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MOLECULAR CHARACTERIZATION OF PHENOTHIAZINES IN EXPERIMENTAL CANCER THERAPY - NEW TRICKS OF AN OLD DRUG REVEALED

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"chance favors the prepared mind"

Louis Pasteur (1854)

To my beloved family who always supported and believed in me

ABSTRACT

Cancer is characterized by uncontrolled malignant proliferation of cells that eventually interfere with tissue/organ functions. Traditionally, cancer is treated with chemo- and/or radiotherapy when surgery is not an option. Unfortunately, the efficacy of conventional anti-cancer chemotherapy is severely limited by therapy resistance. A conceptually appealing strategy to combat tumor resistance is to use chemosensitizers, compounds that selectively sensitize tumor cells to chemotherapy without affecting normal tissue. Phenothiazines belong to a class of "old" drugs that are used clinically to treat psychiatric disorders. In this thesis, we characterized the chemosensitizing potential of phenothiazines in combination with DNA damaging chemotherapeutic drugs. Our primary aims are to elucidate the molecular mechanisms by which phenothiazines impart sensitization and to delineate molecular determinants that predict responsiveness of tumors to phenothiazine-based intervention. In **Paper I**, we confirmed that the phenothiazine compound trifluoperazine (TFP) was a potent sensitizer of bleomycin in human non-small cell lung carcinoma (NSCLC) cells; the likely mechanism being inhibition of repair of DNA single strand breaks (SSB) as well as DNA double strand breaks (DSB). In **Paper II**, we found that TFP delayed the resolution of bleomycin- or cisplatin-induced γH2AX, a marker of unrepaired DNA DSB, prolonged the cell cycle arrest and increased oxidative stress in NSCLC cells. TFP co-treated cells eventually resumed cycling without fully repairing the DNA damage, which led to mitotic defects, secondary checkpoint arrest, exacerbated oxidative stress, organelle dysfunction, caspase activation and ultimately apoptosis. In **Paper III**, we uncovered a possible link between phenothiazines and chromatin remodeling by *in silico* gene expression analysis. We found that TFP and structurally related phenothiazines significantly enhanced the activity DNA-PK/ATM in tumor but not normal fibroblasts in response to DNA DSB-inducing agents, resulting in increased selective phosphorylation of a subset of ATM substrates with chromatin regulatory functions. Notably, this represents an adaptive response which could be targeted by DNA-PK/ATM inhibitors to further enhance TFP-mediated chemosensitization in NSCLC cells. Moreover, we found that wild-type p53 is a potential predictor of unresponsiveness to phenothiazine-based chemosensitization. We further demonstrated that TFP preferentially increased the cytotoxicity of direct-acing DNA damaging agents, but not indirect-acting DNA damaging or non-DNA damaging agents, in p53-deficient tumor cells (NSCLC, breast cancer). In **Paper IV**, we compared the gene expression profile of NSCLC residual clones that survived cisplatin treatment with counterparts that survived cisplatin/TFP co-treatment. We found that survival after cisplatin was associated with enrichment of pathways involved in DNA metabolism/repair, cell cycle and RNA post-translational modification. Pathway analysis showed that several DNA repair genes were concurrently up-regulated in residual clones that survived cisplatin treatment, but not in residual clones that survived cisplatin/TFP co-treatment did not shown such enrichment of DNA repair genes. In summary, our data showed for the first time that inhibition of DNA DSB repair by TFP is related to alterations in DNA-PK/ATM signaling, which led to increased apoptosis in the short term and gene expression changes as well as loss of clonogenicity in the long term. Further, our identification of molecular contexts that predict responsiveness to phenothiazines will aid in the design of future clinical trials.

LIST OF PUBLICATIONS

I. Polischouk AG, Holgersson A, **Zong D**, Stenerlöw B, Karlsson HL, Möller L, Viktorsson K, Lewensohn R. The antipsychotic drug trifluoperazine inhibits DNA repair and sensitizes non small cell lung carcinoma cells to DNA double-strand break induced cell death. *Mol Cancer Ther.* 2007; 6(8):2303-9.

II. **Zong D***, Hååg P, Yakymovych I, Lewensohn R, Viktorsson K*. Chemosensitization by phenothiazines in human lung cancer cells: impaired resolution of γH2AX and increased oxidative stress elicit apoptosis associated with lysosomal expansion and intense vacuolation. *Cell Death Dis*. 2011; 2:e181.

III. **Zong D***, Hååg P, Zielinska-Chomej K, Yakymovych I, Lewensohn R, Viktorsson K*. Identification of phenothiazines as putative regulators of the chromatin response to DNA damage provides a rationale for context-dependent chemosensitization. (*manuscript*)

IV. Salim H[#], **Zong D**[#], Lundholm L[#], Hååg P, Mörk B, Lewensohn R, Viktorsson K. Gene expression analysis reveals DNA repair pathway modulation as a potential mechanism for phenothiazine-mediated long term cisplatin sensitization in NSCLC. (*manuscript*)

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CONTENTS

LIST OF ABBREVIATIONS

AML	Acute myeloid leukemia
A-NHEJ	Alternative NHEJ
ATM	Ataxia telangiectasia mutated
BIR	Break-induced replication
BLM	Bleomycin
B-NHEJ	Backup NHEJ
CaM	Calmodulin
CDDP	Cisplatin
C-NHEJ	Classic NHEJ
CNS	Central nervous system
Cmap	Connectivity Map
CPZ	Chlorpromazine
DDR	DNA damage response
DNA-PK	DNA-dependent protein kinase
DNA-PKcs	DNA-PK catalytic subunit
DSB	Double strand break
DSBR	DSB repair
FA	Fanconi anemia
FANC	Fanconi anemia complementation group
FPZ	Fluphenazine
GO	Gene Ontology
GSEA	Gene set enrichment analysis
HDAC	Histone deacetylase
HDR	Homology-directed repair
HRR	Homologous recombination repair
ICL	Interstrand crosslink
IPA	Ingenuity pathway analysis
IR	Ionizing radiation
LMP	Lysosomal membrane permeabilization
MDR	Multidrug resistance
MMEJ	Microhomology-mediated end joining
MMR	Mismatch repair
MOMP	Mitochondrial outer membrane permeabilization
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
NSCLC	Non-small cell lung carcinoma
OIS	Oncogene-induced senescence
PARP-1	Poly(ADP-ribose) polymerase-1
PCA	Principal component analysis
PFGE	Pulsed-field gel electrophoresis
PIKK	Phosphatidylinositol 3’-kinase-like protein kinase
PRKDC	Protein kinase, DNA-activated, catalytic polypeptide
PTM	Post-translational modification
ROS	Reactive oxygen species

RT-qPCR	Real time quantitative polymerase chain reaction
SAC	Spindle assembly checkpoint
SDSA	Synthesis-dependent strand annealing
SSA	Single strand annealing
SSB	Single strand break
TLS	Translesion synthesis
TFP	Trifluoperazine
TFPZ	Trifluprомazine
VP16	Etoposide
VPA	Valproic acid

1 INTRODUCTION

1.1 DNA damaging chemotherapy in cancer treatment

Cancer is the collective name for a large number of pathologic conditions characterized by uncontrolled malignant proliferation of cells. It may originate from almost any type of tissues in the body and is usually rapidly fatal if not promptly treated. The latest estimates for the global incidence of cancer and its associated mortality are 12.7 million and 7.6 million, respectively, in 2008 [1]. Although cancer can affect people of all ages, there is a steep increase in cancer incidence amongst the elderly (>65 years), suggesting that it is an age-related pathology. Whenever possible, surgery is the treatment of choice because it has the highest chance of achieving a complete cure. If surgery is not an option, for instance due to physical inaccessibility of the tumor site or poor health of the patient, chemotherapy and/or radiotherapy may be considered. Ionizing radiation (IR) and most conventional chemotherapeutic agents directly or indirectly cause DNA damage, which is generally more toxic to proliferating cells. However, as neither of these treatment modalities can discriminate tumor cells from rapidly dividing non-cancerous cells (e.g. bone marrow cells, intestinal cells), their anti-tumor efficacy comes at the cost of significant normal tissue toxicity. Therefore, the development of chemosensitizers, compounds that can selectively enhance the cytotoxicity of chemotherapeutic agents without affecting the sensitivity of normal tissues, is urgently needed.

1.2 The DNA damage response (DDR)

DNA molecules are chemically reactive, and as such are susceptible to attack from endogenous (e.g. reactive oxygen intermediates of mitochondrial aerobic respiration) and/or exogenous DNA damaging agents (e.g. ionizing radiation, chemotherapeutic drugs), which produce a wide spectrum of DNA lesions, ranging from oxidative base damages (e.g. 8-oxoguanine) to breakage of DNA strands (e.g. DNA double strand break, DSB) [2]. The cytotoxic potential of any DNA lesion is influenced by a variety of cell-intrinsic factors, such as lineage, position within the cell cycle, availability of dedicated or redundant DNA repair factors, etc. It is generally accepted that DNA DSB has the highest cytotoxic potential [3]. Different types of DNA lesions can also occur within close proximity of each other (i.e. clustered damage), making them more difficult to repair [4, 5]. Incursion of DNA damage in eukaryotic cells triggers the DNA damage response (DDR), which elicits temporally concerted activation of multiple signaling networks that together bring about cell cycle arrest, cessation of RNA polymerase II-mediated transcription, localized chromatin remodeling and DNA repair [6-11] (Figure 1). If the DNA damage is successfully repaired by the afflicted cells, DDR signaling is shut off allowing cell cycle to recommence. However, if the lesions can not be adequately repaired or if DDR signaling cannot be turned off following completion of repair, persistent DDR signaling can drive cells into senescence or apoptosis [12, 13]. The ultimate goal of DDR is to maintain genomic integrity at the organism level.

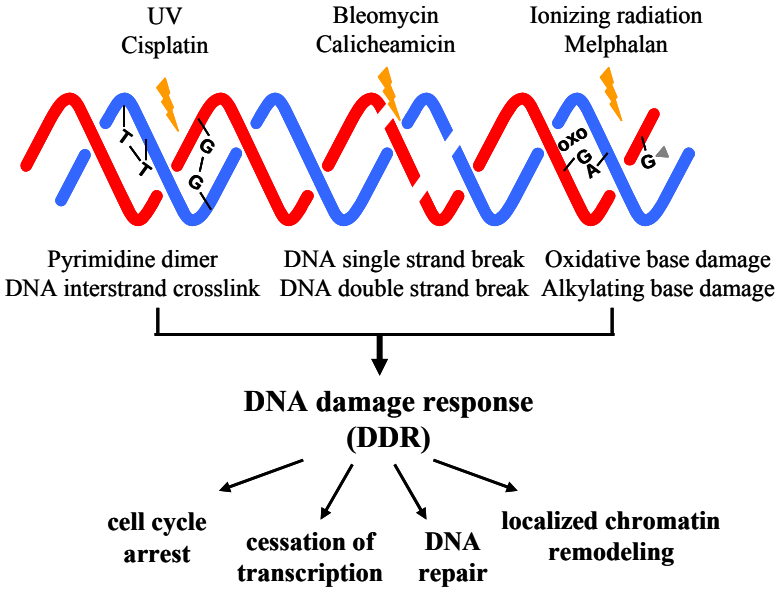

Figure 1. The DNA damage response.

1.2.1 Detection of DNA DSBs

DNA DSB sensors

DNA DSBs are particularly dangerous because they can lead to loss of genetic information and genomic rearrangements [14]. To ensure rapid detection of these potentially deleterious lesions, eukaryotic cells are equipped with a set of damage sensors that can recognize DNA DSBs, including the Ku70/Ku80 (Ku) complex, the Mre11/Rad50/Nbs1 (MRN) complex, poly(ADP-ribose) polymerase 1 (PARP-1), and Rad52 [15-18]. These factors bind to similar double stranded DNA substrates *in vitro* and can under certain circumstances functionally compensate for each other *in vivo*. As of yet, the molecular parameters that determine which of these DNA DSB sensors will be recruited to a given DNA DSB are not well understood. Cell-intrinsic factors such as protein expression level, cell cycle position, chemical milieu in the vicinity of the break and genomic localization of the break may all influence which sensors are preferentially engaged. Regardless, it is clear that the choice of DNA DSB sensor governs repair pathway utilization. In line with this, direct competition between different sensors for binding dsDNA ends has been demonstrated [19-21].

DNA damage-associated chromatin remodeling

The DNA of eukaryotes exists in close association with a large number of proteins that together comprise the chromatin, whose primary functions are to enable efficient packaging of DNA into nucleosomes, to protect DNA from damage and to allow molecular fine-tuning of essential DNA-centered processes such as gene expression and DNA replication [22]. The chief protein components of the chromatin are histones, which contain numerous amino acids in their N-terminus that are amenable to post-translational modifications (PTMs), including phosphorylation, acetylation, methylation, ubiquitination, sumoylation and ADP-ribosylation [23]. Histone PTMs determine the level of chromatin compaction/relaxation as well as the recruitment of non-histone proteins by creating specific docking sites [24]. While some PTMs can occur

independently, others are induced in a synergistic or antagonistic manner. In addition, the structure of chromatin can also be regulated by processes that do not involve histone PTMs, such as CpG island DNA methylation or nucleosome shuffling/eviction by ATP-dependent chromatin remodeling complexes [25, 26].

