis and survival in early-stage breast cancer. *Oncology* **66**, 429-438 (2004).
182. Pedersen, K. B., Nesland, J. M., Fodstad, O. & Maelandsmo, G. M. Expression of S100A4, E-cadherin, alpha- and beta-catenin in breast cancer biopsies. *Br J Cancer* **87**, 1281-1286 (2002).
183. Platt-Higgins, A. M. *et al.* Comparison of the metastasis-inducing protein S100A4 (p9ka) with other prognostic markers in human breast cancer. *Int J Cancer* **89**, 198-208 (2000).
184. Rudland, P. S. *et al.* Prognostic significance of the metastasis-inducing protein S100A4 (p9Ka) in human breast cancer. *Cancer Res* **60**, 1595-1603 (2000).
185. Moriyama-Kita, M. *et al.* Correlation of S100A4 expression with invasion and metastasis in oral squamous cell carcinoma. *Oral Oncol* **40**, 496-500 (2004).
186. Cho, Y. G. *et al.* Overexpression of S100A4 is closely related to the aggressiveness of gastric cancer. *Apmis* **111**, 539-545 (2003).
187. Nakamura, T. *et al.* Prognostic significance of S100A4 expression in gallbladder cancer. *Int J Oncol* **20**, 937-941 (2002).
188. Ninomiya, I. *et al.* Increased expression of S100A4 and its prognostic significance in esophageal squamous cell carcinoma. *Int J Oncol* **18**, 715-720 (2001).
189. Grigorian, M. *et al.* Tumor suppressor p53 protein is a new target for the metastasis-associated Mts1/S100A4 protein: functional consequences of their interaction. *J Biol Chem* **276**, 22699-22708 (2001).

190. Ambartsumian, N. S. *et al.* Metastasis of mammary carcinomas in GRS/A hybrid mice transgenic for the mts1 gene. *Oncogene* **13**, 1621-1630 (1996).
191. Davies, B. R., Davies, M. P., Gibbs, F. E., Barraclough, R. & Rudland, P. S. Induction of the metastatic phenotype by transfection of a benign rat mammary epithelial cell line with the gene for p9Ka, a rat calcium-binding protein, but not with the oncogene EJ-ras-1. *Oncogene* **8**, 999-1008 (1993).
192. Grigorian, M. *et al.* Effect of mts1 (S100A4) expression on the progression of human breast cancer cells. *Int J Cancer* **67**, 831-841 (1996).
193. Maelandsmo, G. M. *et al.* Reversal of the in vivo metastatic phenotype of human tumor cells by an anti-CAPL (mts1) ribozyme. *Cancer Res* **56**, 5490-5498 (1996).
194. Takenaga, K., Nakamura, Y. & Sakiyama, S. Expression of antisense RNA to S100A4 gene encoding an S100-related calcium-binding protein suppresses metastatic potential of high-metastatic Lewis lung carcinoma cells. *Oncogene* **14**, 331-337 (1997).
195. Ford, H. L., Silver, D. L., Kachar, B., Sellers, J. R. & Zain, S. B. Effect of Mts1 on the structure and activity of nonmuscle myosin II. *Biochemistry* **36**, 16321-16327 (1997).
196. Li, Z. H. & Bresnick, A. R. The S100A4 metastasis factor regulates cellular motility via a direct interaction with myosin-IIA. *Cancer Res* **66**, 5173-5180 (2006).
197. Bjornland, K. *et al.* S100A4 involvement in metastasis: deregulation of matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases in osteosarcoma cells transfected with an anti-S100A4 ribozyme. *Cancer Res* **59**, 4702-4708 (1999).
198. Schmidt-Hansen, B. *et al.* Functional significance of metastasis-inducing S100A4(Mts1) in tumor-stroma interplay. *J Biol Chem* **279**, 24498-24504 (2004).
199. Crnogorac-Jurcevic, T. *et al.* Molecular alterations in pancreatic carcinoma: expression profiling shows that dysregulated expression of S100 genes is highly prevalent. *J Pathol* **201**, 63-74 (2003).
200. Ohuchida, K. *et al.* The role of S100A6 in pancreatic cancer development and its clinical implication as a diagnostic marker and therapeutic target. *Clin Cancer Res* **11**, 7785-7793 (2005).
201. Shekouh, A. R. *et al.* Application of laser capture microdissection combined with two-dimensional electrophoresis for the discovery of differentially regulated proteins in pancreatic ductal adenocarcinoma. *Proteomics* **3**, 1988-2001 (2003).
