Chapter 1

Introduction: Signaling Networks in Living Cells

1.1 Brief Introduction to Molecular Cell Biology

The chemistry of life, mostly referred to as biochemistry, determines the fate of all organisms, may they be as small and simple as viruses and bacteria, or as complex as humans and other multicellular animals or plants. Life on a molecular level is characterized by the dynamic synthesis and breakdown of proteins, nucleic acids, carbohydrates, membranes and other cellular components. Although highly demanding with respect to energy consumption and throughput of molecular material, this enables cells or multicellular organisms to cope with environmental changes, challenges and physical threats, and thereby allows them to improve the probability of surviving. Most living organisms form complex and precisely regulated networks of molecular assemblies and functional interactions, the human brain representing probably one of the most complex and impressive achievements in biological evolution.

Amino acids, nucleotides, monosaccharides, lipids and other low-molecular compounds, with the exception of some high-energy intermediates, are very stable due to the nature of the chemical bonds involved. This applies likewise to the linkage of biochemical polymers, such as proteins, nucleic acids and polysaccharides. In fact, the amide bond (or peptide bond) in proteins, the phosphodiester linkage in nucleic acids, and the glycosidic bond in sugar polymers are among the most stable bonds in biochemistry. However, the three-dimensional (3D) structure of proteins and nucleic acids needs additional stabilization by hydrogen bonds, ionic and hydrophobic interactions which are rather weak compared to covalent chemical bonds. Although most proteins in living cells have high turnover rates leading to rapid degradation of functionally inactive protein molecules and their replacement by protein biosynthesis, isolated proteins and other biochemical compounds may exist for long periods of time, unless they are actively damaged or degraded. Likewise, since every living cell contains the complete set of genetic information, cells could in principle generate

offspring almost forever by means of cell division, thus eventually replacing injured cells whenever there is a need. This applies to most single-cell organisms. Higher organisms, however, do not hold the key to endless rounds of cell division and thus cannot multiply indefinitely. Instead multicellular organisms undergo the processes of ageing and death, both on the level of cells and the whole organism. To date, three processes are considered the molecular basis of ageing, each of them resulting in loss of gene function and their main transcripts, the proteins: (i) randomly accumulating mutations in DNA and/or RNA during replication and/or transcription, (ii) DNA damage, followed by impaired replication and cell death due to high-energy radiation, free radicals, often referred to as reactive oxygen species (ROS), and other toxic compounds, and (iii) shortening of chromosomal telomeres upon multiple rounds of replication and cell division. In this model, ageing and death are the direct consequence of the functional knockout of proteins and other vital cellular components.

On a cellular level, DNA damage may trigger cell cycle checkpoints to allow time for DNA repair. However, if the repair machinery fails to restore genetic integrity, the cellular DNA damage response may also induce senescence, a permanent block of cell division, and apoptosis, the cellular suicide program. In addition the cellular diversity of higher organisms includes also cells that divide only within certain time windows during development, and even cells such as some neurons that are thought to remain in a permanent non-dividing state. Interestingly, progress in stem cell research over recent years has provided strong evidence that even in adults certain differentiated damaged cells, including neurons, may become eventually replaced by stem cells present in the tissue. Although this offers the intriguing possibility of ultimate cure from disease and ageing, the pool of adult stem cells apparently ceases over time. Thus, ageing and death may be considered the result of an incorrect or impaired process of constant renewal both on the molecular and cellular level. However, higher eukaryotes hold at least the key to constant renewal on the organism's level, and this is where sexual reproduction comes into play. Each time the parental genetic material stored in the germ cells is combined and transferred to the offspring the molecular/cellular clock of ageing is literally reset to start.

1.1.1 Cell Cycle Regulation and Cancer Development

Cell division, proliferation and differentiation are probably among the most complex molecular processes in living nature. They are not only tightly regulated but also precisely synchronized with vital cellular mechanisms such as replication and DNA repair, or even with the hormone status of a multicellular organism. In higher organisms, the cell cycle follows a scheme with clearly distinct parts or phases, starting with a gap phase (G_1), followed by the phase in which DNA synthesis (replication) takes place (S), a second gap phase (G_2), and the phase of cell division (mitosis, M) (Figure 1).

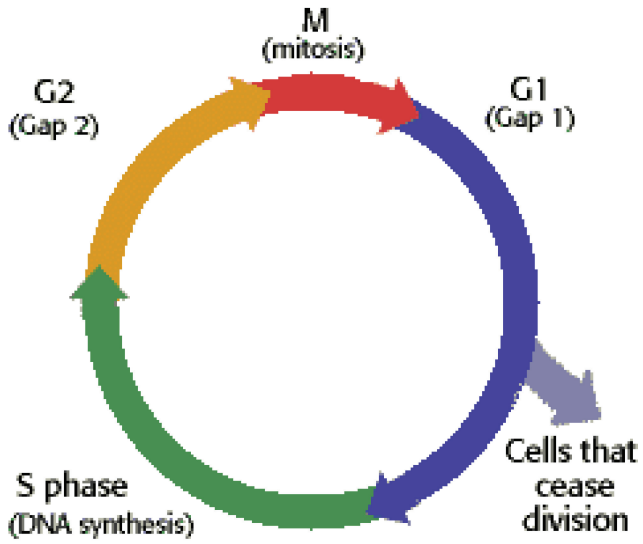

Figure 1: The eukaryotic cell cycle.
Schematic depiction of the different cell cycle phases which comprise DNA replication (green), mitosis (red), and two gap phases G1 and G2 (blue and yellow, respectively). Cell cycle progression is tightly linked to DNA repair to preserve genomic integrity. Cell cycle checkpoints (G_1-S, intra-S, G2-M, intra-M) functions as molecular emergency brakes in response to DNA damage. Figure adopted from The Biology Project, University of Arizona, Department of Biochemistry and Molecular Biophysics (http://www.biology.arizona.edu/).