The compact organization of eukaryotic chromosomes poses a big challenge to the cellular DNA repair machinery due to its limited accessibility [27]. To ensure rapid detection and repair of genomic lesions, damage-proximal DDR signaling triggers the concerted activation of many proteins with chromatin remodeling functions leading to changes in both local and global chromatin landscape [28, 29] (Figure 2). Localized chromatin relaxation can occur by several parallel mechanisms. Activation of the apical kinase Ataxia Telangiectasia Mutated (ATM) represents one of the earliest events in DDR signaling [30]. Active ATM relocates to sites of DNA DSB where it phosphorylates histone H2AX on serine-139 (aka γH2AX) in the immediate vicinity of the break [31]. The initial ATM-dependent γH2AX formation is facilitated by localized chromatin relaxation following casein kinase 2-mediated release of HP1β from histone H3 trimethylated on lysine 9 (H3K9Me3, a marker of compact chromatin) [32]. This generates a binding site for the Tip60 histone acetyltransferase, which acetylates ATM on lysine-3016 resulting in enhanced ATM kinase activity [33]. Efficient activation of ATM also requires HMGN1-dependent histone acetylation [30, 34]. In turn, γH2AX serve as molecular platforms to help concentrate various checkpoint mediators (e.g. ATM, MDC1, MRN, BRIT1/MCPH1) and ATP-dependent chromatin remodeling complexes (e.g. NuA4, SWI/SNF) at the sites of DNA DSB [24, 35-38]. This creates a self-sustained cycle whereby ATM promotes further spreading of γH2AX along the chromosome [31, 39, 40]. Recruitment of NuA4 and SWI/SNF complexes to γH2AX domains surrounding DSBs leads to local destabilization/disruption of nucleosomes and promotes histone ubiquitination by RNF8/RNF168, which facilitates the subsequent ubiquitination-dependent recruitment of BRCA1 to amplify checkpoint signaling [38, 41-45]. 53BP1 is an important checkpoint mediator whose retention on chromatin in response to DNA damage requires *de novo* histone methylation as well as unmasking of pre-existing ones. Localized methylation of histone H4 on lysine-20 (H4K20Me) and histone H3 on lysine-79 (H3K79Me) are catalyzed by MMSET and Dot1L, respectively [46-49]. Unmasking of pre-existing H4K20Me requires RNF8/RNF168-mediated displacement of JMJD2A and L3MBTL1 [50, 51]. In turn, 53BP1 interacts with the chromatin architectural protein EXPAND/MUM1 to bring about chromosomal decondensation [52]. Moreover, phosphorylation of KAP-1 (S824) by ATM causes chromatin relaxation by disrupting the SUMO-dependent interaction between KAP-1 and CHD3, a component of the Mi-2/NuRD ATP-dependent chromatin remodeling complex [53, 54]. The precise role of ATM-dependent phosphorylation of cohesin components SMC1 (S957, S966) and SMC3 is less clear, but appears to be required for the activation of intra-S checkpoint and reinforcement of chromosome cohesion [55-58]. The latter function may be important for maintaining the positional stability of DNA DSBs [29, 59-61].

In addition to its role in checkpoint regulation, chromatin remodeling is also intimately linked to DNA DSB repair. Localized mono-ubiquitination of histone H2B by RNF20/RNF40 is required for chromatin relaxation and the timely recruitment of several DSB repair factors that participate in non-homologous end joining (NHEJ) as well as homologous recombination repair (HRR) [62-64]. Binding of Ku to damaged DNA is facilitated by acetylation of histones H3 and H4 by the CBP/p300 complex [65]. On the other hand, HDAC1/2-mediated deacetylation of histone H3 on lysine-56 (H3K56Ac) is required for efficient Ku-dependent NHEJ [66]. Direct polyADP-ribosylation (PARylation) of histones by PARP-1 enhances chromatin accessibility [67]. PARP-1 and

Ku also recruit several ATP-dependent chromatin remodeling complexes (e.g. NuRD, ALC1, CHRAC) to facilitate DNA DSB repair [68-71]. Together, these data suggest that chromatin remodeling and loading of repair factors occur in tandem self-amplifying cycles. Finally, a number of proteins implicated in stem cell renewal and over-expressed in tumors, such as BMI1 and Piwil2, have recently been found to function in the chromatin response to DNA damage [72, 73]. This raises the possibility that tumors may utilize lineage/differentiation-related differences in chromatin remodeling to enhance their own tolerance for DNA damaging chemotherapy.

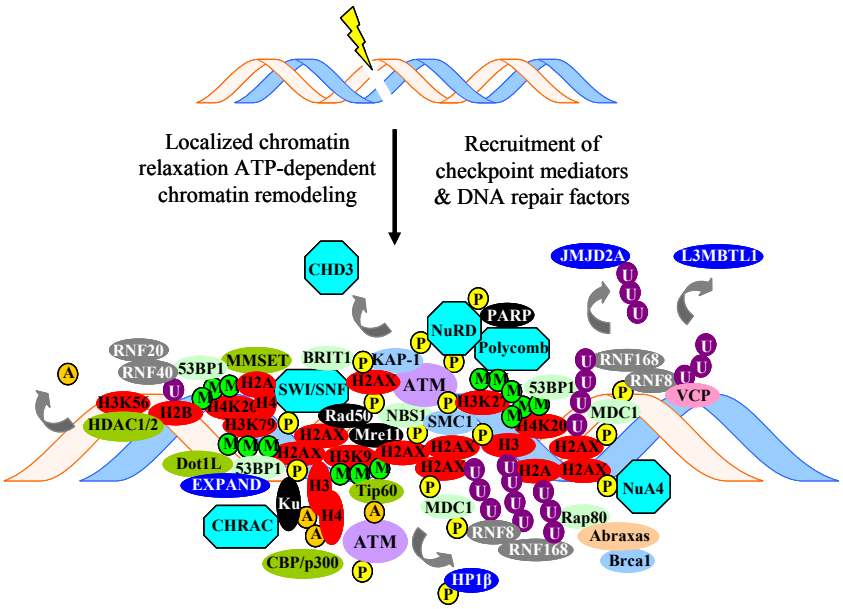

Figure 2. DNA DSB-induced chromatin remodeling. This is a highly complex process involving multiple protein-protein interactions and PTMs but their potential interplay and individual kinetics remain largely undefined.

1.2.2 Cell cycle checkpoints

Cell cycle arrest versus cell death

As mentioned earlier, DDR signaling in response to genotoxic stress triggers the activation of checkpoint proteins that block further progression in the cell cycle, giving the afflicted cell time to repair its damaged DNA [74] (Figure 3). The duration, reversibility and final outcome of any cell cycle arrest is governed by a complex interplay between numerous factors (e.g., cell type, extent of DNA damage, position in the cell cycle when damage was sustained, repair capacity, etc.). In general, relatively transient arrest favors cell survival while prolonged arrest frequently culminates in cell death or premature senescence [75]. There are, however, exceptions where the reverse is true. For instance, radioresistant tumor stem/progenitor cells tend to have a longer and more stable checkpoint response than their more differentiated and radiosensitive progenies [76, 77]. Conversely, chemically-induced G_2 checkpoint abrogation (e.g. Chk1 inhibition) are exceedingly toxic in tumor cells whose ability to enforce G_1 arrest is compromised by p53-deficiency [78, 79].

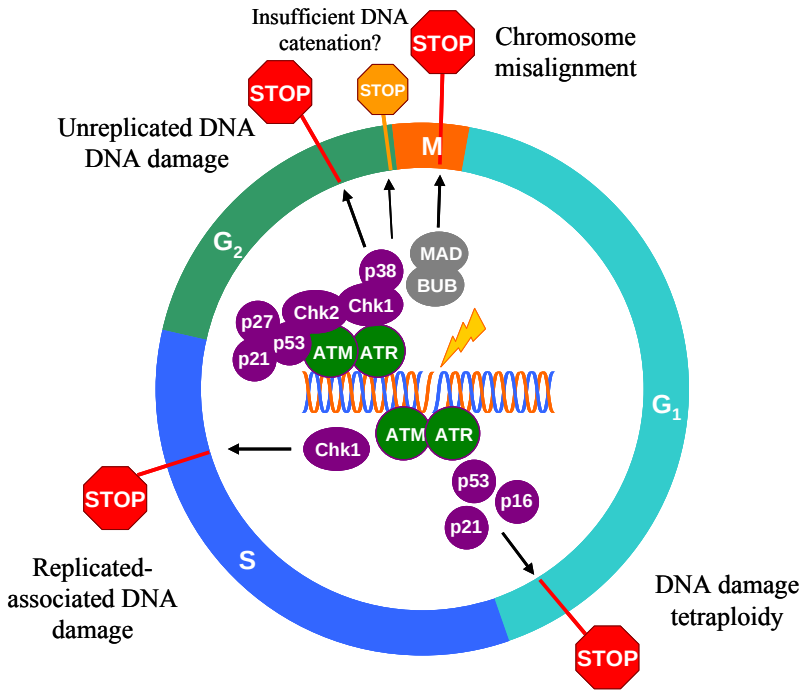

Figure 3. A schematic view of the cell cycle and the main checkpoint regulators. DNA damage activates checkpoint mechanisms in G_1, intra-S, G_2 and M phases to stop cell cycle progression. The existence of the antephase checkpoint (orange) is controversial.

G_1 checkpoint

The G_1 checkpoint is activated during late G_1 to prevent cells with DNA damage from transiting into S phase and initiate DNA replication [80]. The best characterized mechanism by which DNA damage triggers G_1 arrest is mediated by the tumor suppressor protein p53 [80]. Stabilization of p53 and up-regulation of its transcriptional activity can be achieved by multiple PTMs, such as phosphorylation and acetylation. Depending on the precise pattern of PTMs, p53 may selectively transactivate genes that contain high affinity p53-binding sites (e.g., CDKNA1) or broadly transactivate genes containing p53-binding sites of varying affinity, many of which are pro-apoptotic (e.g., PUMA, BAX) [81, 82]. The main effectors of p53-mediated G_1 arrest are $p21^{Cip1/Waf1}$ (encoded by CDKNA1), a universal inhibitor of cyclin-dependent kinases (cdks), and to a lesser degree $p16^{INK4a}$, a predominantly G_1-restricted cdk inhibitor [80]. Suppression of the kinase activities of G_1 cyclin-cdk complexes by $p21^{Cip1/Waf1}$ (cyclin D-cdk4/6, cyclin E-cdk2) and $p16^{INK4a}$ (cyclin D-cdk4/6) prevents the phosphorylation-dependent release of E2F family transcription factors from the Pocket proteins (pRb, p107, p130) and inhibits the E2F-mediated transactivation of genes required for S phase entry. Transient G_1 checkpoint arrest is usually reversible while prolonged activation of $p21^{Cip1/Waf1}$/$p16^{INK4a}$ can drive cells into premature senescence [83]. Given that the p53 pathway is functionally inactivated in a majority of human tumors [84], the G_1 checkpoint (and hence the ability to senesce) is compromised or lacking in most cancer cells.

Intra-S checkpoint

The intra-S checkpoint (aka replication checkpoint) is activated primarily by disturbances in DNA replication, such as replication stress caused by oncogene-induced

hyper-proliferation and stalling of replication forks on DNA strands damaged by anti-cancer drugs [74, 85, 86]. Regardless of the cause, collapse of replication forks may follow their initial stalling leading to the exposure of single stranded DNA (ssDNA), a potent signal for the activation of ATR [87]. ATRIP recruits ATR to RPA-coated ssDNA where Rad9-Rad1-Hus1 (9-1-1 complex), claspin and TopBP1 collaborate to promote ATR activation and phosphorylation of the downstream checkpoint effector kinase Chk1. In turn, Chk1 phosphorylates Cdc25A leading to the latter's proteasomal degradation and/or cytoplasmic sequestration by 14-3-3 proteins [88-92]. The loss of Cdc25A phosphatase activity prevents the activation of cyclin A-cdk1/2, which is required for completion of S phase and entry into G_2.

G_2 checkpoint

The G_2 checkpoint (aka G_2-M checkpoint) functions to prevent cells from entering mitosis. DNA damage detected in G_2 activates both ATM-Chk2 and ATR-Chk1 arms of the DDR, with the net result being the inactivation of Cdc25 family of protein phosphatases (Cdc25A/B/C) and inhibition of cyclin B-cdk1 whose activity is required for mitotic entry [74, 93, 94]. The p53-p21$^{Cip1/Waf1}$ pathway in p53 wild-type tumors and the p38MAPK-MK2 pathway in p53-deficient tumors have also been implicated in the enforcement of G_2 checkpoint arrest [95-97]. It is worth pointing out that in human tumors, the ATM/R-Chk1/2-Cdc25 pathway may be incapacitated by over-expression of Akt or polo-like kinases (e.g. Plk-1), while both the p53-p21$^{Cip1/Waf1}$ and the p38MAPK-MK2 pathways may also be attenuated [84, 98-103]. Therefore, the G_2 checkpoint may be compromised or sub-optimal in a large proportion of tumor cells, rendering them more susceptible to pharmacologically-induced checkpoint abrogation [78, 79].

Antephase checkpoint

The term "antephase" refers the relatively short window of time from late G_2, when chromosome condensation first becomes visible, until commitment to mitosis. Based on this definition, the antephase checkpoint is a putative checkpoint that blocks cell cycle progression somewhere between late G_2 and early prophase [104]. Signals that activate the antephase checkpoint appear to be topologic disturbances in the DNA molecule rather than *bona fide* DNA damage [104, 105]. In this respect, it is similar to another putative checkpoint that operates at the G_2-M border, the decatenation checkpoint, which is activated in response to chromosome entanglement (e.g. topoisomerase II inhibition) [106, 107]. It is not clear whether the antephase checkpoint represents an extension of the "conventional" DNA damage-induced G_2 checkpoint or is in fact a separate entity with its own distinct molecular control mechanisms. It is however possible that there is a temporal constraint on the activation of the conventional G_2 checkpoint and that the antephase checkpoint represents a last attempt by the cell to arrest before progressing into mitosis. So far, p38MAPK and CHFR are the only proteins that have been implicated in the antephase checkpoint [105, 108].

Spindle assembly checkpoint

During late G_2 and early prophase, the nuclear membrane breaks down and the chromatin undergoes marked condensation accompanied by phosphorylation of histone H3 on serine-10 [109]. From late prophase through metaphase, sister chromatids become attached to the mitotic spindle at their kinetochores, which are specialized protein structures that form at centromeres. Sister chromatids align at the metaphase plate but

are prevented from separating by the spindle assembly checkpoint (SAC, aka mitotic checkpoint). The SAC is an active signal generated by mitotic spindle microtubules that lack tension at unattached or improperly attached kinetochores [110, 111]. A host of proteins have been implicated in the execution of SAC, including MAD1, MAD2, MAD3, BUB1, BUB3, BUBR1 and MPS1. SAC blocks anaphase by negatively regulating CDC20, a component of the anaphase promoting complex/cyclosome (APC/C) [112]. Once correct bi-orientation of sister chromatids has been achieved, SAC is turned off and its inhibition on APC/C is lifted. The E3 ubiquitin-ligase activity of APC/C is required to target securin and cyclin B for proteasomal degradation. This in turn frees a protease called separase to cleave the cohesin ring that holds sister chromatids together. Loss of cohesion then allows the sister chromatids to segregate towards opposite spindle poles to complete anaphase. This is followed by telophase and cytokinesis in which the nuclear membrane reforms around the newly-separated chromosomes and the cytoplasm is cleaved along the mitotic furrow to produce two daughter cells. A functional SAC is therefore vital for the maintenance of genome integrity by ensuring faithful division of genetic material between two daughter cells. Mutation of SAC components is quite common in human cancers and compromised SAC function is thought to be a major cause of aneuploidy [113, 114]. However, even a fully functional SAC cannot arrest cells in mitosis indefinitely and mitotic slippage can occur, especially in cancer cells, as a result of checkpoint adaptation [115-117]. Since prolonged mitotic arrest promotes apoptosis, blocking mitotic exit may be a promising strategy to selectively sensitize tumor cells to anti-mitotic drugs such as taxanes [75, 116, 118-120].