202. Vimalachandran, D. *et al.* High nuclear S100A6 (Calcyclin) is significantly associated with poor survival in pancreatic cancer patients. *Cancer Res* **65**, 3218-3225 (2005).
203. Bronckart, Y. *et al.* Development and progression of malignancy in human colon tissues are correlated with expression of specific Ca(2+)-binding S100 proteins. *Histol Histopathol* **16**, 707-712 (2001).
204. Komatsu, K. *et al.* Increased expression of S100A6 (Calcyclin), a calcium-binding protein of the S100 family, in human colorectal adenocarcinomas. *Clin Cancer Res* **6**, 172-177 (2000).
205. Komatsu, K. *et al.* Increased expression of S100A6 at the invading fronts of the primary lesion and liver metastasis in patients with colorectal adenocarcinoma. *Br J Cancer* **83**, 769-774 (2000).
206. Komatsu, K. *et al.* Expression of S100A6 and S100A4 in matched samples of human colorectal mucosa, primary colorectal adenocarcinomas and liver metastases. *Oncology* **63**, 192-200 (2002).
207. Stulik, J. *et al.* Differential expression of the Ca2+ binding S100A6 protein in normal, preneoplastic and neoplastic colon mucosa. *Eur J Cancer* **36**, 1050-1059 (2000).
208. Brown, L. M. *et al.* Quantitative and qualitative differences in protein expression between papillary thyroid carcinoma and normal thyroid tissue. *Mol Carcinog*, (2006).

209. Ito, Y. *et al.* Expression of S100A2 and S100A6 in thyroid carcinomas. *Histopathology* **46**, 569-575 (2005).
210. Yang, Y. Q. *et al.* Upregulated expression of S100A6 in human gastric cancer. *J Dig Dis* **8**, 186-193 (2007).
211. Cross, S. S., Hamdy, F. C., Deloulme, J. C. & Rehman, I. Expression of S100 proteins in normal human tissues and common cancers using tissue microarrays: S100A6, S100A8, S100A9 and S100A11 are all overexpressed in common cancers. *Histopathology* **46**, 256-269 (2005).
212. Fullen, D. R., Garrisi, A. J., Sanders, D. & Thomas, D. Expression of S100A6 protein in a broad spectrum of cutaneous tumors using tissue microarrays. *J Cutan Pathol*, (2008).
213. Luu, H. H. *et al.* Increased expression of S100A6 is associated with decreased metastasis and inhibition of cell migration and anchorage independent growth in human osteosarcoma. *Cancer Lett* **229**, 135-148 (2005).
214. Rehman, I. *et al.* S100A6 (Calcyclin) is a prostate basal cell marker absent in prostate cancer and its precursors. *Br J Cancer* **91**, 739-744 (2004).
215. Ribe, A. & McNutt, N. S. S100A6 protein expression is different in Spitz nevi and melanomas. *Mod Pathol* **16**, 505-511 (2003).
216. Rehman, I. *et al.* Promoter hyper-methylation of calcium binding proteins S100A6 and S100A2 in human prostate cancer. *Prostate* **65**, 322-330 (2005).
217. Lindsey, J. C. *et al.* Epigenetic deregulation of multiple S100 gene family members by differential hypomethylation and hypermethylation events in medulloblastoma. *Br J Cancer* **97**, 267-274 (2007).
218. Emberley, E. D., Alowami, S., Snell, L., Murphy, L. C. & Watson, P. H. S100A7 (psoriasin) expression is associated with aggressive features and alteration of Jab1 in ductal carcinoma in situ of the breast. *Breast Cancer Res* **6**, R308-315 (2004).
219. Jiang, W. G., Watkins, G., Douglas-Jones, A. & Mansel, R. E. Psoriasin is aberrantly expressed in human breast cancer and is related to clinical outcomes. *Int J Oncol* **25**, 81-85 (2004).
220. Leygue, E. *et al.* Differential expression of psoriasin messenger RNA between in situ and invasive human breast carcinoma. *Cancer Res* **56**, 4606-4609 (1996).
221. Emberley, E. D. *et al.* The S100A7-c-Jun activation domain binding protein 1 pathway enhances prosurvival pathways in breast cancer. *Cancer Res* **65**, 5696-5702 (2005).
222. Emberley, E. D. *et al.* Psoriasin interacts with Jab1 and influences breast cancer progression. *Cancer Res* **63**, 1954-1961 (2003).
223. Celis, J. E. *et al.* Bladder squamous cell carcinomas express psoriasin and externalize it to the urine. *J Urol* **155**, 2105-2112 (1996).