The by far most critical phases are S and M, in which the chromosomal DNA is duplicated and transferred to the daughter cells, respectively. To ensure that these processes proceed with the highest possible fidelity, cell cycle checkpoints are established at the G_1-S and the G_2-M transitions. If DNA synthesis is expected to be error-prone, cells are arrested at G_1-S. Similarly, the G_2-M arrest prevents cells from starting cell division if DNA synthesis and repair is not fully completed. In addition the intra-S checkpoint is activated in response to DNA damage or if the replication fork stalls and DNA synthesis is impaired, whereas the fidelity of chromosome separation during mitosis is monitored by the metaphase (mitotic spindle assembly) checkpoint at the metaphase to anaphase transition which effectively surveys the attachment of the mitotic spindle to the kinetochore.

Cancer development appears to be closely linked to a permanent failure of cell cycle regulation and the loss of cellular fail-safe mechanisms such as senescence and apoptosis to avoid oncogenic transformation. Most cancer types become invasive due to checkpoint inactivation, ionizating radiation-resistant DNA synthesis and premature mitosis. This is most deleterious if the DNA damage repair machinery is impaired or error-prone as failure of these molecular emergency breaks allows mutations to be transferred to daughter chromatides and cellular offspring. Cancer cells are thus characterized by increased mutation rates, uncontrolled cell division and genomic instability. In fact, genomic instability is considered both the consequence and the prerequisite of high mutation rates. This ultimately generates genetically diverse daughter cells which may gain resistance to the deadly toxic chemicals administered to affected patients in the course of chemotherapy.

Cell-Cycle Regulatory Circuits

Although the underlying mechanism of cell division and cell cycle regulation is very similar in various cell types, the expression profile and molecular function of cell-type specific tumor suppressor proteins may differ. Cell viability is closely linked to the fine-tuning of pro-/anti-*apoptotic* signals modulated by the antagonistic effects of pro-/anti-*proliferative* signals (Figure 2). Overriding pro-mitotic/oncogenic signals are coupled to regulatory circuits that activate cell cycle checkpoints, thereby inducing ***quiescence*** (reversible cell cycle arrest), ***senescence*** (permanent cell cycle arrest) or even ***apoptosis*** (programmed cell death). By that, cell fate apparently relies on the balance between proliferation, cell cycle arrest, senescence and apoptosis, and thus critically depends on the innate network of signal transduction pathways and the cellular microenvironment [1-4].

The transcription factor p53 apparently plays the role of a master regulator in response to different types of cellular stress [1,5]. Most signal transduction networks driving cell proliferation are part of intricate regulatory circuits that couple cell division closely to growth inhibition and apoptosis (Figure 2a). Thus, multiple pathways are required to promote either cell growth or apoptosis, and activation of a single anti-apoptotic, mitogenic pathway upon oncogenic mutations is effectively trapped by counter-acting pathways that induce senescence and apoptosis to block neoplastic progression. Interestingly, during development signals may also lead to cell cycle arrest and even cell death.