Checkpoint recovery

Checkpoint recovery refers to the resumption of cell cycle progression after dissipation of signals that maintain cell cycle arrest. Recovery from DNA damage-induced checkpoint arrest occurs when genomic lesions have been repaired with sufficient fidelity that they no longer engage the DDR machinery. As human tumors show a high propensity to harbor mutations that inactivate the G_1 checkpoint, considerable efforts have been devoted to the elucidation of regulatory mechanisms that control recovery from G_2 arrest [121-125]. These mechanisms can be broadly divided into three categories: (1) epigenetic modifications, (2) inactivation of checkpoint signaling and (3) transcriptional responses. The best characterized epigenetic modifications that accompany checkpoint recovery are γH2AX dephosphorylation by protein serine/threonine phosphatases (e.g., PP2A, PP4, PP6, Wip1) and re-acetylation of H3K56 by histone chaperones/histone acetylases [126-131]. Meanwhile, checkpoint signaling can be shut off by several mitotic kinases (e.g., Plk1, Aurora A) that block the activation of checkpoint kinases while promoting Cdk1 activation [132-134]. Finally, a number of transcription factors (e.g., B-Myb, FoxM1) have been shown to facilitate checkpoint recovery by transcriptionally up-regulating genes that are essential for mitotic entry while repressing genes that are involved in checkpoint control [135, 136]. It is conceivable that these mechanisms act in a non-mutually exclusive manner and may promote and/or cooperate with each other. The complex network of molecular interactions that regulates checkpoint recovery is only partially understood.

Checkpoint adaptation

Cells that cannot adequately repair its damaged DNA can still resume cell cycle progression by undergoing a process called checkpoint adaptation, which involves

active shutdown of checkpoint signaling in the face of residual DNA damage [121, 122, 137]. Checkpoint adaptation was originally observed in yeast and was thought to be limited to unicellular organisms, since it carries a high risk for mutations [138]. Nevertheless, checkpoint adaptation has since been found in human cancer cells after exposure to chemo- and/or radiotherapy [139]. The molecular pathways involved in G_2 checkpoint adaptation appear to overlap with those that control checkpoint recovery [121, 122, 137]. In particular, Plk1 has been implicated in the adaptation to the G_2 checkpoint. Given the fact that human tumors frequently over-express Plk1, checkpoint adaptation may be expected to play a critical role in promoting tumor survival and genomic instability, making it a promising target for anti-cancer intervention [140].

1.3 DNA DSB repair

Since the DNA molecule can incur a broad spectrum of lesions, eukaryotes have evolved an elaborate set of DNA repair mechanisms (Figure 4). Two principal modes of DNA DSB repair, namely NHEJ and HRR, are utilized by eukaryotic cells. In NHEJ, the two ends of a DNA DSB are directly rejoined with little or no requirement for terminal homology [141]. In HRR, homologous sequences on another chromosome (usually sister chromatids) is used as a template to restore the lost genomic information across the DNA DSB [142]. Notably, DNA DSB repair is frequently intersected by other DNA repair pathways because genomic lesions tend to be structurally and chemically heterogeneous, requiring additional factors to process the damaged DNA into substrates that can be used by NHEJ or HRR. This scenario is neatly illustrated the cooperation between Fanconi anemia (FA) pathway and HRR (see below). There is evidence that NHEJ and HRR can function cooperatively, independently/sequentially or antagonistically [143-146]. Moreover, both NHEJ and HRR contain several competing sub-modules that differ in repair efficiency as well as fidelity. In general, low fidelity sub-modules are mostly employed as backups that are negatively regulated by high fidelity ones [147, 148].

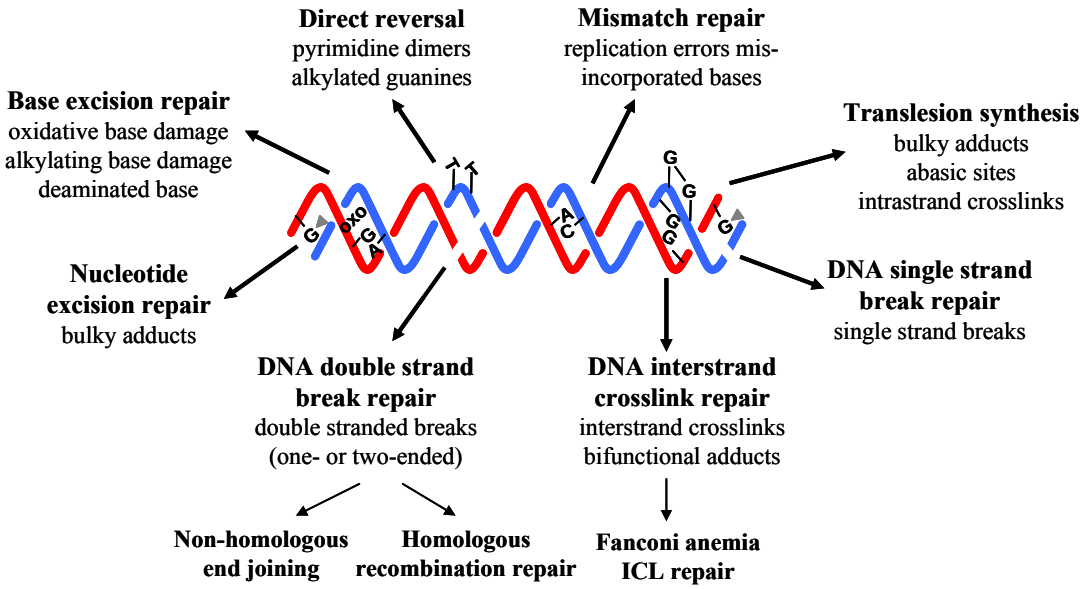

Figure 4. An overview of DNA repair pathways in eukaryotes.

1.3.1 Non-homologous end joining

Classic NHEJ

Classic NHEJ is initiated by Ku, which binds DNA DSB with high avidity in a sequence- and homology-independent manner by forming a ring shaped structure that encloses the DNA double helix [149] (Figure 5). Ku in turn recruits the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) to form the DNA-PK holoenzyme [17]. The physical interaction between two DNA-PK molecules on either side of a DNA DSB helps align the ends in a synapse that facilitates end joining [150, 151]. Binding to Ku and DNA greatly stimulates its serine/threonine kinase activity, resulting in substrate phosphorylation (including autophosphorylation) which is required for efficient NHEJ [152, 153]. DNA-PKcs also causes Ku to slide inwards away from the DNA termini allowing chromatin access to several end-processing enzymes (e.g. Artemis, DNA polymerase μ, DNA polymerase λ, polynucleotide kinase/phosphatase) which together with Ku convert DNA ends into chemical configurations suitable for ligation [141, 154-158]. The final resealing of the broken DNA ends is carried out by a ternary complex consisting of XRCC4, XLF and DNA Ligase IV [154]. Although the above description gives the impression that NHEJ follows a highly ordered sequence of events, substantial amount of mechanistic flexibility are observed during NHEJ *in vivo* whereby the order of recruitment of different factors may be more varied and the two broken strands can be rejoined independent of each other [154, 159]. Moreover, several NHEJ proteins, including Ku and DNA-PKcs, are mobile and dynamically exchange between chromatin-bound and soluble forms [160, 161]. It is not yet clear at what stage of NHEJ DSB repair proteins are released from the chromatin. The available data suggest that DNA-PKcs dissociates from the DNA DSB in an autophosphorylation-dependent manner and that the Ku complex is removed from DNA following RNF8-dependent K48-linked poly-ubiquitination and subsequently degraded [162-164]. In the budding yeast, release of Ku from DNA requires the nuclease activity of Mre11, but it is not known whether Mre11 has a similar function in mammalian cells [165].

Backup NHEJ

Backup NHEJ (B-NHEJ) refers to cellular processes that promote rejoining of DNA DSBs when classic NHEJ (C-NHEJ) is absent or functional inactivated [166]. The molecular composition of B-NHEJ is largely undefined and might possibly encompass several sub-pathways with overlapping activities. For instance, it is not known whether alternative NHEJ (A-NHEJ), microhomology-mediated end joining (MMEJ) and B-NHEJ are simply different terms used to describe the same pathway or in fact represent distinct sub-pathways that operate (at least partially) independent of each other [167-169]. For the purpose of this thesis, however, all end joining events supported by non-canonical NHEJ will be collectively called B-NHEJ. There is substantial experimental evidence demonstrating that B-NHEJ is normally suppressed by DNA-PK and that B-NHEJ utilizes a separate set of DNA repair proteins such as PARP-1, Mre11, CtIP and XRCC1/DNA Ligase III [20, 147, 167, 170-177] (Figure 5). In comparison to C-NHEJ, which is usually rapid and relatively accurate, DSB repair via B-NHEJ is both slower and generally more error-prone, which increases the likelihood of chromosomal translocations especially involving the immunoglobulin heavy chain (IgH) locus in developing lymphocytes [14, 166, 175, 177-180]. Moreover, increased activity of B-NHEJ has been observed in human cancers, such as bladder, breast and acute/chronic myeloid leukemia, concomitant with a reduced ability to perform C-NHEJ [14, 174, 179, 180]. These

data suggest that de-regulation of NHEJ may favor mutation-driven tumor evolution and adversely affect clinical treatment responses.

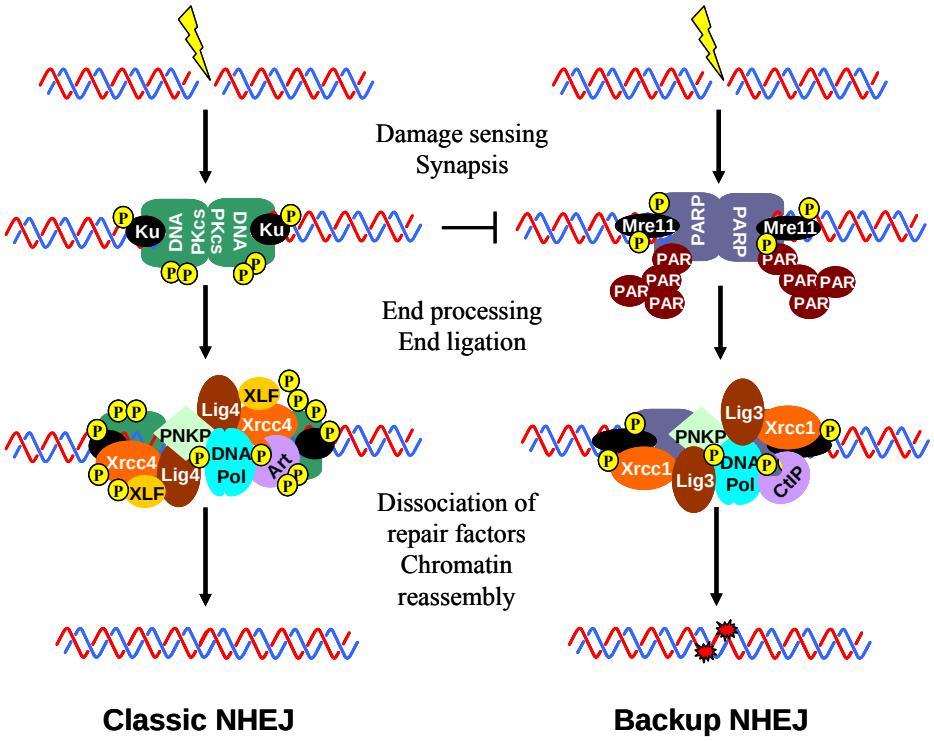

Figure 5. A schematic view of the classic (canonical) and backup (alternative) pathways of NHEJ.