224. Moubayed, N. *et al.* Psoriasin (S100A7) is significantly up-regulated in human epithelial skin tumours. *J Cancer Res Clin Oncol* **133**, 253-261 (2007).
225. Zhang, H. *et al.* Identification and validation of S100A7 associated with lung squamous cell carcinoma metastasis to brain. *Lung Cancer* **57**, 37-45 (2007).
226. Zhang, H. *et al.* Selective expression of S100A7 in lung squamous cell carcinomas and large cell carcinomas but not in adenocarcinomas and small cell carcinomas. *Thorax* **63**, 352-359 (2008).
227. Hermani, A. *et al.* Calcium-binding proteins S100A8 and S100A9 as novel diagnostic markers in human prostate cancer. *Clin Cancer Res* **11**, 5146-5152 (2005).
228. Tolson, J. P. *et al.* Differential detection of S100A8 in transitional cell carcinoma of the bladder by pair wise tissue proteomic and immunohistochemical analysis. *Proteomics* **6**, 697-708 (2006).
229. Guo, J. *et al.* A strategy for high-resolution protein identification in surface-enhanced laser desorption/ionization mass spectrometry: calgranulin A and chaperonin 10 as protein markers for endometrial carcinoma. *Proteomics* **5**, 1953-1966 (2005).
230. Arai, K., Teratani, T., Nozawa, R. & Yamada, T. Immunohistochemical investigation of S100A9 expression in pulmonary adenocarcinoma: S100A9

expression is associated with tumor differentiation. *Oncol Rep* **8**, 591-596 (2001).
231. Arai, K., Yamada, T. & Nozawa, R. Immunohistochemical investigation of migration inhibitory factor-related protein (MRP)-14 expression in hepatocellular carcinoma. *Med Oncol* **17**, 183-188 (2000).
232. Ito, Y. *et al.* S100A9 expression is significantly linked to dedifferentiation of thyroid carcinoma. *Pathol Res Pract* **201**, 551-556 (2005).
233. Arai, K., Teratani, T., Kuruto-Niwa, R., Yamada, T. & Nozawa, R. S100A9 expression in invasive ductal carcinoma of the breast: S100A9 expression in adenocarcinoma is closely associated with poor tumour differentiation. *Eur J Cancer* **40**, 1179-1187 (2004).
234. Stulik, J. *et al.* The analysis of S100A9 and S100A8 expression in matched sets of macroscopically normal colon mucosa and colorectal carcinoma: the S100A9 and S100A8 positive cells underlie and invade tumor mass. *Electrophoresis* **20**, 1047-1054 (1999).
235. Roesch Ely, M. *et al.* Transcript and proteome analysis reveals reduced expression of calgranulins in head and neck squamous cell carcinoma. *Eur J Cell Biol* **84**, 431-444 (2005).
236. Luo, A. *et al.* Discovery of Ca2+-relevant and differentiation-associated genes downregulated in esophageal squamous cell carcinoma using cDNA microarray. *Oncogene* **23**, 1291-1299 (2004).
237. Memon, A. A. *et al.* Down-regulation of S100C is associated with bladder cancer progression and poor survival. *Clin Cancer Res* **11**, 606-611 (2005).
238. Miyazaki, M. *et al.* Involvement of interferon regulatory factor 1 and S100C/A11 in growth inhibition by transforming growth factor beta 1 in human hepatocellular carcinoma cells. *Cancer Res* **64**, 4155-4161 (2004).
239. Tian, T. *et al.* Determination of metastasis-associated proteins in non-small cell lung cancer by comparative proteomic analysis. *Cancer Sci* **98**, 1265-1274 (2007).
240. Rehman, I. *et al.* Dysregulated expression of S100A11 (calgizzarin) in prostate cancer and precursor lesions. *Hum Pathol* **35**, 1385-1391 (2004).
241. Wang, G. *et al.* Colorectal cancer progression correlates with upregulation of S100A11 expression in tumor tissues. *Int J Colorectal Dis*, (2008).
242. Andres, R. *et al.* Prognostic value of serum S-100B in malignant melanoma. *Tumori* **90**, 607-610 (2004).
243. Bonfrer, J. M., Korse, C. M., Nieweg, O. E. & Rankin, E. M. The luminescence immunoassay S-100: a sensitive test to measure circulating S-100B: its prognostic value in malignant melanoma. *Br J Cancer* **77**, 2210-2214 (1998).
244. Boni, R. *et al.* Immunohistochemical localization of the Ca2+ binding S100 proteins in normal human skin and melanocytic lesions. *Br J Dermatol* **137**, 39-43 (1997).