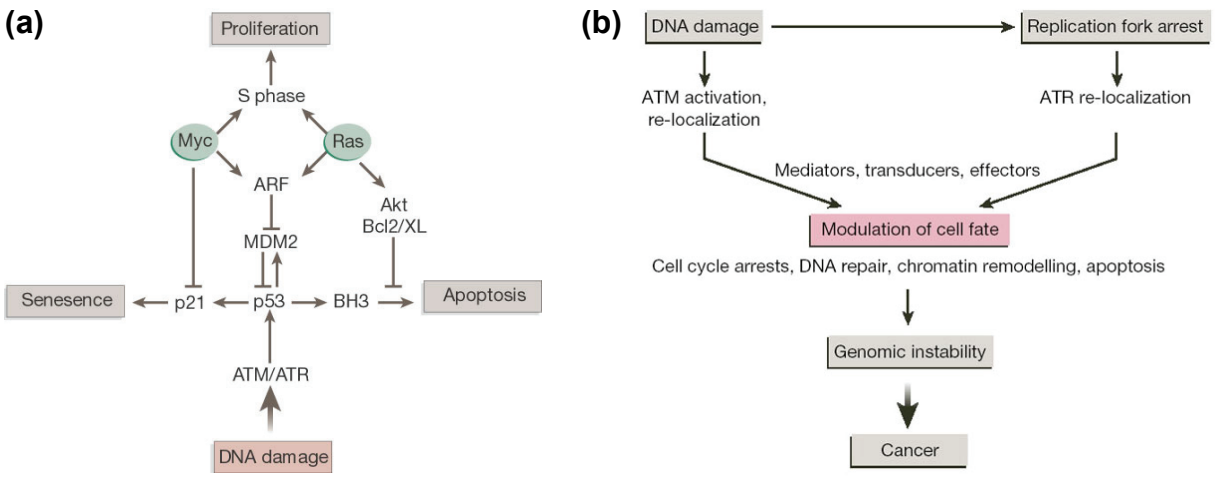

Figure 2: DNA damage, cell cycle regulatory circuits and cancer development.
DNA lesions activate signal transduction pathways that are coupled to cell cycle regulation, DNA repair, and cell fate modulation. **(a)** DNA damage leads to ATM and ATR activation and subsequent phosphorylation of down-stream signal transducers which are functionally linked to cell cycle regulation and DNA repair. Myc and Ras may drive proliferation or trigger apoptosis and senescence, depending on the balance of pro-/anti-*proliferative* and pro-/anti-*apoptotic* signals and the cellular microenvironment. **(b)** The DNA damage response results in cell cycle checkpoint activation and triggers DNA repair mechanisms. Impaired or error-prone DNA repair may results in genetic instability and cancer. Figures adopted from [1,2].

Caretakers and Gatekeepers

The current model of cancer development implies that accumulation of DNA damages ultimately results in the functional knock-out of key proteins involved in DNA repair and/or cell cycle regulation. According to the classification of Vogelstein and Kinzler, proteins harboring tumor suppressor functions may act as ***caretakers*** or as ***gatekeepers*** [6,7].

The former care for the genomic integrity and prevent the DNA from accumulating mutations and replication errors. Caretaker proteins mostly belong to the DNA repair machinery. They assist in homologous recombination (HR), sister chromatid exchange (SEC), mismatch and base excision repair, or other molecular mechanisms involved to protect and/or cure the DNA from radiation damage, free radicals and other toxic compounds entering the cell. In contrast, gatekeeper proteins act on the cellular level by performing cell cycle control functions. They build an intricate network of cell cycle checkpoint proteins comprising kinases and phosphatases, proteases, transcription factors, and others, many of which are still not even known. The activity of cell cycle checkpoint proteins is precisely regulated by phosphorylation/de-phosphorylation or other mechanisms of transient activation/de-activation. They may form multi-protein complexes to synchronize high-fidelity DNA-repair with replication, mitosis and cell division.

1.1.2 The DNA Damage Response

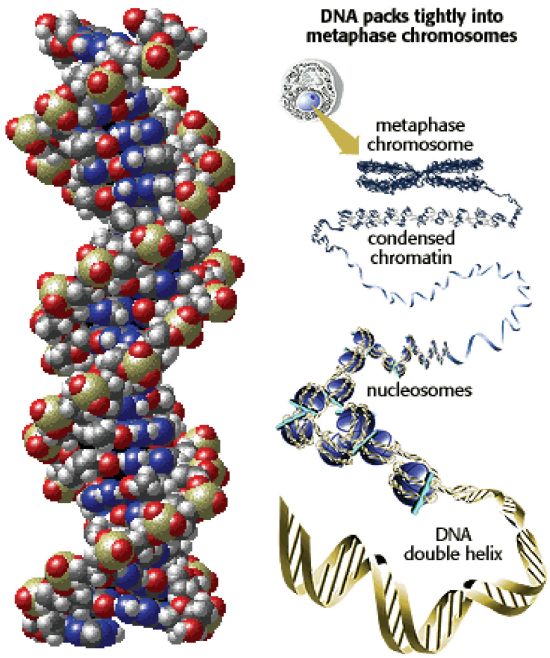

Figure 3: Molecular structure and chromatid organization of DNA.
Space-filling model of double-stranded B-DNA (left) and different levels of DNA compaction in the cell nucleus (right). The double helix is coiled up on nucleosomes to form higher order structures, such as condensed chromatin and chromosomes. Figure adopted from: The Biology Project, University of Arizona, Department of Biochemistry and Molecular Biophysics (http://www.biology.arizona.edu/).

Precisely regulated cell division and accurate transmission of genetic information is vital to the survival of every organism. However, DNA may be subject to replication errors, double-strand breaks (DSBs) and other defects caused by the deleterious effects of free radicals, radioactive decay products, ultra-violet (UV) or ionizating radiation (IR). To minimize errors during replication, chromosome segregation and daughter cell separation, cells employ a number of molecular surveillance mechanisms that align cell cycle progression with DNA repair. Therefore the cellular DNA damage response may be considered a network of signal transduction pathways initiated by different kinds of DNA damage which together function as a cell cycle emergency brake to allow time for repair. There is growing evidence that in addition to checkpoint activation and cell cycle arrest, the DNA damage response involves also the immediate recruitment and assembly of repair proteins at the site(s) of DNA damage. In the case of a virtual overkill of DNA damage and the functional knock-out of the repair machinery, cells may undergo apoptosis which although fatal for the affected cell, prevents accumulation of mutations and oncogenic transformation, thus allowing the organism to survive as a whole.