1.3.2 Homologous recombination repair

Homologous recombination repair (HRR), aka homology-directed repair (HDR), is actually a collective term for a number of functionally overlapping DSB repair sub-modules that all use a homologous template to restore lost genetic information [142] (Figure 6). Unlike MMEJ, which is facilitated by short terminal homologies (2-3 nucleotide), all HRR sub-pathways require significant longer (>100 nucleotides) homology tracts on the template, which is usually but not always provided by the sister chromatid [142, 169, 181]. HRR begins with DNA DSB end resection, whereby stretches of the 5'-strand is removed by the concerted actions of Mre11, CtIP and BLM-Exo1 to form long single stranded 3'-DNA (ssDNA) tails which becomes coated with RPA [182-184]. DNA DSB end resection is most efficient during S and G_2, when it is stimulated by CDK2-mediated phosphorylation, but is significantly suppressed by Ku during G_1 [185-187]. Next, Rad51 is recruited to the RPA-coated ssDNA where it forms Rad51 nucleofilament by displacing RPA [188]. Loading of Rad51 is facilitated by Rad52, BRCA2, PALB2 and the Rad51 paralogs but the molecular details of Rad51 nucleofilament formation are not fully defined [142, 189-192]. Regardless, Rad51 then catalyzes a homology-dependent strand exchange (or invasion) reaction in which the 3'-overhang of a resected ssDNA end becomes annealed to the 5'-strand of an intact template DNA molecule, resulting in the formation of a displacement loop (D-loop) connected by a Holliday junction (HJ) [142, 188]. This is followed by end extension through polymerase-mediated DNA synthesis and D-loop expansion. After this stage, the HRR sub-modules become distinct. In the DSB repair (DSBR, aka gene conversion) pathway, Rad52 promotes the annealing of a second resected ssDNA end to the displaced strand of the template molecule in a reaction known as second-end

capture to form a stabilized joint molecule with double HJs [193]. Rad54 has the ability to promote ATP-dependent branch migration of HJs along the joint molecule in a bi-directional manner, which determines the extent of heteroduplex formation and hence the amount of genetic information that is transferred during recombination [194]. Upon completion of repair-associated DNA synthesis and ligation of the broken strands, the double HJs are resolved by specialized endonucleases such as GEN1 and SLX1-SLX4 [195, 196]. Depending on which strands of the HJ the enzymatic nicks occur, crossover or non-crossover recombinants will be produced. DSBR is thought to be the predominant HRR pathway during meosis due to its propensity to generate crossover recombinants. Conversely, the synthesis-dependent strand annealing (SDSA) and break-induced replication (BIR) pathways are primarily used to repair DSBs in proliferating somatic cells. In SDSA, where only a single HJ is formed, end extension is coupled to branch migration followed by D-loop dissociation such that the newly synthesized 3'-end of the invading strand is annealed to the 3'-overhang of the second resected end. Any gaps or flaps that may arise are subsequently filled in/resealed and excised by polymerases and nucleases, respectively. The SDSA pathway generates only non-crossover products [142]. DSBR and SDSA are generally perceived as conservative HRR pathways because recombination occurs preferentially between sister chromatids [190]. However, mitotic HRR can also occur between allelic loci located on homologous chromosomes, which may lead to loss of heterozygosity (LOH) if the homologues are polymorphic. Moreover, on relatively rare occasions (<2% compared to gene conversion) when the other end of the DSB is either missing or is heterologous, DNA synthesis on the invading strand primes the assembly of replication forks, leading to extensive DNA

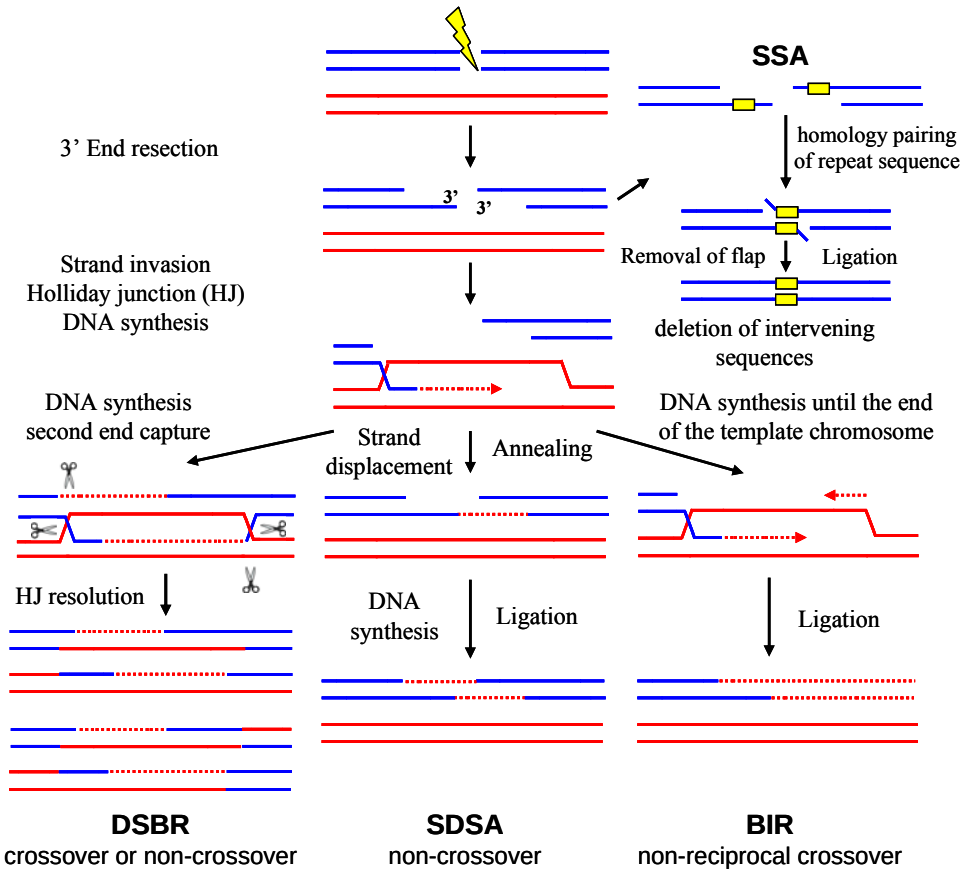

Figure 6. A schematic view of the different sub-modules of homologous recombination repair (HRR). DSBR and SDSA are considered to be error-free as long as the sister chromatid is used as the template for repair. BIR and SSA are considerd to be mutagenic.

replication to the end of the template chromosome. This process, termed break-induced replication (BIR), always leads to non-reciprocal crossover and as such may lead to LOH or even chromosomal translocation [197]. Unlike DSBR, SDSA and BIR, which involve inter-chromosomal recombination events, repetitive sequences within the vicinity of the break on the same chromosome can also be used for DSB repair. This pathway, termed single strand annealing (SSA), is initiated by Rad52-mediated homologous pairing of two complementary repeat sequences exposed by DNA end resection [142]. This annealing reaction produces non-homologous 3'-overhangs that are cut by nucleases such as ERCC1-XPF and SLX1-SLX4. DNA synthesis then fills in any gaps and ligation re-seals the broken strands. Since one of the two repeats along with the intervening sequences in-between are lost during the process, SSA should always be considered as mutagenic.

1.3.3 Fanconi anemia pathway

The Fanconi anemia (FA) pathway is involved in the repair of DNA DSBs and interstrand crosslinks (ICLs) [198] (Figure 7). ICLs, which can be induced by platinum- and nitrogen mustard-based chemotherapeutic drugs, covalently connects two guanines located on opposite strands of the DNA molecule and thereby block any DNA transaction that require strand separation, such as DNA replication and transcription [199, 200]. ICLs are therefore exceeding toxic in proliferating cells. Thirteen FANC proteins have been identified to date, namely FANCA, FANCB, FANCC, FANCD1/BRCA2, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCJ/BRIP1, FANCL, FANCM, FANCN/PALB2 [201]. ICLs physically block the progress of replication forks during S phase, causing the fork to stall. This in turn leads to activation of ATR signaling, which engages the intra-S checkpoint, stabilizes the fork and coordinates the recruitment of repair proteins to the site of DNA damage [202]. Incision on either side of the lesion on one DNA strand by ERCC1-XPF helps unhook the ICL. The gap generated by unhooking is filled in by translesion synthesis (TLS) utilizing error-prone DNA polymerases that can bypass the lesion, and the unhooked adduct is subsequently removed by nucleotide excision repair (NER) [199, 200, 203]. This process creates a DNA DSB at the same time, which undergoes end resection and is then channeled into HRR. It is thought that the primary function of FA in ICL repair is to couple DSB generation to HRR, although the precise mechanisms are still being investigated [203, 204]. Eight of the identified FA proteins, FANCA, B, C, E, F, G, L and M, form a nuclear complex (the FA core complex) and together with two other non-FA proteins FAAP24 and FAAP100 promotes the mono-ubiquitination of FANCD2 and FANCI, which is critical for mediating cellular resistance of ICL-inducing agents, such as cisplatin or mitomycin C [198, 203-205]. FANCD2 and FANCI can also be phosphorylated by ATM/ATR in response to replication and/or oxidative stress although its significance to activation of the FA pathway is not yet clear. Identified interaction partners of FA proteins are implicated in a number of cellular processes including DNA repair, chromatin remodeling and anti-oxidant defense, suggesting that the FA pathway may broadly influence several aspects of the DDR [206, 207]. Consistent with this notion, loss-of-function mutation in any of the known components of FA causes Fanconi anemia, a pediatric cancer disposition syndrome characterized by bone marrow failure, developmental defects and a high incidence of hematologic malignancies [201].

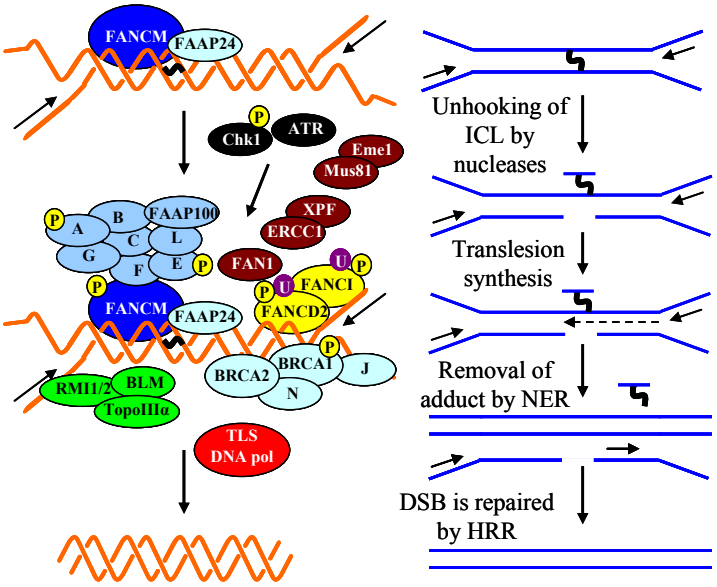

Figure 7. A schematic view of the Fanconi anemia pathway and its intersection with HRR. The different sub-units of the FA core complex (except FANCM) are depicted by single letters to indicate the complementation groups they belong to.

1.4 DNA DSB and the fate of cells with damage

The fate of cells that incur DNA DSBs is determined by a complex interplay between many factors [208]. First, the intrinsic ability of cells to maintain cell cycle arrest, which provides time for DNA repair, has a major impact on cell survival. For instance, acute myeloid leukemia (AML) cell lines that exhibit stringent G_2 checkpoint responses are more resistant to chemotherapy than counterparts that show transient G_2 arrest [77]. Likewise, glioma progenitor cells have a more robust G_2 checkpoint response than non-progenitor cells and are consequently more radioresistant [76, 209]. However, prolonged checkpoint activation can also be detrimental to cells in certain settings due to depletion of anti-apoptotic factors with short half-lives (e.g. Mcl-1) and in the extreme case, cause permanent loss of proliferative capacity [12, 13, 119]. Second, the ability of cells to accurately repair DNA DSBs influences whether cells can survive DNA damaging treatment. This point is particularly well illustrated by the extreme radio- and chemosensitivity of mammalian cells that are genetically deficient in DNA DSB repair [210]. Moreover, the developmental identities of cells are important determinants of fate by impinging on the proficiency of DSB repair. Thus for a given lineage, DNA repair is generally more precise in stem/progenitor cells than in more differentiated progenies [211-213]. In addition, mesenchymal cells tend to be more resistant than epithelial or hematopoietic cells due to inherent differences in growth rate and/or apoptotic threshold. Fourth, microenvironmental factors including autocrine/paracrine growth factor signaling, oxygenation/hypoxia and pH can greatly influence the susceptibility/resistance to DNA damage [214, 215]. Finally, mutations and/or de-regulated expression of certain proteins can sever (e.g. p53) or strengthen (e.g. E2F1) the signaling link between the DDR and cell death pathways, leading to either decreased or increased sensitivity to DNA damage [216, 217].

1.4.1 Cell survival

In order for a cell to survive DNA damage, several conditions must be satisfied. First, the amount of DNA damage incurred must not be large enough to trigger cell death outright [218-221]. Second, checkpoint mechanisms need to kick in promptly and provide time for DNA repair [79]. Third, genomic lesions need to be repaired with sufficient fidelity such that residual damage, if any, no longer sustains checkpoint signaling (recovery) [121, 122, 222, 223]. Alternatively, checkpoint signaling must be actively shut off by pro-survival signaling (adaptation) if efficient repair of DNA damage cannot be accomplished [121, 122, 137]. Fourth, cells must maintain low level expression of essential cell cycle regulators during checkpoint arrest allowing them to retain cell cycle competency following checkpoint release [135]. Fifth, any residual DNA damage should not elicit (prolonged) DDR signaling in the next cell cycle phase or overtly interfere with mitotic chromosome segregation [119]. Finally, the complement of genetic material inherited by each daughter cell after mitosis must be of sufficient quality to support various cellular processes that are essential for long-term viability [221]. Hence, cell survival following complete and faithful repair of all DNA lesions represents the best case scenario of a continuum of possible scenarios, where variable but relatively low levels of residual DNA damage may exist in viable cycling cells. As such, cells that survive DNA damage carry an increased risk for the development of delayed chromosomal instability.

1.4.2 Cell death

A failure to meet one or more of the above-mentioned conditions for survival significantly increases the likelihood that a cell will succumb to DNA damage-induced cell death, which has been shown to occur by multiple modes [220, 224-226]. Apoptosis and necrosis are two unequivocal cell death mechanisms. On the other hand, autophagy and mitotic catastrophe are more ambiguous in that they can also promote survival under certain conditions [227-231]. Senescence is not strictly a cell death mode since senescent cells remain metabolically active albeit being clonogenically dead [232]. While apoptosis and necrosis are generally considered to be irreversible, both autophagy and senescence are clearly reversible [233, 234]. Notably, multiple cell death modes can be triggered by different genotoxic stimuli, the same stimulus at different strengths, the same stimulus at identical strength in different cells or even transition into each other in the same cell [235-240].