245. Hauschild, A. *et al.* Prognostic significance of serum S100B detection compared with routine blood parameters in advanced metastatic melanoma patients. *Melanoma Res* **9**, 155-161 (1999).
246. Schlagenhauff, B. *et al.* Significance of serum protein S100 levels in screening for melanoma metastasis: does protein S100 enable early detection of melanoma recurrence? *Melanoma Res* **10**, 451-459 (2000).
247. Hamberg, A. P., Korse, C. M., Bonfrer, J. M. & de Gast, G. C. Serum S100B is suitable for prediction and monitoring of response to chemoimmunotherapy in metastatic malignant melanoma. *Melanoma Res* **13**, 45-49 (2003).
248. Hauschild, A. *et al.* Predictive value of serum S100B for monitoring patients with metastatic melanoma during chemotherapy and/or immunotherapy. *Br J Dermatol* **140**, 1065-1071 (1999).
249. Baudier, J., Delphin, C., Grunwald, D., Khochbin, S. & Lawrence, J. J. Characterization of the tumor suppressor protein p53 as a protein kinase C substrate and a S100b-binding protein. *Proc Natl Acad Sci U S A* **89**, 11627-11631 (1992).
250. Rustandi, R. R., Baldisseri, D. M. & Weber, D. J. Structure of the negative regulatory domain of p53 bound to S100B(betabeta). *Nat Struct Biol* **7**, 570-574 (2000).

251. Lin, J. *et al.* Inhibition of p53 transcriptional activity by the S100B calcium-binding protein. *J Biol Chem* **276**, 35037-35041 (2001).
252. Lin, J. *et al.* Inhibiting S100B restores p53 levels in primary malignant melanoma cancer cells. *J Biol Chem* **279**, 34071-34077 (2004).
253. Calabretta, B., Battini, R., Kaczmarek, L., de Riel, J. K. & Baserga, R. Molecular cloning of the cDNA for a growth factor-inducible gene with strong homology to S-100, a calcium-binding protein. *J Biol Chem* **261**, 12628-12632 (1986).
254. Hirschhorn, R. R., Aller, P., Yuan, Z. A., Gibson, C. W. & Baserga, R. Cell-cycle-specific cDNAs from mammalian cells temperature sensitive for growth. *Proc Natl Acad Sci U S A* **81**, 6004-6008 (1984).
255. Calabretta, B. *et al.* Cell-cycle-specific genes differentially expressed in human leukemias. *Proc Natl Acad Sci U S A* **82**, 4463-4467 (1985).
256. Calabretta, B. *et al.* Altered expression of G1-specific genes in human malignant myeloid cells. *Proc Natl Acad Sci U S A* **83**, 1495-1498 (1986).
257. Ferrari, S. *et al.* Structural and functional analysis of a growth-regulated gene, the human calcyclin. *J Biol Chem* **262**, 8325-8332 (1987).
258. Kuznicki, J. & Filipek, A. Purification and properties of a novel Ca2+-binding protein (10.5 kDa) from Ehrlich-ascites-tumour cells. *Biochem J* **247**, 663-667 (1987).
259. Murphy, L. C., Murphy, L. J., Tsuyuki, D., Duckworth, M. L. & Shiu, R. P. Cloning and characterization of a cDNA encoding a highly conserved, putative calcium binding protein, identified by an anti-prolactin receptor antiserum. *J Biol Chem* **263**, 2397-2401 (1988).
260. Schafer, B. W., Wicki, R., Engelkamp, D., Mattei, M. G. & Heizmann, C. W. Isolation of a YAC clone covering a cluster of nine S100 genes on human chromosome 1q21: rationale for a new nomenclature of the S100 calcium-binding protein family. *Genomics* **25**, 638-643 (1995).
261. Potts, B. C. *et al.* The structure of calcyclin reveals a novel homodimeric fold for S100 Ca(2+)-binding proteins. *Nat Struct Biol* **2**, 790-796 (1995).
262. Sastry, M. *et al.* The three-dimensional structure of Ca(2+)-bound calcyclin: implications for Ca(2+)-signal transduction by S100 proteins. *Structure* **6**, 223-231 (1998).
263. Otterbein, L. R., Kordowska, J., Witte-Hoffmann, C., Wang, C. L. & Dominguez, R. Crystal structures of S100A6 in the Ca(2+)-free and Ca(2+)-bound states: the calcium sensor mechanism of S100 proteins revealed at atomic resolution. *Structure* **10**, 557-567 (2002).
264. Deloulme, J. C. *et al.* S100A6 and S100A11 are specific targets of the calcium- and zinc-binding S100B protein in vivo. *J Biol Chem* **275**, 35302-35310 (2000).