DNA damage signaling involves different proteins that become consecutively activated to trigger a cellular response [8]. On the top are ***DNA damage sensors*** that probably become activated upon binding to aberrant DNA structures (Figure 4). However, the identity of DNA damage sensors still largely remains a matter of uncertainty. Candidate sensor proteins are expected to recognize non-native or damaged DNA structures, such as DSBs and single-stranded DNA, and to induce DNA damage response pathways, typically through phosphorylation of ATM and ATR kinases (ataxia telangiectasia-mutated and ataxia telangiectasia- and RAD3-related, respectively). A series of reconstitution experiments identified components of the Mre11-Rad50-Nbs1 (MRN) complex as potential DBS sensor. DNA damage sensors in turn activate ***signal transducers***, typically through phosphorylation of key Ser/Thr residues. Transducers again contain binding modules or domains that specifically interact with phosphorylated peptide motifs present in other (activated) signaling proteins. Finally, signal transducers activate ***effectors*** that give rise to the cellular response to DNA damage. Recent results suggest that phosphopeptide recognition and binding by FHA (Forkhead-associated) and BRCT (BRCA1 C-terminal) domains is of central importance for the signal transduction in response to DNA damage.

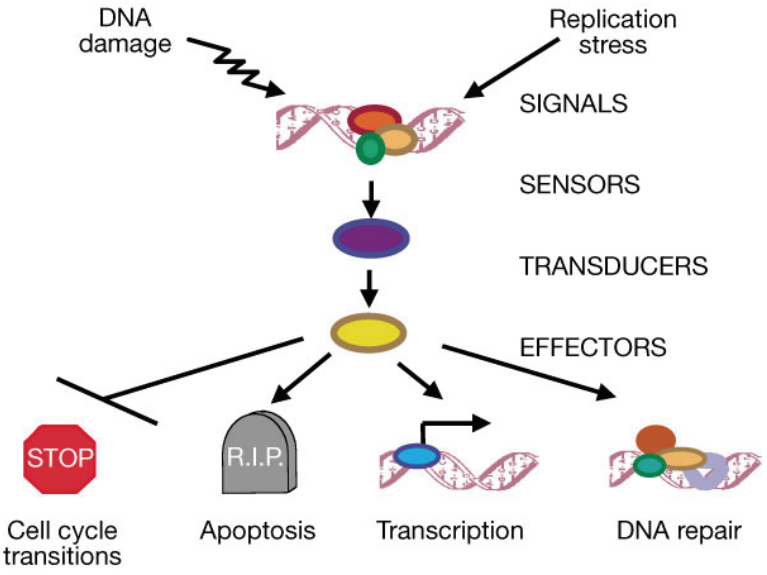

Figure 4: General features of the DNA damage response.
Replication stress and DNA damage may induce various cellular responses, such as checkpoint activation to allow time for DNA repair, regulation of transcription, and apoptosis, the cellular suicide program. For the purpose of simplicity, the network of interacting pathways is depicted as a linear pathway consisting of signals, sensors, transducers and effectors. Figure adopted from [8].

Although a number of key mediators and effectors that become activated in response to DNA damage have been identified, the nature of the DNA damage sensors is largely elusive. Recent data suggest that the trimeric MRN complex may take this role in the case of DNA

double strand breaks [9]. However, the molecular mechanisms inducing the DNA damage response are not fully understood. One key question addresses the issue of DNA damage recognition and asks how the primary DNA lesion is detected. Up to now it is not clear how DNA damage sensors are actually directed to the points of DNA damage, how they detect aberrant DNA structures and how they activate downstream mediator kinases of the cellular DNA damage response, such as the phosphoinositide kinase (PIK) related protein kinases ATM, ATR and the DNA-dependent protein kinase (DNA-PK). Recent results identified conserved C-terminal sequence motifs in Nbs1, ATRIP (ATR interacting protein), and Ku80 proteins which are required for the recruitment and activation of ATM, ATR, and the catalytic subunit of DNA-PK (DNA-PKcs), respectively [10]. Further questions are how lesion-specific DNA repair proteins are recruited to the point of damage, and how the DNA repair process is monitored and ultimately coupled to DNA replication and cell-cycle regulation.

In mammalian cells, ATM and ATR are rapidly activated upon DNA damage (Figures 2 and 5). ATM and ATR may be considered "master kinases" that seem to be on top of partially overlapping signaling pathways which involve phosphorylation and recruitment of signal transducers to the sites of DNA damage. Activated ATM in turn promotes the assembly of the trimeric MRN complex. The latter is part of the BASC complex (BRCA1-associated genome surveillance complex) which involves also ATM, BRCA1, the mismatch repair proteins MSH2/6 and MLH2, Bloom's helicase BLM, and replication factor C (RFC) [11].