Apoptosis

Apoptosis is a form of programmed cell death that is evolutionarily conserved from yeast to humans. Morphologically, apoptosis is characterized by cell shrinkage, membrane blebbing, chromatin condensation and DNA fragmentation that culminate in the formation of apoptotic bodies and rapid removal by professional phagocytes or neighboring cells [241, 242]. The main cellular enforcers of apoptosis are a family of cysteine proteases known as caspases [243] . These are synthesized as inactive zymogens containing an N-terminal pro-domain of variable length and a C-terminal protease domain. Depending on the length of their pro-domains, caspases are subdivided into two categories: initiator and effector (executioner). Initiator caspases (e.g. caspases-2, -8, -9) have large pro-domains containing specialized protein-protein interaction motifs (e.g. caspase recruitment domain, CARD; death effector domain, DED) that enable their recruitment into multi-protein complexes (e.g. PIDDosome, death-inducing signaling complex, apoptosome) through homotypic interactions with adaptor

molecules (e.g. RAIDD, FADD, Apaf-1) [244]. Within these complexes, inactive pro-caspases undergo conformational changes and/or auto-processing leading to their activation. By contrast, effector caspases (e.g. caspases-3, -6, -7) have short pro-domains. Activation of these caspases requires proteolytic maturation by either initiator caspases or other proteases (e.g. cathepsins) [245]. Enzymatically active effector caspases cleave protein substrates that function in a wide variety of cellular processes, such as structural maintenance (e.g. lamins), adhesion (e.g. focal adhesion kinase), anti-apoptotic signaling (e.g. Bcl-2) and DNA repair (e.g. DNA-PKcs, PARP-1), to bring about cell demise in an ordered and energy-dependent manner [246, 247]. There are two main apoptotic pathways operating in mammalian cells which are activated predominantly by extracellular (extrinsic) and intracellular (intrinsic) stimuli, respectively (Figure 8). In the extrinsic pathway, binding of extracellular death ligands (e.g. FasL/CD95L, TRAIL) to cell surface death receptors triggers receptor clustering and the formation of a death-inducing signaling complex (DISC) leading to the recruitment and activation of caspase-8, which in turn activates caspase-3 [248]. In the intrinsic pathway, intracellular stress signal (e.g. DNA damage) trigger multiple pro-apoptotic signaling modules (e.g. JNK, p38) to bring about the conformational activation of pro-apoptotic Bcl-2 family members Bak and Bax, leading to mitochondrial outer membrane permeabilization (MOMP) and the release of cytochrome c [249, 250] Roos, 2006 #737]. Once in the cytosol, cytochrome c and Apaf-1 assemble into a complex called the apoptosome that leads to the recruitment and activation of caspase-9, which in turn activates caspase-3. In addition, there is substantial cross-talk between these two pathways. For instance, cleavage of the pro-apoptotic Bcl-2 family member Bid by caspase-8 produces a truncated molecule (tBid) that translocates to the mitochondrial membrane where it contributes to the activation of Bak and Bax [251]. Conversely, caspase-3 activated through the intrinsic pathway can also cleave caspase-8 to further amplify the apoptotic cascade [252]. Moreover, many noxious stimuli are known to induce oxidative stress, causing damage to intracellular organelles such as lysosomes and endoplasmic reticulum (ER) [253, 254]. In turn, lysosomal membrane permeabilization (LMP) and ER stress could lead to activation of

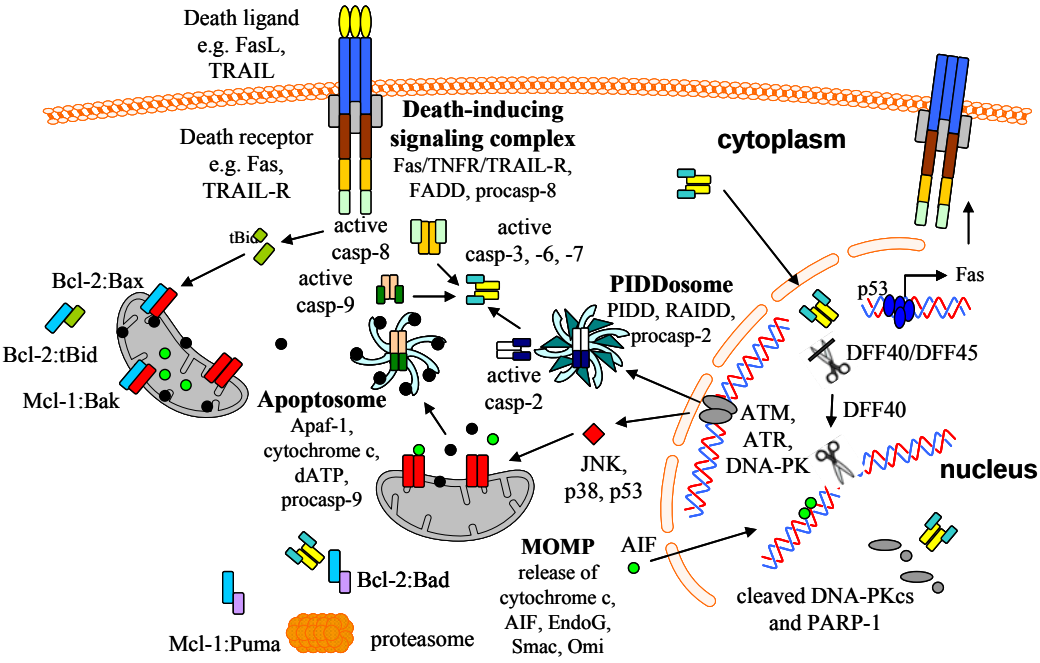

Figure 8. A schematic overview of the apoptotic pathways. DNA damage has been shown to activate both the extrinsic death receptor-mediated pathway and the intrinsic mitochondrial pathway. Casp, caspase.

cathepsins and calpains, which in some experimental models have been shown to activate effector caspases [255, 256]. Unlike caspases-3, -8 and -9, the role of caspase-2 in DNA damage-induced apoptosis is not clearly defined [257-261]. Although its structure resembles that of an initiator caspase, the phenotype of caspase-2-deficient cells suggests that it may have a more prominent role in regulating the G_2 checkpoint rather than apoptosis.

Because inappropriate activation of caspases carries potentially deleterious consequences, mammalian cells are equipped with a number of braking mechanisms that inhibit caspase activation and/or activity. Activation of caspase-8 within the DISC can be suppressed by c-FLIP, a DED domain-containing caspase homolog that lacks catalytic activity [262]. Anti-apoptotic Bcl-2 family members such as Bcl-2, Bcl-X_L and Mcl-1 normally hold Bak and Bax in inactive complexes, thereby preventing them from inducing MOMP [263]. Furthermore, the pro-apoptotic Bcl-2 family member Bad is phosphorylated by Akt, which leads to its sequestration by 14-3-3 proteins in the cytosol [264]. Accidental activation of caspases is also prevented by the inhibitor of apoptosis (IAP) family of anti-apoptotic proteins (e.g. XIAP, c-IAP1/2, survivin) which bind to caspases and suppress their activity. In response to DNA damage, p53 becomes stabilized and transcriptionally active, leading to the increased expression of a variety of pro-apoptotic proteins, such as FasL, death receptor-4/TRAIL receptor-1 (DR4/TRAIL-R1), Apaf-1 and the pro-apoptotic Bcl-2 family proteins Puma, Noxa and Bax [265]. Moreover, cytosolic p53 appears to directly induce MOMP by activating Bak and Bax [266, 267], which can release mitochondrial SMAC/Diablo and Htr2a/Omi to inactivate IAPs. Human tumor cells frequently show increased expression of various anti-apoptotic proteins as well as functional inactivation of p53. As expected, the apoptotic propensity of p53 deficient cells in response to DNA damage is markedly reduced [216]. In these cells, however, p53-independent apoptosis can still occur, but the molecular mechanisms are not well understood.

Necrosis

Necrosis is a form of cell death characterized by swelling and rupture of organelles as well as cells that leads to the release of noxious pro-inflammatory mediators and injury to surrounding tissues [241, 242]. In the context of chemotherapy, it is generally believed that the probability of triggering necrosis increases with increasing doses. Historically, cell death by necrosis is considered to be un-regulated and pathologic, caused by massive irreparable damage to cellular components. However, evidence has emerged over the past decade suggesting that necrosis can also be highly regulated. PARP appears to be an important regulator of necrosis. Over-stimulation of PARP activity by excessive alkylating or oxidative DNA damage causes rapid depletion of intracellular ATP and NAD(+), resulting in the shutdown of metabolism and caspase-independent programmed necrosis (aka necroptosis) [268] (Figure 9). As the name indicates, necroptosis share some features with apoptosis, in that its main effector apoptosis-inducing factor (AIF) needs to be released from mitochondria via a process that depends on calpain and Bax. AIF then translocates into the nucleus where it induces chromatinolysis (but not internucleosomal DNA cleavage as in apoptosis) by interacting with H2AX [268]. Two additional forms of programmed necrosis have been described recently (Figure 9). In one model, necrotic cell death after DNA damage was regulated by p53-dependent up-regulation of lysosomal cathepsin Q and reactive oxygen species (ROS) independently of Bak/Bax-mediated MOMP [269]. In the other model, necrotic cell death induced by tumor necrosis factor-α (TNFα) was shown to involve RIP1/RIP3-dependent formation of a “necrosome” complex that enhances ROS

production, calcium mobilization and lysosomal dysfunction [270]. Moreover, apoptotic cells can undergo secondary necrosis, especially in the absence of phagocytes as under most *in vitro* culture conditions [271]. The processes of apoptosis and necrosis are clearly more intertwined than previously thought and both are likely to contribute to chemotherapy-induced cell death *in vivo* [233].

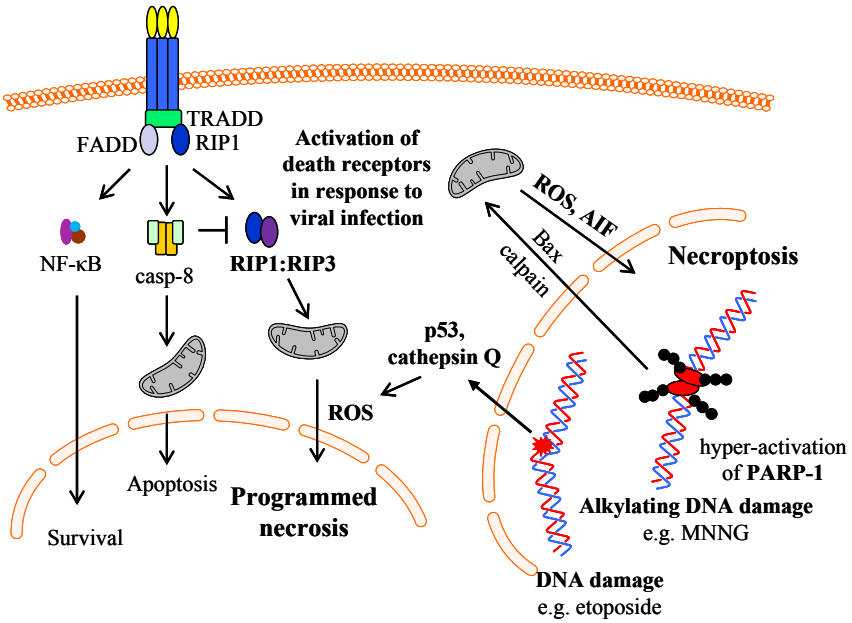

Figure 9. A schematic overview of the pathways implicated in programmed necrosis. DNA damage has been shown to induce necrotic cell death by at least two mechanisms via p53/cathepsin Q/ROS or PARP-1/Calpain/Bax/ROS/AIF. Depending on the adaptor proteins and cellular context, signaling from death receptors may lead to necrosis, apoptosis or survival.

Mitotic catastrophe

Mitotic catastrophe is a term used to describe highly disorganized and defective mitosis [225, 242, 272]. It is not clear whether mitotic catastrophe represents a distinct mode of cell death because there are no molecules exclusively linked to the execution of mitotic catastrophe. As such, mitotic catastrophe is more likely a physical manifestation of failed mitosis that is severe enough to trigger cell death by any mode(s) [225] (Figure 10). Mitotic catastrophe appears to be more easily invoked in tumor cells with compromised cell cycle checkpoints due to p53 deficiency or pharmacologically induced checkpoint abrogation [225, 273, 274]. Hallmarks of apoptosis (e.g., caspase activation) are frequently observed following mitotic catastrophe, suggesting that severe mitotic defects can trigger apoptotic cell death [275]. However, there is also evidence implicating an adaptive and pro-survival role of mitotic catastrophe [276] (Figure 10). Notably, severe DNA damage can drive p53-deficient tumor cells into the endocycle, whereby cells continuously replicate their DNA without undergoing cytokinesis leading to high-level ploidy (endopolyploidy) and formation of giant cells. While the vast majority of endopolypoid tumor cells are not clonogenically viable, a small fraction has been shown to up-regulate genes that control embryonic self-renewal, such as Oct4, Sox2 and Nanog, resulting in transient acquisition of stem cell properties and resistance to apoptosis [277]. Subsequent depolyploidization and reorganization of the genetic material can produce seemingly diploid daughter cells that are clonogenic. The process of

depolyploidization appears to be regulated by a set of genes normally involved in meiosis, a form of reduction cell division that generates haploid gametes (i.e., spermatogonia and oocytes) [278]. Alternatively, before they die non-viable polyploid giant cells produced from mitotic catastrophe may also give rise to mitotically viable daughter cells via nuclear budding and asymmetric cytokinesis in a phenomenon termed neosis [279]. Importantly, endopolyploidization-depolyploidization and neosis-like events have been observed during tumorigenesis as well as in response to DNA damaging treatment, suggesting that these processes have the potential to promote tumor heterogeneity and drug resistance [229, 230, 276, 278, 280, 281].

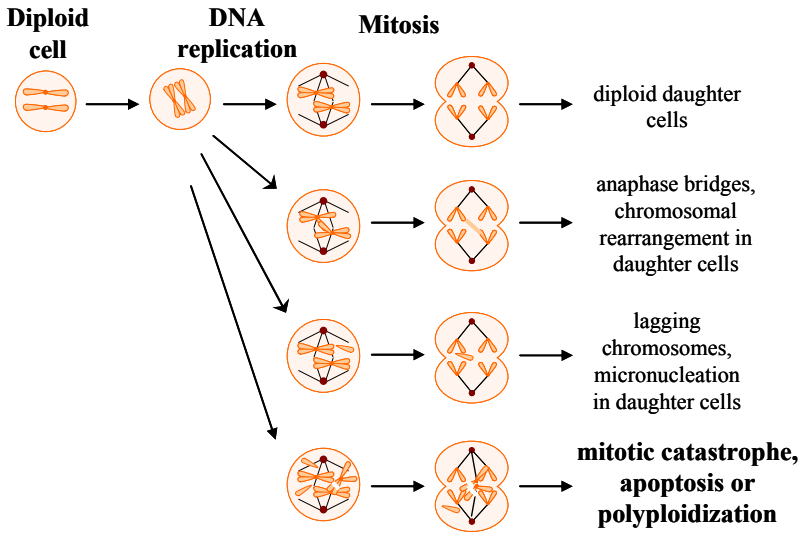

Figure 10. A schematic overview of mitosis. Depending on the severity, unrepaired DNA damage may cause chromosomal segregation defects or mitotic catastrophe. The latter can drive cells into apoptosis or the endocycle leading to high-level polyploidization.