265. Yang, Q., O'Hanlon, D., Heizmann, C. W. & Marks, A. Demonstration of heterodimer formation between S100B and S100A6 in the yeast two-hybrid system and human melanoma. *Exp Cell Res* **246**, 501-509 (1999).
266. Kordowska, J., Stafford, W. F. & Wang, C. L. Ca2+ and Zn2+ bind to different sites and induce different conformational changes in human calcyclin. *Eur J Biochem* **253**, 57-66 (1998).
267. Pedrocchi, M., Schafer, B. W., Durussel, I., Cox, J. A. & Heizmann, C. W. Purification and characterization of the recombinant human calcium-binding S100 proteins CAPL and CACY. *Biochemistry* **33**, 6732-6738 (1994).
268. Guo, X. J. *et al.* Identification of a serum-inducible messenger RNA (5B10) as the mouse homologue of calcyclin: tissue distribution and expression in metastatic, ras-transformed NIH 3T3 cells. *Cell Growth Differ* **1**, 333-338 (1990).
269. Kuznicki, J., Kordowska, J., Puzianowska, M. & Wozniewicz, B. M. Calcyclin as a marker of human epithelial cells and fibroblasts. *Exp Cell Res* **200**, 425-430 (1992).
270. Filipek, A., Puzianowska, M., Cieslak, B. & Kuznicki, J. Calcyclin--Ca(2+)-binding protein homologous to glial S-100 beta is present in neurones. *Neuroreport* **4**, 383-386 (1993).
271. Yamashita, N., Ilg, E. C., Schafer, B. W., Heizmann, C. W. & Kosaka, T. Distribution of a specific calcium-binding protein of the S100 protein family,

S100A6 (calcyclin), in subpopulations of neurons and glial cells of the adult rat nervous system. *J Comp Neurol* **404**, 235-257 (1999).
272. Ghezzo, F., Lauret, E., Ferrari, S. & Baserga, R. Growth factor regulation of the promoter for calcyclin, a growth-regulated gene. *J Biol Chem* **263**, 4758-4763 (1988).
273. Bottini, F., Mazzocco, K., Abbondi, T. & Tonini, G. P. Identification of an AP-1-like sequence in the promoter region of calcyclin, a S-100-like gene. Enhancement of binding during retinoic acid-induced neuroblastoma cell differentiation. *Neurosci Lett* **181**, 35-38 (1994).
274. Lesniak, W., Jezierska, A. & Kuznicki, J. Upstream stimulatory factor is involved in the regulation of the human calcyclin (S100A6) gene. *Biochim Biophys Acta* **1517**, 73-81 (2000).
275. Lesniak, W. & Kuznicki, J. Binding and functional characteristics of two E-box motifs within the S100A6 (calcyclin) gene promoter. *J Cell Biochem* **97**, 1017-1024 (2006).
276. Lesniak, W., Szczepanska, A. & Kuznicki, J. Calcyclin (S100A6) expression is stimulated by agents evoking oxidative stress via the antioxidant response element. *Biochim Biophys Acta* **1744**, 29-37 (2005).
277. Joo, J. H. *et al.* Involvement of NF-kappaB in the regulation of S100A6 gene expression in human hepatoblastoma cell line HepG2. *Biochem Biophys Res Commun* **307**, 274-280 (2003).
278. Lesniak, W., Slomnicki, L. P. & Kuznicki, J. Epigenetic control of the S100A6 (calcyclin) gene expression. *J Invest Dermatol* **127**, 2307-2314 (2007).
279. Filipek, A., Wojda, U. & Lesniak, W. Interaction of calcyclin and its cyanogen bromide fragments with annexin II and glyceraldehyde 3-phosphate dehydrogenase. *Int J Biochem Cell Biol* **27**, 1123-1131 (1995).
280. Zeng, F. Y., Gerke, V. & Gabius, H. J. Identification of annexin II, annexin VI and glyceraldehyde-3-phosphate dehydrogenase as calcyclin-binding proteins in bovine heart. *Int J Biochem* **25**, 1019-1027 (1993).
281. Minami, H. *et al.* Specific binding of CAP-50 to calcyclin. *FEBS Lett* **305**, 217-219 (1992).
282. Tokumitsu, H., Mizutani, A., Minami, H., Kobayashi, R. & Hidaka, H. A calcyclin-associated protein is a newly identified member of the Ca2+/phospholipid-binding proteins, annexin family. *J Biol Chem* **267**, 8919-8924 (1992).