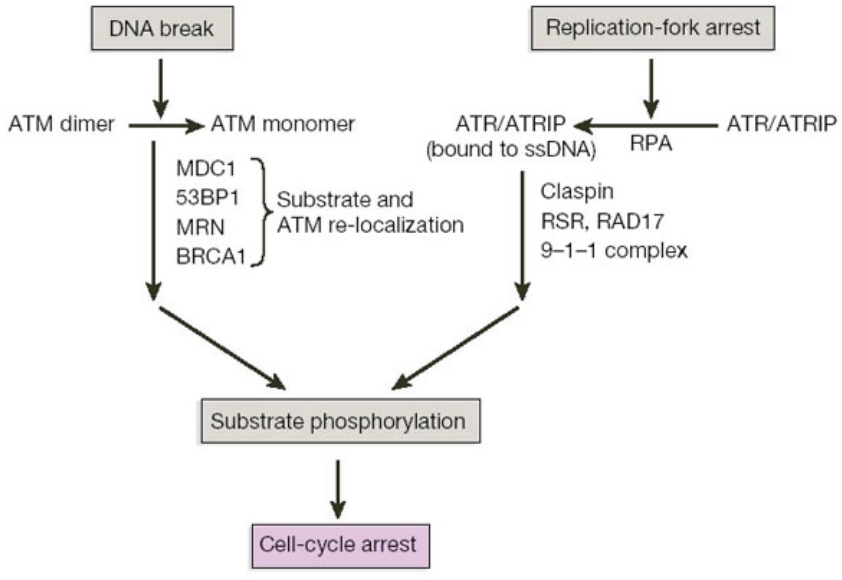

Figure 5: DNA damage induced signal transduction pathways.
Initial DNA damage signals trigger the recruitment and assembly of DNA damage sensors and activation of the master kinases ATM and ATR. Subsequent phosphorylation of signal transducers, such as MDC1, BRCA1 and 53BP1, mediates the signal to downstream effectors (not shown) and induces a cellular response including cell-cycle arrest, DNA repair, or, in rare cases, apoptosis. ATM and ATR dependent pathways are partially over-lapping, thus suggesting functional cross-talk and coupled regulation. Figure adopted from [2].

The MRN complex has been shown to assemble at sites of DNA damage and to activate ATM kinase in response to DSBs [12], probably through a C-terminal segment of the Nbs1 component [13,14]. Moreover, degradation of the MRN complex prevents auto-phosphorylation of ATM and ATM-dependent G_2-M checkpoint activation. MRN functions upstream of ATM and localizes to sites of DNA lesions very early upon damaging. Thus, MRN is considered a candidate DNA damage sensor. This view is supported by earlier results showing that the MRN complex binds to double strand breaks *in vitro* [15]. DNA repair may involve homologous recombination (HR) and/or non-homologous end joining (NHEJ) in the case of DSBs, but also base excision repair of cyclobutane dimers. Notably, the tumor suppressor protein BRCA1 is not only involved in DNA damage signaling but also in DNA repair by supporting HR [16,17]. DNA repair may additionally require up-regulation of dNTP synthesis and the activation of cell cycle checkpoint proteins.

In general, regulation takes place both on the level of gene expression and protein biosynthesis and turnover. Activation/inactivation of signal transduction pathways is accomplished by the action of kinases/phosphatases and phosphorylation/de-phosphorylation of transducer and effector proteins. In some cases, such as with the universal tumor suppressor protein p53, target inactivation is mediated by ubiquitinylation and subsequent proteolytic degradation. Interestingly, BRCA1 exhibits an E3 ubiquitin ligase activity in complex with the BRCA1-associated RING domain protein 1 (BARD1) [18-20]. The intricate network of DNA repair mechanisms is schematically depicted in Figure 6.

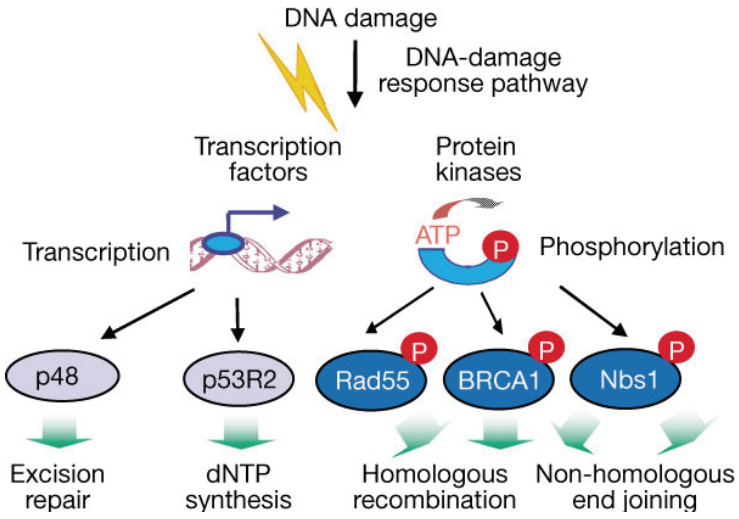

Figure 6: The regulatory network of DNA repair mechanisms.
Phosphorylation of BRCT proteins, such as BRCA1 and Nbs1, by the phosphoinositide kinase (PIK) related protein kinases ATM and ATR is central to DNA damage signaling and activation of DNA repair. DNA double-strand breaks (DSBs) finally induce homologous recombination (HR) and/or non-homologous end joining (NHEJ), whereas base excision repair is employed to remove cyclobutane dimers, the reaction products of UV-induced crosslinks between adjacent nucleobases. Figure adopted from [8].