Autophagic cell death

Autophagy involves the sequestration of cellular proteins and organelles into specialized double-membraned vesicles (autophagosomes) which subsequently fuse with lysosomes resulting in proteolytic degradation of the vesicular contents [282] (Figure 11). Autophagy is an essential physiologic process during development and its de-regulation has been demonstrated in a variety of human pathologies, including neurodegenerative diseases and cancer [283-285]. Autophagy is also elicited by ionizing radiation and many clinically used chemotherapeutic agents, such as temozolomide and etoposide [227, 286-288] (Figure 11). However, the consequence of drug-induced autophagy may be either cytoprotective or cytotoxic, depending on the cellular and experimental context under which it was induced [233]. In general, transient induction of autophagy is beneficial for tumor cells by isolating damaged cell components (e.g., mitochondria) and recycling limiting resources (e.g. nucleotides, amino acids, lipids). On the contrary, persistent autophagy is usually detrimental and leads to cell death. Notably, autophagic cell death often displays certain hallmarks of apoptosis (e.g., caspase activation), suggesting that these cell death modes may share some common features [289]. Inhibition of apoptotic effectors can sometimes trigger autophagic cell death [290]. Furthermore, apoptosis and autophagy appears to be co-regulated by Bcl-2 family members Bcl-2, Bcl-X_L and Beclin-1 at the level of mitochondria and ER [291, 292]. Therefore, autophagic cell death and apoptosis probably exist in a continuum and both of these cell death

modes can be induced in a given tumor. Interestingly, autophagy plays a dual role in tumorigenesis, serving as a barrier against the initiation of incipient tumors while enhancing the survival of established tumors in the face of metabolic crisis or drug treatment [293, 294]. Consistent with this notion, autophagy inhibition has emerged recently as a promising strategy for combating cancer [295, 296].

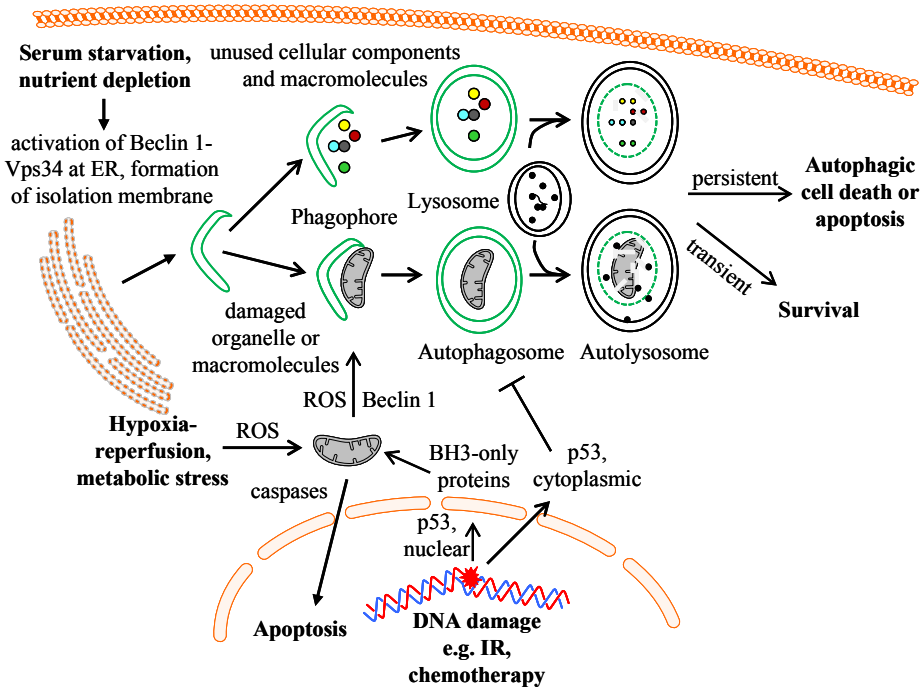

Figure 11. A schematic overview of autophagy. Autophagy is a normally a homeostatic process by which cells degrade and recycle unused or damaged organelles and macromolecules. Persistent autophagy may lead to autophagic cell death, which shares many features with apoptosis.

Senescence

Senescence refers to a state where cells remain metabolic active but are unable to proliferate. These cells are typically larger, appear flattened and express senescence-associated β-galactosidase [297]. The replicative life span of mammalian cells is limited by their ability to maintain telomere length [298]. The telomere is a region of highly repetitive nucleotide sequence at the end of a chromosome which interacts with specialized protein complexes (i.e. shelterin) to form a protective cap that prevent chromosome end-to-end fusion [299]. During each round of cell cycle, the telomeres become successively shorter because DNA polymerases cannot replicate DNA all the way to the end of the chromosomes. Critically shortened telomeres trigger a p53-dependent DNA damage response leading to permanent cell cycle arrest or replicative senescence that is maintained by $p21^{Cip1/Waf1}$ and $p16^{INK4a}$ [12] (Figure 12). Adult tissue stem cells typically express the enzyme telomerase and are therefore able to delay the onset of replicative senescence [300]. By contrast, differentiated cells normally do not express telomerase and consequently have a relatively short proliferative life span [301]. Notably, human tumors frequently re-activate telomerase expression giving them the ability to sustain proliferation indefinitely [232]. In telomerase-negative tumors, alternative means to maintain telomere length (e.g. BIR) have been described [302].

De-regulated oncogenic signaling and chemotherapy have been shown to induce terminal growth arrest in tumor cells by two related processes called oncogene-induced senescence (OIS) and premature senescence (aka accelerated senescence), respectively [303, 304] (Figure 12). Unlike replicative senescence, OIS and premature senescence are triggered by DNA damage that is not necessarily caused by telomere dysfunction. DDR signaling appears to plays a key role in the enforcement of senescence program [305], but there is no strict requirement on the p53-p21$^{Cip1/Waf1}$/p16^{INK4a} pathway [306, 307]. OIS constitutes an early barrier against tumorigenesis that indolent tumors must overcome to achieve full-blown malignancy [308]. Drug-induced premature senescence is an important route for tumor clearance *in vivo* [309, 310] and its evasion may limit the efficacy of cancer treatment [311]. However, senescence induction is a double-edged sword because senescent tumor cells and fibroblasts have been shown to secrete pro-inflammatory cytokines, proteases and mitogenic factors that lead to tissue remodeling in the microenvironment and accelerated tumor growth [312]. Hence, novel agents that can prevent the acquisition of senescence-associated secretory phenotype (SASP) have the potential to significant improve tumor eradication by conventional chemotherapy.

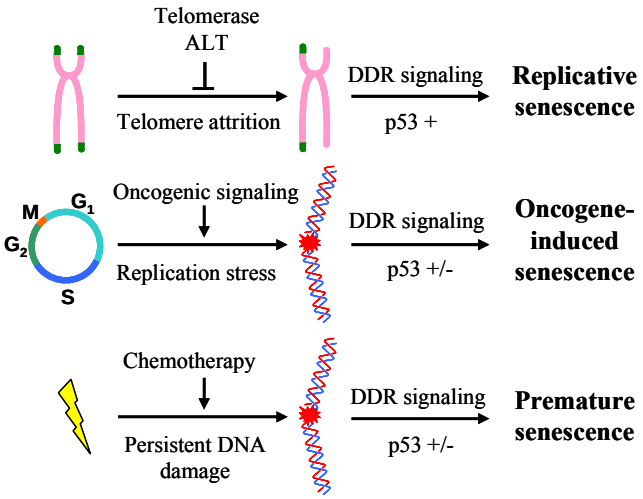

Figure 12. A schematic overview of pro-senescence pathways induced by telomere attrition, oncogene-induced replication stress or chemotherapy. ALT, alternative lengthening of telomeres.

1.5 DDR signaling in cancer

1.5.1 DDR as a barrier against tumorigenesis

There is ample evidence to suggest that the DNA damage response constitutes an early barrier against tumorigenesis [305, 313]. In pre-neoplastic tissue, oncogene-driven hyper-proliferation causes significant replication stress leading to chronic activation of the DDR machinery, orchestrated by ATR-Chk1 and ATM-Chk2 [314, 315]. Persistent DDR signaling induces senescence or death of oncogene-transformed cells resulting in delayed tumor formation. To achieve full-blown malignancy, pre-neoplastic cells must breach this early DDR-imposed barrier, which would explain the propensity of human malignant tumors, including lung cancer, to harbor genetic and/or epigenetic defects that inactivate key components of the DDR network, including ATM, Chk2, H2AX, 53BP1 and p53 [316-318].

1.5.2 DNA DSB repair deficiency and cancer predisposition

A significant number of (rare) inherited human syndromes are linked to deficient expression of genes encoding proteins that participate in DNA DSB repair and/or checkpoint regulation (Table 1). This list is likely to grow over time as research into the intricacy of the DDR network continues to reveal novel factors not previously known to function in DNA DSB repair. A common characteristic of these familial syndromes is hypersensitivity to endogenous (and exogenous) DNA damage, which in certain instances may predispose afflicted individuals to cancer and/or premature aging. Some inherited DNA DSB repair deficiencies also cause developmental abnormalities, mental retardation and/or immunodeficiency. Complete bi-allelic inactivation of DSB repair genes is relatively uncommon because it is usually not conducive to survival. Instead, most clinically relevant DNA DSB repair deficiencies stem from hypomorphic mutations which allow the mutant proteins to retain some residual activity. Notably, many genes implicated in DNA DSB repair, especially those involved in the ATR network, are haploinsufficient [319], suggesting that even partially compromised DNA DSB repair may precipitate the development of human pathologies. In addition to these familial cancer predisposition syndromes, somatic mutations and single nucleotide polymorphisms (SNPs) in DSB repair genes have also been implicated in tumorigenesis [320-325]. Finally, the DSB repair machinery itself appears to become less efficient and more error-prone during physiological aging, which may account for the age-dependent increases in cancer incidence [326].

Table 1. Human familial syndromes associated with DNA DSB repair deficiency.

Disorder	Gene	Radiation sensitivity	Cancer predisposition	Reference
Ataxia telangictasia	ATM	yes	yes	327
ATR-Seckel syndrome	ATR	yes	?	328
Nijmegen breakage syndrome	NBN [a]	yes	yes	329
AT-like disorder	MRE11	yes	?	330
Li-Fraumeni syndrome	TP53, CHEK2 [a]	yes	yes	331
Hereditary breast/ovarian cancer syndrome	BRCA1, BARD1, BRCA2, PALB2	yes	yes	332-334
Primary Microcephaly	MCPH1 [a]	yes	?	335
RS-SCID	PRKDC [a]	yes	?	336
LIG4 syndrome	LIG4	yes	?	328
Omenn syndrome (RS-SCID)	DCLRE1C [a]	yes	yes	328
NHEJ1 syndrome (RS-SCID)	NHEJ1 [a]	yes	?	337
RIDDLE syndrome	RNF168	yes	?	338
Bloom syndrome	BLM	yes	yes	339
Werner syndrome	WRN	yes	yes	339
Rothmund-Thomson syndrome	RECQL4	yes	no	339
Fanconi anemia	FANC(A-N)	yes	yes	199

Abbreviations: SCID, severe combined immunodeficiency.

[a] *NBN, CHEK2, DCLRE1C, MCPH1, NHEJ1 and PRKDC encode for Nbs1, Chk2, Artemis, microcephalin, XLF and DNA-PKcs, respectively.*

1.5.3 De-regulation of DNA DSB repair in cancer

As partial inactivation of DDR signaling during early stages of tumorigenesis is required for the transition into full-blown malignancy [314, 315], DNA DSB repair pathways in established human tumors are likely to be profoundly different from their normal counterparts. For instance, DNA repair in bladder cancer is often characterized by low fidelity NHEJ caused by diminished Ku DNA-binding activity [340, 341]. In leukemia, expression of oncogenic (fusion) tyrosine kinases (e.g. FLT3-ITD, BCR-ABL, TEL-JAK2, etc.) causes a shift from C-NHEJ to DNA Ligase III-mediated B-NHEJ [342, 343]. Multiple myelomas also show impaired NHEJ function although these cells seem to become genomically instable due to over-activation of HRR [344, 345]. Over-expression of Rad51 has been linked to hyper-recombination and/or increased usage of alternative HRR in a variety of cancers, including breast, pancreatic and BCR-ABL$^+$ CML [321, 346-349]. Similarly, loss of BRCA1/2 in breast and ovarian cancer compromises error-free HRR and causes up-regulation of mutagenic SSA [148]. Moreover, large tumors with hypoxic regions may resort to error-prone DSB repair due to reduced expression of HRR factors [350]. Finally, B-NHEJ can promote fusion of dysfunctional telomeres while BIR may facilitate telomerase-independent alternative lengthening of telomeres (ALT) [302, 351]. De-regulation of DNA repair is thus a double-edged sword; on the one hand promoting genomic instability to fuel the malignant evolution of tumors, while on the other hand imposing over-reliance on inefficient repair and checkpoint mechanisms for survival. As such, many tumors may exist in a primed-for-death state whereby sub-optimal DNA repair just barely manages to hold the mutation load at a survivable level. Consequently, therapeutics that abolish DNA checkpoint and/or repair have the potential to tip this precarious balance and selectively eradicate tumor cells [352, 353].

1.5.4 DNA DSB repair and tumor chemosensitivity

The relationship between DNA DSB repair and chemosensitivity is very complex and most likely context-dependent. In terms of relative mRNA and protein expression, different tumors have been found to produce higher, comparable or lower levels of certain DNA DSB repair factors than adjacent normal tissues (Table 2). However, depending on their histological origin and/or stage, such expression changes may or may not be associated with tumor aggressiveness or chemosensitivity. In addition, SNPs in DSB repair genes may also have important bearings on the outcome of treatment [322, 354].

1.6 Resistance to DNA damaging chemotherapy

1.6.1 Mechanisms of resistance

The underlying causes of resistance, whether intrinsic or acquired, can be pleiotropic and different for each chemotherapeutic agent. Intrinsic resistance refers to profound drug insensitivity that is apparent already at initial exposure to a particular treatment and is usually linked to processes involved in tumorigenesis itself. By contrast, acquired resistance is by definition induced upon (recurrent) drug treatment, whereby tumors cells that are initially sensitive become progressively more resistant to subsequent treatment. Drug resistance mechanisms can evolve independently or in tandem in a given tumor, and can be transient or long-lasting. Moreover, exposure to a single chemotherapeutic agent may elicit cellular defense mechanisms that render tumor cells cross-resistant to a multitude of other drugs, a condition termed multidrug

resistance (MDR). Because they obvious impact on treatment outcome, resistance mechanisms that human tumors utilize to survive conventional DNA damaging chemotherapy have been the subject of intense research. Known causes of chemoresistance can be broadly divided into four categories: (1) failure of the drug to reach its intended target, the DNA (pre-target resistance), (2) inability of the drug to efficiently induce DNA lesions (on-target resistance), (3) malfunctioning of cell death pathways (post-target resistance) and (4) constitutive activation of pro-survival signaling pathways that are unrelated to the drug treatment *per se* but which can abolish its death-inducing capacity (off-target resistance). A few examples clinically relevant resistance mechanisms are provided below.