283. Sudo, T. & Hidaka, H. Regulation of calcyclin (S100A6) binding by alternative splicing in the N-terminal regulatory domain of annexin XI isoforms. *J Biol Chem* **273**, 6351-6357 (1998).
284. Sudo, T. & Hidaka, H. Characterization of the calcyclin (S100A6) binding site of annexin XI-A by site-directed mutagenesis. *FEBS Lett* **444**, 11-14 (1999).
285. Mani, R. S. & Kay, C. M. Effect of caltropin on caldesmon-actin interaction. *J Biol Chem* **270**, 6658-6663 (1995).
286. Mani, R. S., McCubbin, W. D. & Kay, C. M. Calcium-dependent regulation of caldesmon by an 11-kDa smooth muscle calcium-binding protein, caltropin. *Biochemistry* **31**, 11896-11901 (1992).
287. Filipek, A., Zasada, A., Wojda, U., Makuch, R. & Dabrowska, R. Characterization of chicken gizzard calcyclin and examination of its interaction with caldesmon. *Comp Biochem Physiol B Biochem Mol Biol* **113**, 745-752 (1996).
288. Golitsina, N. L., Kordowska, J., Wang, C. L. & Lehrer, S. S. Ca2+-dependent binding of calcyclin to muscle tropomyosin. *Biochem Biophys Res Commun* **220**, 360-365 (1996).
289. Breen, E. C. & Tang, K. Calcyclin (S100A6) regulates pulmonary fibroblast proliferation, morphology, and cytoskeletal organization in vitro. *J Cell Biochem* **88**, 848-854 (2003).
290. Wills, F. L., McCubbin, W. D. & Kay, C. M. Smooth muscle calponin-caltropin interaction: effect on biological activity and stability of calponin. *Biochemistry* **33**, 5562-5569 (1994).
291. Filipek, A. & Wojda, U. Chicken gizzard calcyclin--distribution and potential target proteins. *Biochem Biophys Res Commun* **225**, 151-154 (1996).

292. Filipek, A. & Wojda, U. p30, a novel protein target of mouse calcyclin (S100A6). *Biochem J* **320 (Pt 2)**, 585-587 (1996).
293. Filipek, A. & Kuznicki, J. Molecular cloning and expression of a mouse brain cDNA encoding a novel protein target of calcyclin. *J Neurochem* **70**, 1793-1798 (1998).
294. Nowotny, M. *et al.* Characterization of the interaction of calcyclin (S100A6) and calcyclin-binding protein. *J Biol Chem* **275**, 31178-31182 (2000).
295. Matsuzawa, S. I. & Reed, J. C. Siah-1, SIP, and Ebi collaborate in a novel pathway for beta-catenin degradation linked to p53 responses. *Mol Cell* **7**, 915-926 (2001).
296. Ning, X. *et al.* Calcyclin-binding protein inhibits proliferation, tumorigenicity, and invasion of gastric cancer. *Mol Cancer Res* **5**, 1254-1262 (2007).
297. Sun, S. *et al.* Overexpressed CacyBP/SIP leads to the suppression of growth in renal cell carcinoma. *Biochem Biophys Res Commun* **356**, 864-871 (2007).
298. Filipek, A., Jastrzebska, B., Nowotny, M. & Kuznicki, J. CacyBP/SIP, a calcyclin and Siah-1-interacting protein, binds EF-hand proteins of the S100 family. *J Biol Chem* **277**, 28848-28852 (2002).
299. Nowotny, M. *et al.* Calcium-regulated interaction of Sgt1 with S100A6 (calcyclin) and other S100 proteins. *J Biol Chem* **278**, 26923-26928 (2003).
300. Niki, I., Okazaki, K., Iino, S., Kobayashi, S. & Hidaka, H. Calcyclin, a calcium-binding protein, which regulates insulin secretion from the permeabilized pancreatic beta-cell. *Adv Exp Med Biol* **426**, 85-89 (1997).
301. Okazaki, K., Niki, I., Iino, S., Kobayashi, S. & Hidaka, H. A role of calcyclin, a Ca(2+)-binding protein, on the Ca(2+)-dependent insulin release from the pancreatic beta cell. *J Biol Chem* **269**, 6149-6152 (1994).
302. Farnsworth, R. L. & Talamantes, F. Calcyclin in the mouse decidua: expression and effects on placental lactogen secretion. *Biol Reprod* **59**, 546-552 (1998).
303. Thordarson, G., Southard, J. N. & Talamantes, F. Purification and characterization of mouse decidual calcyclin: a novel stimulator of mouse placental lactogen-II secretion. *Endocrinology* **129**, 1257-1265 (1991).