Chemotherapy and Cancer Treatment

The cellular suicide program apoptosis may be considered a safety mechanism for cells suffering from extensive DNA damage and impaired replication to avoid neoplastic transformation and cancer development. Unfortunately tumor cells often acquire mutations that inactivate pro-apoptotic pathways. Most drugs used in chemotherapy have pronounced cytotoxic and/or cytostatic effects, causing severe DNA damage and detrimental interference with vital cellular functions such as metabolism, DNA replication and cell division. Based on early observations that tumor cells proliferate faster than other cells, chemotherapy has been (and still is) often considered a rational approach for the treatment of cancer. However, chemotherapeutic drugs are by no means specific for cancer cells, thus affecting basically every (proliferating) cell in the organism. Although this kind of shotgun method has been proven fairly successful in the case of testicular cancer and some forms of leukemia, the results with most other (solid) tumors are far from what might be considered a successful "therapy", let alone a true cure of disease. This is due to a number of "side-effects", such as the lack of specificity and consequently the general toxicity of chemotherapeutic drugs, and the occurrence of drug resistant tumor cells that may evolve due to their higher mutation rate and the increased adaptability to cellular stress. The latter may cause a cellular response that is different in normal and neoplastic cells. The most critical response to DNA damage and cellular stress is cell cycle arrest and, in the case of severe DNA lesions, apoptosis. Although tightly regulated in normal cells, the functional knockout of pro-apoptotic signaling, combined with oncogene activation and loss of cell cycle checkpoint functions, confers a selective growth advantage to cancer cells and strongly promotes further genomic instability [21]. Thus, despite the cytostatic effect of anticancer drugs, the increase in tumor burden and the genetic diversity of cellular offspring may result in the selection of resistant and thereby more malignant subclones with a defect in drug-induced apoptotic signaling. The challenges of modern cancer treatment are reviewed in [22].

Following these considerations, it is highly desirable to identify the regulatory circuits that become activated upon drug-induced cellular stress, and to get a detailed knowledge about the nature of death pathways and response mechanisms that confer drug resistance. This is even more important as some anti-cancer drugs are known to be completely ineffective in promoting cell death in certain resistant tumor cells which have acquired genetic modifications and therefore do not rely on the specific drug target. Thus, successful cancer treatment in the future might consider the genetic background of the tumor and the use of tumor-type specific drugs if chemotherapy is to be applied. This view is further explored in [23-26].

1.1.3 BRCT Proteins: Molecular Transducers of DNA Damage Signaling

Growing evidence strongly points at BRCT proteins as components of central importance for DNA damage signaling and cell cycle regulation. BRCT domains were originally identified as a novel domain family present in key proteins that are involved in DNA repair and/or cell cycle regulation, such as the breast cancer associated tumor suppressor protein BRCA1, the cell-cycle checkpoint protein MDC1, the p53-binding protein 53BP1, and Nbs1 (Nijmegen breakage syndrome protein 1) that is part of the MRN complex, a candidate DNA damage sensor (Figure 6). The key function of tandem BRCT repeat modules appears to be in the assembly of protein complexes through interaction with phosphorylated peptide (pPep) motifs [27]. The conserved recognition sequence comprises phosphoserine (pSer) or phosphothreonine (pThr), and at least one additional, usually hydrophobic amino acid residue at or near the position pSer/Thr(+3). Many BRCT proteins are implicated in DNA repair, cell cycle regulation and tumor suppression, such as BRCA1, MDC1, 53BP1, and Nbs1. Mutations in the BRCT region are frequently related to loss of checkpoint activation, genomic instability and cancer development. In principle the cell-type specific expression profile of the phosphopeptide binding modules might be used for precise fine-tuning of the DNA damage signal transduction and checkpoint activation in different tissues.

Recent studies show MDC1 to act as key mediator of ATM-dependent DNA damage signals up-stream of BRCA1 and other components of the cellular DNA damage response [28,29]. MDC1 interacts with ATM kinase and phosphorylated histone H2AX through the tandem BRCT repeat and its FHA domain, respectively. The proposed model involves a DNA damage sensor, possibly the MRN complex which recruits ATM to DSBs [30]. Recruitment of ATM kinase to damaged DNA sites upon initial DNA damage signals leads to autophosphorylation and activation of the ATM kinase function, possibly through the interaction with Nbs1 [13], followed by rapid expansion of H2AX phosphorylation. This in turn changes the nearby chromatin structure, allowing direct access of additional signal transducers and repair proteins. Thus MDC1 mediates amplification of the DNA damage signal by acting as a molecular bridge between ATM and H2AX, thereby accumulating ATM and its downstream substrates (BRCA1, 53BP1, and others) at the sites of DNA damage (Figure 6). The DNA damage induced signaling cascade in turn provokes a cellular response comprising DNA repair and cell cycle checkpoint activation.

The Tumor Suppressor Protein BRCA1

In 1994, the highly competitive search for genes correlated with the development of breast and ovarian cancer led to the discovery of *BRCA1* (breast cancer susceptibility gene 1) and its primary transcript, the tumor suppressor protein BRCA1 [31]. Sequence analysis of the 1863 amino-acid polypeptide first revealed two regions that were predicted to be potentially structured: (i) a RING domain near the N-terminus, and (ii) a tandem repeat of two homologous domains close to the C-terminus of BRCA1 [32,33]. The latter were considered novel domains, termed BRCT domains (BRCA1 C-terminal domains), and were found to be unrelated in sequence to any other known protein domains.