Table 2. Alterations in the expression of DNA DSB repair factors and their impact on tumor progression and resistance.

Tumor type	Transcript expression	Protein expression	Resistance [a]	Reference
T-cell lymphomas B-cell lymphomas Multiple myeloma	decreased XRCC6 [b] and MRE11A; increased XRCC4 and Rad50 in MM	n.d.	n.d.	355
Multiple myeloma	increased endo-/ exonucleases, helicases, ERCC1, Rad23, Rad50, Rad51, Rad51B, Rad51C, Rad51D and XRCC3	increased Rad51, Rad51B, Rad51C and Rad51D	n.d.	344
Diffuse large B-cell lymphoma	decreased 53BP1 in a subset of tumors	n.d.	n.d.	356
Chronic myeloid leukemia	n.d.	Increased DNA-PKcs	chlorambucil	357
Breast cancer Bladder cancer	n.d.	increased Ku70/80 only in breast cancer	n.d.	358
Breast cancer	n.d.	increased Rad51 with tumor grade in sporadic cases; increased Rad51 in BRCA1-deficient cases	n.d.	359, 360
Cervical cancer Esophageal cancer	n.d.	increased DNA-PKcs and Ku70/80 in residual tumors	expression correlates with IR resistance	361, 362

Abbreviation: n.d., not determined.
[a] *Resistance to chemo-/radiotherapy;* [b] *XRCC6 encodes forKu70.*

Altered drug transport and detoxification as examples of pre-target resistance

Most if not all chemotherapeutics with intracellular targets require some types of transport mechanism en route to their destination. Alterations in drug transport can thus be an important contributor of resistance by preventing chemotherapeutics from reaching their intended targets. For instance, tumor cells may acquire resistance to platinum-based compounds (e.g. cisplatin) as a result of reduced drug uptake, which can occur by drug-induced down-regulation of the copper influx transporter hCtr1 or enhanced expulsion of internalized drug by the copper efflux transporters ATP7A/B [363]. Likewise, intracellular accumulation of anthracyclines (e.g. doxorubicin) is hampered

by over-expression of several efflux pumps of the ATP-binding cassette (ABC) transporter superfamily (e.g. p-glycoprotein) [364].

Detoxification refers to processes by which cells inactivate xenobiotics either enzymatically or by sequestering them away from their intended targets. Given that many anti-cancer drugs (e.g. bleomycin, cisplatin) trigger the production of ROS through iron-catalyzed redox reactions and/or disruption of mitochondrial respiratory chain, it is perhaps not surprising that increased expression of proteins involved in the cellular antioxidant defense (e.g. glutathione S-transferases, thioredoxin reductase, superoxide dismutase) is frequently observed in chemoresistant tumors [365]. In addition, increased acidification of intracellular organelles (e.g. lysosomes, endosomes, *trans*-Golgi network) with concomitant cytoplasmic alkalinization is a recurrent feature in many tumors. This would allow the membrane-permeable neutral form of weakly basic drugs (e.g. anthracyclines) to diffuse into acidic organelles where they become protonated and trapped, thereby reducing the intracellular concentration of drugs at their nuclear target sites [366].

Enhanced DNA repair as an example of on-target resistance

DNA represents the major intracellular target of many commonly used chemotherapeutic agents. Unrepaired DNA damage is highly cytotoxic, as demonstrated by the extreme radiosensitivity in patients afflicted with rare human syndromes caused by inherited mutations in DNA repair genes, such as ataxia telangiectasia, Fanconi anemia and xeroderma pigmentosum [367, 368]. It is generally believed that elevated DNA repair capacity correlates with diminished tumor sensitivity to DNA damaging chemotherapy. For instance, high expression of the nucleotide excision repair (NER) and HRR component ERCC1 is associated with platinum resistance in non-small cell lung carcinoma (NSCLC) [369, 370]. Similarly, high expression of the DNA repair protein MGMT is associated with resistance to alkylating agents, in particular temozolomide, and conversely MGMT promoter methylation correlates with increased drug sensitivity [371]. Enhanced FA/BRCA-mediated ICL repair contributes to melphalan resistance in multiple myeloma and there is evidence suggesting that high expression/activity of DNA-PK is associated with chlorambucil resistance in B-cell chronic lymphocytic leukemia (B-CLL) [357, 372-374]. The epidermal growth factor receptor (EGFR), which is frequently over-expressed in NSCLC and gliomas, is a positive regulator of DNA-PK activity and this may account for the observed benefit of adding an EGFR inhibitor to standard platinum-based chemotherapy [375-377]. Finally, Rad51 is over-expressed in many human tumors; the available data suggest that Rad51 may confer chemoresistance as well as genomic re-stabilization of previously instable tumors [346, 378].

The cellular DNA repair machinery is subject to complex regulation by growth receptor signaling. For instance, both the EGFR and IGF1R exert cytoprotective effects in lung cancer cells by promoting NHEJ [376, 379]. Essentially all growth factor receptor studied in connection with DDR have been shown to enhance DNA repair, including c-Met, TrkA, Her2/ErbB2 and the aforementioned EGFR and IGF1R [376, 379-383]. In addition, growth factor receptor signaling almost invariably leads to activation of the phosphatidylinositol 3'-kinase (PI-3K)/Akt pathway, which is a potent inhibitor of both apoptosis and checkpoint responses [103, 264]. Since over-expression of growth factor receptors is a fairly common phenomenon in human tumors, paracrine growth factor signaling could have a major influence on chemoresistance by promoting DNA repair, checkpoint override and apoptosis evasion.

Defective apoptotic signaling as an example of post-target resistance

Human tumors can acquire resistance to apoptosis through de-regulation of the extrinsic and intrinsic apoptotic pathways as well as their upstream regulatory networks [384, 385]. For instance, reduced apoptosis sensitivity of cisplatin-treated NSCLC cells has been shown to result from diminished expression of Fas, pro-caspase-8, pro-caspase-9, XIAP, Bcl-2 and Bcl-X_L [386]. Similarly, over-expression of c-FLIP in breast cancer cells confers resistance to doxorubicin/interferon-γ co-treatment [387]. Increased expression of anti-apoptotic Bcl-2 family members (e.g. Mcl-1) is associated with poor prognosis as well as chemoresistance (e.g. etoposide, doxorubicin) in both neuroblastoma and leukemia, while decreased expression/activation of their pro-apoptotic counterparts Bax and Bak in colon cancer and NSCLC resulted in resistance to etoposide and IR, respectively [388-391]. Elevated expression of IAPs and heat shock proteins, which negatively regulate caspase activity, has also been implicated in chemoresistance in some tumors [384, 392]. Furthermore, hyper-activity of Akt, caused by de-regulated upstream signaling from receptor tyrosine kinases (e.g. EGFR), Ras and PI-3K or deletion of its negative regulator PTEN, is observed in a large proportion of tumors and contributes to suppression of chemotherapy-induced apoptosis [385, 389, 393]. In fact, defective apoptotic signaling is a recurrent feature for most if not all human solid tumors [394].

Hypoxia, quiescence and EMT as examples of off-target resistance

Insufficient oxygen delivery is fairly common in solid tumors, especially when a tumor has reached beyond the size of 3 cm. In these cases, the cells located in the center of the tumor experiences hypoxia, which has significant impact on chemosensitivity [395]. First, the lack of adequate perfusion of blood results in diminished drug delivery to the hypoxic region of the tumor. Second, hypoxia limits the formation of cytotoxic ROS that is induced by many chemotherapeutic drugs. Third, hypoxic cells often enter quiescence and become insensitive towards drugs that selectively target DNA replication-related processes. Finally, hypoxia directly shifts the balance of DNA repair from error-free HRR to error-prone NHEJ [350, 395]. This decrease in repair accuracy in tandem with increased resistance to cell death can cause genomic instability, rapid tumor evolution and metastasis.

Cancer stem cells (CSCs, aka tumor-initiating cells, TICs) belong to a rare sub-population of cells within the tumor bulk endowed with the capacity to self-renew and sustain tissue ontogeny, a defining property of normal embryonic/adult stem cells [396, 397]. CSCs typically activate transcriptional modules associated with maintenance of pluripotency (e.g. Oct4, Sox2, Nanog) and express similar surface markers as normal stem cells (e.g. CD133, CD44) [398-400]. CSCs generally show increased chemo- and radioresistance as compared to their more differentiated counterparts, which is due partly to their superior capacity in DNA repair and/or checkpoint arrest and partly to their quiescent state [72, 76, 209, 401-406]. Quiescence refers to a state of dormancy during which cells resides in G_0. Since many chemotherapeutic agents (e.g. cisplatin, gemcitabine) preferentially kill fast proliferating tumor cells, the lack of cell cycle progression as a result of quiescence largely negates the activity of S-phase targeting drugs. Quiescence can also be reversibly induced in differentiated cells by certain anti-cancer treatments and might represent an adaptive response to adverse metabolic conditions [407].

Epithelial-mesenchymal transition (EMT) refers to a developmental process in which cells with a predominantly epithelial identity acquire features consistent with a predominantly mesenchymal identity, characterized by decreased cell adhesion (loss of E-cadherin) and increased motility [408]. Although EMT is physiologically important during normal embryonal development, organ formation and wound healing, it can also be pathologically induced in tumors by de-regulated oncogenic signaling pathways (e.g. growth factors, Ras, Wnt/β-catenin and Notch) [408]. EMT is controlled by a number of transcription factors (e.g. Snail, Slug, Twist, Smads) that collectively promote survival signaling, acquisition of stem cell properties, increased DNA repair (e.g. up-regulation of ERCC1) and metastasis [409-411].

1.7 DDR signaling as a target for chemotherapy sensitization

1.7.1 Abrogation of cell cycle checkpoints

Functional inactivation of the tumor suppressor protein p53 is a recurrent feature in human tumors [84, 412]. This can happen through deletion of the chromosomal arm where p53 resides, inactivating mutation of p53 itself or over-expression of its chief negative regulator MDM2, which targets p53 for proteasomal degradation. Loss of p53 functions usually lead to increased tumor resistance to DNA damaging agents due to the absence of p53-dependent pro-apoptotic signaling [413]. However, these tumors also lack the ability to initiate checkpoint arrest in G_1, a largely p53-dependent process and become overly reliant on checkpoints in S and G_2 to deal with endogenous DNA damage and replication stress associated with hyper-proliferation [80]. Although p53 is not absolutely required for enforcing cell cycle arrest in S and G_2, it does modulate the functions of these checkpoints, which tend to become less stringent in its absence [74, 95]. It is therefore anticipated that combining DNA damaging chemotherapy with drugs that abrogate the intra-S and G_2 checkpoints would overwhelm the DNA repair machineries of p53-mutant tumors, leading to enhanced therapeutic efficacy [414, 415] (Figure 13). The validity of this hypothesis is currently being tested for a number of small molecule inhibitors of Chk1/2 that recently entered clinical trials [78, 79].

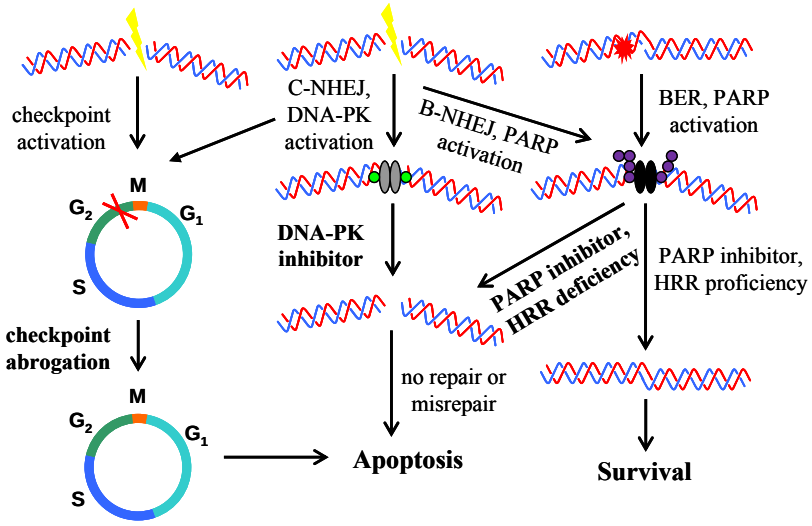

Figure 13. Examples of strategies for targeting DNA DSB repair.

1.7.2 Inhibition of DNA DSB repair

As most conventional chemotherapeutic agents kill tumor cells by inducing DNA damage, it is widely expected that their efficacy should be enhanced by inactivation of the cellular DNA repair machinery. While this assumption is intuitively attractive, it also presents a number of potential pitfalls. For instance, lowering the DNA repair capacity indiscriminately may cause significant collateral damage to highly DNA damage-sensitive normal proliferative tissues such as the colon mucosa and bone marrow, while inducing mutagenesis in other tissues that are not apoptosis-prone. Moreover, as human cells routinely employ multiple DNA repair pathways in response to chemotherapy, targeting a single pathway may not be sufficient to markedly augment tumor eradication [416]. In addition, there is evidence suggesting that functional DNA damage signaling might be required to trigger cell death, particularly when damage is extensive [218, 219]. Nevertheless, the concept of targeting DNA DSB repair as a means to enhance chemotherapy does have merits and may be highly effective in certain context (see below).