304. Timmons, P. M., Chan, C. T., Rigby, P. W. & Poirier, F. The gene encoding the calcium binding protein calcyclin is expressed at sites of exocytosis in the mouse. *J Cell Sci* **104 (Pt 1)**, 187-196 (1993).
305. Mbeunkui, F., Metge, B. J., Shevde, L. A. & Pannell, L. K. Identification of differentially secreted biomarkers using LC-MS/MS in isogenic cell lines representing a progression of breast cancer. *J Proteome Res* **6**, 2993-3002 (2007).
306. Leclerc, E., Fritz, G., Weibel, M., Heizmann, C. W. & Galichet, A. S100B and S100A6 differentially modulate cell survival by interacting with distinct RAGE (receptor for advanced glycation end products) immunoglobulin domains. *J Biol Chem* **282**, 31317-31331 (2007).
307. Huttunen, H. J. *et al.* Coregulation of neurite outgrowth and cell survival by amphoterin and S100 proteins through receptor for advanced glycation end products (RAGE) activation. *J Biol Chem* **275**, 40096-40105 (2000).
308. Ghavami, S. *et al.* S100A8/A9 at low concentration promotes tumor cell growth via RAGE ligation and MAP kinase-dependent pathway. *J Leukoc Biol*, (2008).
309. Joo, J. H. *et al.* S100A6 (calcyclin) enhances the sensitivity to apoptosis via the upregulation of caspase-3 activity in Hep3B cells. *J Cell Biochem* **103**, 1183-1197 (2008).
310. Leong, S., Christopherson, R. I. & Baxter, R. C. Profiling of apoptotic changes in human breast cancer cells using SELDI-TOF mass spectrometry. *Cell Physiol Biochem* **20**, 579-590 (2007).
311. Bunz, F. *et al.* Requirement for p53 and p21 to sustain G2 arrest after DNA damage. *Science* **282**, 1497-1501 (1998).
312. Cox, J. & Mann, M. Is proteomics the new genomics? *Cell* **130**, 395-398 (2007).
313. Orre, L. M. *et al.* Rad51-related changes in global gene expression. *Biochem Biophys Res Commun* **341**, 334-342 (2006).

314. Linke, S. P. *et al.* p53 interacts with hRAD51 and hRAD54, and directly modulates homologous recombination. *Cancer Res* **63**, 2596-2605 (2003).
315. Yun, S., Lie, A. Cheong C. & Porter, A. C. Discriminatory suppression of homologous recombination by p53. *Nucleic Acids Res* **32**, 6479-6489 (2004).
316. Klein, H. L. The consequences of Rad51 overexpression for normal and tumor cells. *DNA Repair (Amst)*, (2008).
317. Bree, R. T., Neary, C., Samali, A. & Lowndes, N. F. The switch from survival responses to apoptosis after chromosomal breaks. *DNA Repair (Amst)* **3**, 989-995 (2004).
318. Mine, J. *et al.* Real-time measurements of the nucleation, growth and dissociation of single Rad51-DNA nucleoprotein filaments. *Nucleic Acids Res* **35**, 7171-7187 (2007).
319. Arias-Lopez, C. *et al.* p53 modulates homologous recombination by transcriptional regulation of the RAD51 gene. *EMBO Rep* **7**, 219-224 (2006).
320. Kovalenko, O. V. *et al.* Mammalian ubiquitin-conjugating enzyme Ubc9 interacts with Rad51 recombination protein and localizes in synaptonemal complexes. *Proc Natl Acad Sci U S A* **93**, 2958-2963 (1996).
321. Shen, Z., Pardington-Purtymun, P. E., Comeaux, J. C., Moyzis, R. K. & Chen, D. J. UBL1, a human ubiquitin-like protein associating with human RAD51/RAD52 proteins. *Genomics* **36**, 271-279 (1996).
322. Bennett, B. T. & Knight, K. L. Cellular localization of human Rad51C and regulation of ubiquitin-mediated proteolysis of Rad51. *J Cell Biochem* **96**, 1095-1109 (2005).
323. Sargent, R. G., Brenneman, M. A. & Wilson, J. H. Repair of site-specific double-strand breaks in a mammalian chromosome by homologous and illegitimate recombination. *Mol Cell Biol* **17**, 267-277 (1997).
324. Bohnke, A., Westphal, F., Schmidt, A., El-Awady, R. A. & Dahm-Daphi, J. Role of p53 mutations, protein function and DNA damage for the radiosensitivity of human tumour cells. *Int J Radiat Biol* **80**, 53-63 (2004).