Since the discovery of BRCA1 and other proteins harboring BRCT sequence motifs, scientists aimed at the elucidation of both the molecular properties and the cellular functions of BRCT domains. Early studies revealed that the BRCT repeats from BRCA1 may activate transcription when fused to the DNA binding domain (DBD) of the yeast transcription factor Gal4, and that this function was lost when breast cancer associated mutations were present in the BRCT repeats [34-36]. In addition, the second BRCT domain (referred to here as BRCT-c) was found to possess a (reduced) transcriptional activation function on its own, i.e. in the complete absence of the first BRCT domain (referred to here as BRCT-n). Intriguingly, mutagenesis studies showed that, in the case of BRCT tandem repeats of BRCA1, some cancer-associated mutations in BRCT-n led to a complete loss of activity even in the presence of intact BRCT-c, thus indicating a close structural and functional interaction between the two BRCT domains.

BRCA1 was shown to play a crucial role in vital cellular functions, such as DNA damage response and DNA repair, homologous recombination, chromatin remodeling, caretaking of genomic integrity, cell cycle checkpoint activation, cancer suppression, stress response and apoptosis [37] (Figure 7). Intriguingly, many of these cellular functions associated with BRCA1 are mediated by the BRCT repeat region. In the search for the molecular features of the BRCA1 BRCT repeats a number of putative binding partners were identified, such as the DNA repair-associated protein BACH1, the retinoblastoma suppressor-associated protein RbAp46, the histone deacetylases HDAC1 and HDAC2, the transcriptional co-repressor CtIP, and the key tumor-suppressor protein p53. In addition, the tandem BRCT repeats were suggested to interact with ends of double-stranded DNA, a structural feature of DSBs. However, the molecular mechanism of BRCT-mediated cell cycle checkpoint activation was largely a black spot when this thesis work was started. As numerous mutations in the BRCT region were strongly correlated to early onset of breast and ovarian cancer the structural features of these novel domains were expected to provide clues as to the clinical impact of

individual mutations. In principle, mutations on the gene level and consequently amino acid changes on the protein level may interfere with protein folding or, alternatively, may abolish essential interactions with target effector molecules, both of which would result in the functional knock-out of the protein. Structure determination of the BRCT domains and the location of binding interfaces therefore might provide a powerful approach toward a deeper understanding of the molecular basis of cancer suppression and the impact of disease related mutations. In addition, knowledge about the structure and function of the BRCA1 BRCT domains might be used to assess unclassified mutations in the *BRCA1* gene and to improve the predictive power of genetic screening tests. This is especially important as it may allow the discrimination between harmless single nucleotide polymorphisms (SNPs) and clinically relevant cancer-associated mutations.

Given the vital role of BRCT domains as transducers of DNA damage signaling and cell cycle checkpoint activation, structural information about the BRCA1 BRCT domains may help to identify conserved sequence and structure motifs which determine the BRCT fold in general and its cellular functions as part of caretaker and gatekeeper proteins. Interestingly, sequence alignment revealed that the fraction of conserved amino acid residues in the BRCT domain family is rather low [32,33]. Although counter-intuitive at first glance, the low sequence homology implicates that the BRCT fold requires rather few conserved amino acid residues to fold properly, leaving much of the sequence free to explore sequence variations that mediate functional diversity.

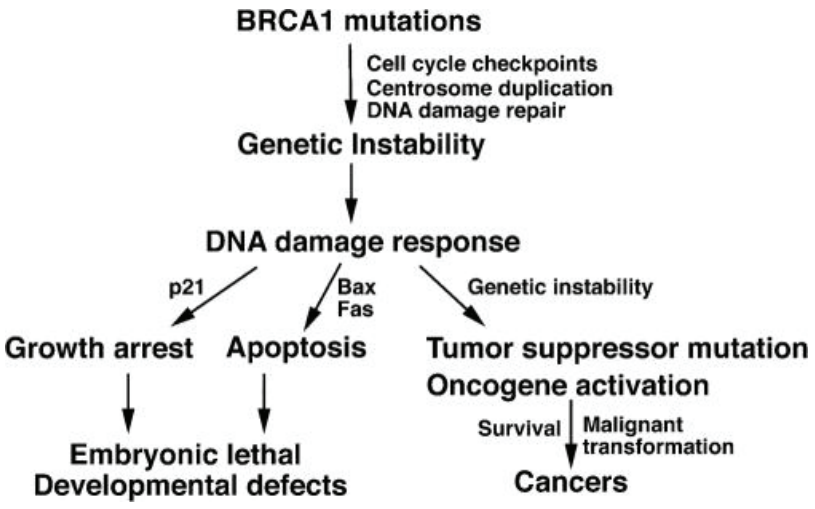

Figure 7: Cellular effects of BRCA1 inactivation.
The schematic model accounts for the central role of BRCA1 as caretaker of genetic integrity and the deleterious effects of BRCA1 mutations. Functional inactivation of BRCA1 causes genetic instability, impaired DNA repair and cell-cycle checkpoint activation. Alternatively, the DNA damage response may turn on growth arrest to allow time for DNA repair, and/or pro-apoptotic pathways. Deficiency of tumor suppressors, such as BRCA1, may stimulate oncogenic transformation and cancer development. Figure adopted from [38].

1.2 Carbohydrate Processing Enzymes

Carbohydrates are the primary reaction products of light-driven photosynthesis and the main energy source for living organisms on earth. Monomeric sugars, such as glucose, may become covalently linked through glycosidic bonds, thus yielding high-molecular weight polysaccharides which are used for energy storage. Chemical breakdown of polysaccharides by enzymatic hydrolysis allows rapid access to glucose and other high-energy compounds.