325. Kennedy, A. S. *et al.* Survival of colorectal cancer cell lines treated with paclitaxel, radiation, and 5-FU: effect of TP53 or hMLH1 deficiency. *Int J Cancer* **90**, 175-185 (2000).
326. Cimoli, G. *et al.* Meta-analysis of the role of p53 status in isogenic systems tested for sensitivity to cytotoxic antineoplastic drugs. *Biochim Biophys Acta* **1705**, 103-120 (2004).
327. Yuan, Z. M. *et al.* p73 is regulated by tyrosine kinase c-Abl in the apoptotic response to DNA damage. *Nature* **399**, 814-817 (1999).
328. Irwin, M. S. Family feud in chemosensitvity: p73 and mutant p53. *Cell Cycle* **3**, 319-323 (2004).
329. Ianzini, F., Bertoldo, A., Kosmacek, E. A., Phillips, S. L. & Mackey, M. A. Lack of p53 function promotes radiation-induced mitotic catastrophe in mouse embryonic fibroblast cells. *Cancer Cell Int* **6**, 11 (2006).
330. Ianzini, F., Domann, F. E., Kosmacek, E. A., Phillips, S. L. & Mackey, M. A. Human glioblastoma U87MG cells transduced with a dominant negative p53 (TP53) adenovirus construct undergo radiation-induced mitotic catastrophe. *Radiat Res* **168**, 183-192 (2007).
331. Mueller, A., Bachi, T., Hochli, M., Schafer, B. W. & Heizmann, C. W. Subcellular distribution of S100 proteins in tumor cells and their relocation in response to calcium activation. *Histochem Cell Biol* **111**, 453-459 (1999).
332. Stradal, T. B. & Gimona, M. Ca(2+)-dependent association of S100A6 (Calcyclin) with the plasma membrane and the nuclear envelope. *J Biol Chem* **274**, 31593-31596 (1999).
333. Garrett, S. C., Varney, K. M., Weber, D. J. & Bresnick, A. R. S100A4, a mediator of metastasis. *J Biol Chem* **281**, 677-680 (2006).
334. Soussi, T. p53 alterations in human cancer: more questions than answers. *Oncogene* **26**, 2145-2156 (2007).
335. Burke, L. *et al.* Prognostic implications of molecular and immunohistochemical profiles of the Rb and p53 cell cycle regulatory pathways in primary non-small cell lung carcinoma. *Clin Cancer Res* **11**, 232-241 (2005).

336. Tomizawa, Y. *et al.* Correlation between the status of the p53 gene and survival in patients with stage I non-small cell lung carcinoma. *Oncogene* **18**, 1007-1014 (1999).
337. Kwiatkowski, D. J. *et al.* Molecular pathologic substaging in 244 stage I non-small-cell lung cancer patients: clinical implications. *J Clin Oncol* **16**, 2468-2477 (1998).
338. Quinlan, D. C., Davidson, A. G., Summers, C. L., Warden, H. E. & Doshi, H. M. Accumulation of p53 protein correlates with a poor prognosis in human lung cancer. *Cancer Res* **52**, 4828-4831 (1992).
339. Kleijnen, M. F., Alarcon, R. M. & Howley, P. M. The ubiquitin-associated domain of hPLIC-2 interacts with the proteasome. *Mol Biol Cell* **14**, 3868-3875 (2003).
340. Kleijnen, M. F. *et al.* The hPLIC proteins may provide a link between the ubiquitination machinery and the proteasome. *Mol Cell* **6**, 409-419 (2000).
341. Feng, P. *et al.* Kaposi's sarcoma-associated herpesvirus K7 protein targets a ubiquitin-like/ubiquitin-associated domain-containing protein to promote protein degradation. *Mol Cell Biol* **24**, 3938-3948 (2004).
342. Bohuslav, J., Chen, L. F., Kwon, H., Mu, Y. & Greene, W. C. p53 induces NF-kappaB activation by an IkappaB kinase-independent mechanism involving phosphorylation of p65 by ribosomal S6 kinase 1. *J Biol Chem* **279**, 26115-26125 (2004).
343. Ryan, K. M., Ernst, M. K., Rice, N. R. & Vousden, K. H. Role of NF-kappaB in p53-mediated programmed cell death. *Nature* **404**, 892-897 (2000).
344. Shirangi, T. R., Zaika, A. & Moll, U. M. Nuclear degradation of p53 occurs during down-regulation of the p53 response after DNA damage. *Faseb J* **16**, 420-422 (2002).
345. Wang, Y. *et al.* NF-kappa B2 p100 is a pro-apoptotic protein with anti-oncogenic function. *Nat Cell Biol* **4**, 888-893 (2002).