Polysaccharides differ with respect to the sugar-type of the monomeric building blocks, the linkage-type of the glycosidic bond, and the configuration at the anomeric carbon (C^1). To perform sugar-type specific (de-)polymerization, molecular evolution invented a number of different classes of carbohydrate processing enzymes which selectively act on target substrates with defined stereochemical properties (i.e. anomeric configuration, linkage-type).

1.2.1 Carbohydrate Depolymerization by Retaining β-Glucanases

The mechanistic model for the hydrolysis of glycosidic bonds by retaining glucanohydrolases (β-glucanases) involves two S_n2-type nucleophilic substitutions at the anomeric carbon (C^1) with an overall retention of configuration. The first step (glycosylation) involves the attack on C^1 by the catalytic nucleophile resulting in a covalent enzyme-substrate intermediate, followed by the second step (deglycosylation) that involves the hydrolysis of the protein-carbohydrate intermediate by an activated water molecule. The glycosylation reaction is rate-limiting and catalyzed by a general acid, whereas deglycosylation requires a general base catalyst. Each function usually resides in a single catalytic residue positioned in the active site. In bacterial β-glucanases both the catalytic nucleophile and the acid/base catalyst are usually glutamic or aspartic residues which are involved in a network with other auxiliary residues. A comprehensive review of enzymatic carbohydrate depolymerization is presented in [39,40].

The Hybrid 1,3-1,4-β-Glucanase H(A16-M)

H(A16-M) is a highly specific, engineered β-glucanase. The protein is designed as hybrid enzyme which was derived from the 1,3-1,4-β-glucanase from *Bacillus macerans*. In H(A16-M) the 16 amino-terminal residues have been replaced by the respective residues from the 1,3-1,4-β-glucanase from *Bacillus amyloliquefaciens*. As such, H(A16-M) belongs to the class of 1,3-1,4-β-glucanases (lichenases) which selectively hydrolyze β-1,4 glycosidic bonds in 3-O-substituted glucopyranosyl units. The catalytic nucleophile and general acid/base in

H(A16-M) were identified as Glu105 and Glu109, respectively. The substrate binding site consists of six glucosyl-binding subsites, of which four subsites (–IV to –I) are on the non-reducing end from the scissile glycosidic bond, and two subsites (+I/II) on the reducing end.

The hybrid enzyme H(A16-M) was originally created in the search for bacterial β-glucanases with improved thermostability which might be used for industrial purposes. In addition, 1,3-1,4-β-glucanases turned out to be very promising targets for crystallographic studies, and a number of structures had been determined and analyzed, including circularly permuted variants (reviewed in [39]). However, with the exception of the crystal structure of a covalent enzyme-inhibitor (E·I) complex [41], structural information about enzyme-carbohydrate interactions of 1,3-1,4-β-glucanases was restricted to models based on kinetic and mutational data. To elucidate the molecular basis of the highly selective substrate recognition and binding, crystallization and structure analysis of H(A16-M) in complex with carbohydrate ligands were envisioned in this thesis project.

1.3 Aims of the Thesis Work

The main objective of this thesis work was to determine the structure of two different proteins and to draw conclusions about the molecular basis for the observed cellular functions. The subjects of this structural biological study were

(i) The BRCT region of the human tumor suppressor protein BRCA1, and

(ii) The bacterial 1,3-1,4-β-glucanase H(A16-M) in complex with natural substrates.

1.3.1 Structure and Function of the BRCA1 BRCT Region

The major aim of this research project was to determine the 3D structure of the BRCT domains from human BRCA1, and to analyze the molecular basis for BRCT-mediated DNA damage signaling and cell cycle checkpoint activation. Furthermore, as information about candidate binding partners became available, the association behavior of the BRCT region was studied. This research project was initiated by preliminary results from sequence analysis by Peer Bork and co-workers which suggested for the first time the physical existence of BRCT domains in BRCA1 and other DNA damage-responsive cell cycle checkpoint proteins [32]. Thus, the BRCT project was started without any knowledge of the biochemical or biophysical properties of the molecular target. The first part of this project was concerned with the molecular cloning and characterization of various BRCT constructs. In a second part, the work focused on the structure determination of the BRCA1 BRCT region by X-ray crystallography and/or solution nuclear magnetic resonance (NMR) spectroscopy. The results are presented in Chapter 3 and in two corresponding publications [42,43] (Chapter 5).

1.3.2 Carbohydrate Binding and Substrate Specificity of H(A16-M)

Another thesis project aimed at the identification of enzyme-ligand interactions between the engineered bacterial *endo*-1,3-1,4-β-glucanase H(A16-M) and a natural oligosaccharide substrate. The enzyme binds polysaccharides with mixed β-1,3 and β-1,4 glycosidic linkages, accommodating six glucosyl residues in the substrate binding cleft. Despite intensive research on H(A16-M) variants and related β-glucanases, the structural basis for substrate specificity and the nature of enzyme-sugar interactions at the site of catalysis had been elusive. Thus, the major aim of this thesis project was to analyze enzyme-carbohydrate interactions by X-ray crystallography, and to compare the structural results with previous mutagenesis data which assigned a number of key residues as important for efficient substrate binding and catalysis. The results are presented in Chapter 4 and in the corresponding publication [44] (Chapter 5).