PARP inhibitors and synthetic lethality

Synthetic lethality refers to the state whereby individual inactivation of two genes is tolerated but their compound inactivation causes lethality. The concept of synthetic lethality has gained widespread attention since the discovery of BRCAness in breast and ovarian cancers, a term that has been used to describe functional inactivation of BRCA1/2 or their associated network. BRCAness, which is characterized by deficiencies in HRR, renders tumor cells highly susceptible to PARP inhibition because the loss of PARP-dependent base excision repair (BER) causes the accumulation of DNA single strand breaks (SSB) and their conversion into replication-associated DNA DSBs. Such lesions are normally dealt with by HRR during S phase, but remain unrepaired or misrepaired by NHEJ in BRCA-deficient cells leading to apoptosis [417-420] (Figure 13). A multitude of clinical trials have since been initiated to examine the feasibility of using PARP inhibitors as mono-therapy as well as in combination with conventional chemo/radiotherapy. To date, the main focus has been on breast and ovarian cancer where BRCA1/2 deficiency is relatively common, but the scope is beginning to widen following the elucidation of the FA pathway as part of the BRCA network [332, 421, 422]. Certain tumor-specific mutations and drug treatment also appear to induce conditions akin to BRCAness which are potentially amenable to PARP inhibition [423, 424]. Finally, several recent studies identified synthetic lethal interactions between PARP and many protein factors not known to participate in BRCA-dependent HRR [425-427]. Collectively, these data suggest that PARP inhibitors may be useful for targeting human tumors with a wide spectrum of DNA repair defects. In addition, the utility of PARP inhibitors in repair-proficient tumors also warrant further investigation, especially in combination with another DNA repair inhibitor [428].

DNA-PK modulators

Given the key role of NHEJ for promoting efficient DNA DSB repair, substantial efforts have been invested in the research of DNA-PK inhibitors as novel chemosensitizers [429]. Unfortunately, the development of DNA-PK inhibitors has stagnated over the past few years, with no compound yet having been approved for clinical trials even though pre-clinical testing has yielded promising results [357, 430-437]. The main obstacle facing at least one class of experimental small molecule DNA-PK inhibitors appears to be related to poor solubility and short metabolic half-lives [438].

DNA-PK inhibitors are thought to potentiate chemo/radiotherapy by increasing the longevity of unrepaired DNA as a result of impaired NHEJ and possibly also HRR [439] (Figure 13). Interestingly, an alternative approach involving pharmacologically-induced hyper-activation of DNA-PK has been recently demonstrated to chemosensitize tumor cells [440, 441]. This strategy employs double-strand DNA bait molecules (Dbait) that act as decoys creating a molecular sink that drains the cellular pool of DNA-PK prior to DNA damaging treatment. Considering that hypo- as well as hyper-phosphorylation of DNA-PK increased cellular DNA damage sensitivity, it follows that perturbation of DNA-PK kinase activity through either inhibition or hyper-activation may have the potential to enhance chemosensitivity in tumors [442].

1.8 Phenothiazines – What are they and what can they do?

Phenothiazines are a class of heterocyclic dopamine receptor antagonists widely used as anti-psychotic medication and as anti-emetics to relieve post-operative or chemotherapy-induced vomiting [443-445] (Figure 14). They are commonly referred to as typical antipsychotics (aka first generation antipsychotics) to distinguish them from atypical antipsychotics (aka second generation antipsychotics), such as clozapine and risperidone, which target the dopamine pathway more specifically and are considered to have superior safety profiles [443]. Common mild side effects of phenothiazines include dry mouth (anti-cholinergic effect), sedation (anti-histaminergic effect), weight gain (metabolic effect) and skin photosensitivity [446, 447]. Long-term treatment with phenothiazines are also associated with the development of extra-pyramidal symptoms (EPS), including akathisia/dyskinesia, muscle tremor and Parkinsonism (anti-dopaminergic effect), which are more debilitating and usually require therapeutic intervention [448]. Neuroleptic malignant syndrome (NMS) and agranulocytosis are two rare but potentially fatal conditions that can be induced by phenothiazine antipsychotics [449, 450]. The spectrum of adverse effects associated with phenothiazine treatment indicate that these compounds are likely to impact on a multitude of physiological processes in addition to their activity in the nervous system.

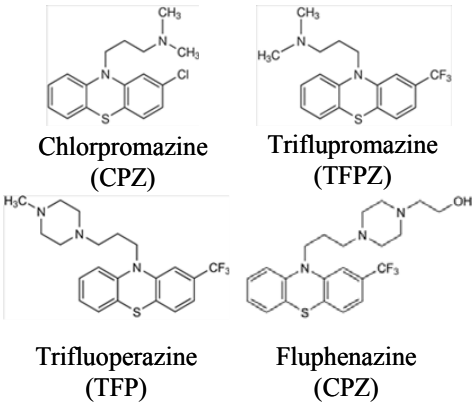

Figure 14. Chemical structures of some common phenothiazine compounds.

1.8.1 Molecular targets of phenothiazines

Neurotransmitter receptors

One of the most important clinical indications for phenothiazine-based medication is the treatment of psychotic disorders, which is thought to be mediated selectively through dopamine D_2 receptor antagonism [443]. However, phenothiazines also bind with significant affinity to several other types of neurotransmitter (e.g. acetylcholine, histamine, serotonin) receptors and the moderate anti-emetic activities of many phenothiazines are likely derived from antagonism of serotonin receptors [451]. Notably, high expression levels of neurotransmitter receptors are not restricted to neuronal or endocrine cells, but are also found in a variety of human (tumor) cells of non-neuroendocrine origin (Human Protein Atlas, http://www.hpr.se). Therefore, phenothiazines can theoretically influence the behavior of many cell types in a highly complex manner. Interestingly, a recent report showed that phenothiazines can inhibit the ability of neural stem cells and primary brain tumor cells to form neurospheres, suggesting that normal as well as malignant stem/progenitor cells may be sensitive to perturbations in neurotransmission pathways [452].

Ion channels

The effect of phenothiazines on ion channels has mostly been investigated in the context of its possible contribution to cardiotoxicity in patients on antipsychotic medication. Human cardiomyocytes express a number of ion channels of which hERG, a potassium channel, appears to be selectively inhibited by phenothiazines [453]. Similar results were also observed in HEK293 and Chinese hamster ovary (CHO) cells transfected with hERG cDNA [453, 454]. Interestingly, hERG channels are expressed in a variety of tumor cells, including lung carcinomas, and hERG inhibitors have been shown to be of therapeutic value in cancer therapy [455, 456].

Drug efflux pumps

The ABC transporters belong to a superfamily of transmembrane proteins that utilize ATP hydrolysis to translocate a variety of substances, including solutes, nutrients and xenobiotics, across biological membranes [364]. Over-expression of certain ABC transporters, such as p-glycoprotein (pgp), is thought to increase therapy resistance in human tumors by facilitating drug efflux [457]. It is therefore anticipated that efflux blockers may enhance chemotherapy and considerable efforts have been devoted to their development. Early studies showed that many calcium channel blockers (e.g. verapamil) and Ca^{2+}/CaM antagonists (e.g. phenothiazines) interfere with pgp-mediated transport of vinca alkaloids (e.g. vincristine) and anthracyclines (e.g. doxorubicin) [458]. Mechanistically, phenothiazines appear to directly bind pgp, possibly at the same site(s) as its natural substrates, although an additional indirect effect due to perturbation of plasma membrane fluidity cannot be excluded [459]. TFP has been reported to increase the permeability of the blood-brain barrier (BBB), where pgp is abundantly expressed, to a number of compounds which normally have limited accessibility to the CNS, such as etoposide and ivermectin [460, 461]. In another study, however, TFP had no effect on the distribution of vinblastine [462], suggesting that phenothiazines may not universally enhance CNS penetration of all chemotherapeutic agents that are substrates of pgp. Nevertheless, phenothiazines could enhance plasma retention of doxorubicin and etoposide in patients receiving chemotherapy, probably by inhibiting its clearance via pgp-expressing cells in the renal proximal tubules [463, 464]. Furthermore, phenothiazines

have been shown to reverse drug resistance in MDR tumor cell lines that over-express different ATP-dependent efflux pumps [459]. While these data clearly demonstrate that phenothiazines are capable of antagonizing drug efflux *in vitro* as well as *in vivo*, several early clinical trials failed to prove conclusively that phenothiazines are useful therapeutically as efflux blockers of anti-cancer drugs [465-468]. Although the focus of research on phenothiazines have since shifted from cancer treatment to management of MDR in bacteria, some new phenothiazine derivatives with promising anti-tumor MDR reversing activities did emerge recently [469-472].

Calcium/calmodulin

Calmodulin (CaM) is a ubiquitously expressed protein that acts as a multifunctional calcium sensor and signal transducer. CaM regulates a variety of cellular processes, including proliferation, metabolism, inflammation, and cell death. It is not clear whether CaM antagonism may underline some aspects of neuromodulation by phenothiazines in psychiatric disorders. However, CaM antagonism appears to a major contributor of phenothiazine-induced cytotoxicity and the order of anti-proliferative potency of phenothiazines closely mirrors their CaM antagonistic activity [473, 474]. Phenothiazines have also been reported to inhibit the functions of some non-CaM-regulated calcium-binding proteins (e.g. protein kinase C, troponin C) [475, 476].

1.8.2 Cellular processes that are affected by phenothiazines

Membrane fluidity

The amphiphilic nature of phenothiazines favors molecular interactions with zwitterionic lipids in biological membranes, such as the plasma membrane [477] (Figure 15). Insertion of phenothiazines into the lipid bilayer enhances the fluidity and permeability of plasma membrane of epithelial cells and causes shape alterations in erythrocytes [478, 479]. In line with this, phenothiazines appear to affect cholesterol homeostasis by modulating the expression of genes involved in sterol biosynthesis [480, 481]. As a result, phenothiazines have been reported to induce dissociation of several membrane-bound signaling proteins, including K-Ras and EGFR [482-484]. Furthermore, phenothiazine-induced membrane stress appears to inhibit translation initiation in both yeast and human cells [485, 486].

Cytoskeletal dynamics and cell motility

Phenothiazines have been reported to disrupt the organization of cellular microfilament network and hepatic metabolites of chlorpromazine could induce gelation of actin [487, 488]. Moreover, phenothiazines inhibit the interaction between myosin-IIA and S100A4, a member of the S100 family of small calcium-binding proteins implicated in the regulation of cell motility, suggesting that phenothiazines may suppress metastasis [489]. Interestingly, exposure to phenothiazines resulted in rapid apoptotic cell death in chemotherapy-resistant high-grade primary breast cancer cells that over-express another S100 family member, S100P [490]. Phenothiazines are also known to inhibit migration of lymphocytes and glioblastoma cells, possibly due to impairment of myosin/actin dynamics and/or matrix metalloproteinase activation [491-493]. Finally, phenothiazine treatment enhances the attachment of human non-small cell lung carcinoma (NSCLC) U1810 cells to the plastic substratum of culture dishes, indicating that phenothiazines may also affect cell adhesion (our unpublished data). It is tempting

to speculate that the cytoskeletal effects of phenothiazines are derived from CaM antagonism (Figure 15). However, definitive proof for this assertion is not available at present.

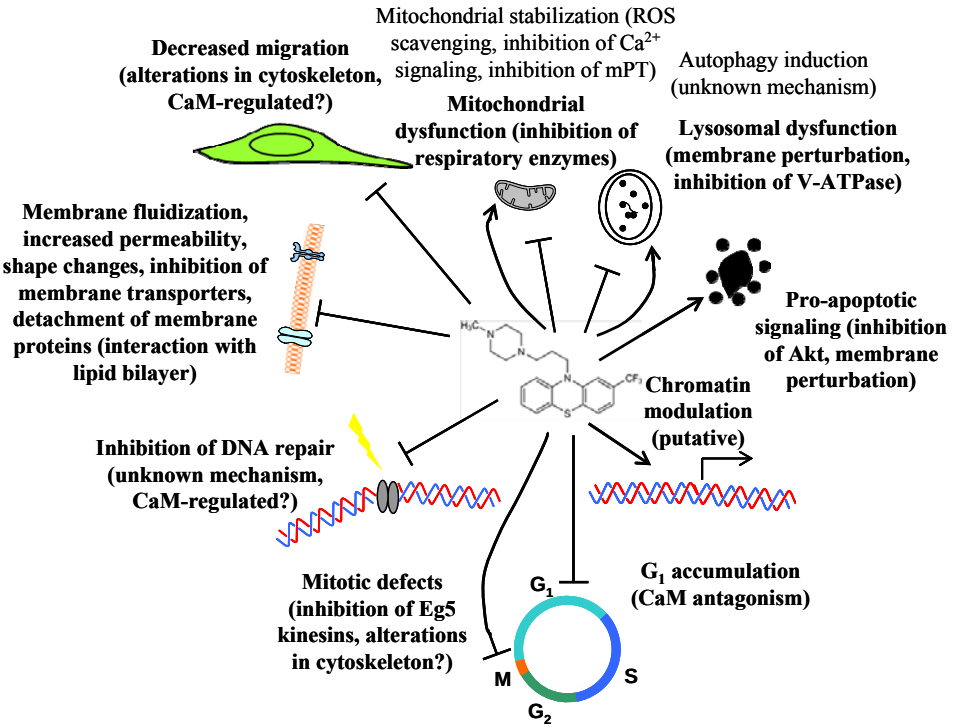

Figure 15. Examples of cellular processes that are modulated by phenothiazines. Processes that may account for phenothiazine-induced cytotoxicity are in highlighted in bold. Other processes have been implicated in phenothiazine-mediated cytoprotection.

Lysosomal function

Phenothiazines are lysosomotropic compounds that contain weakly basic amine groups attached to a core of largely lipophilic tricyclic ring system (Figures 14 and 15). These properties allow uncharged phenothiazines to freely intercalate into biological membranes or cross it. Once inside acidic lumen of lysosomes, the amine groups of phenothiazines become protonated, thus preventing the now positively charged phenothiazines from diffusing out of lysosomes [494]. Accumulation of lysosomotropic compounds within lysosomes may cause a rise in luminal pH and adversely affect the activities of many lysosomal proteins, such as cathepsins and acidic sphingomyelinases. Consistent with this notion, resistance to chronic TFP treatment in *Sacchromyces cerevisiae* has been correlated to over-expression of components of the vacuolar ATPase which might be required to maintain an adequate level of vacuolar acidification [495, 496]. In mammalian cells, lysosomotropic compounds (e.g. chloroquine) can inhibit autophagy due to elevation of lysosomal pH and failure of autophagosome maturation [497]. Paradoxically, phenothiazines have recently been identified as activators of autophagy, suggesting that partial disruption of lysosomal function may stimulate compensatory autophagy, while more severe lysosomal perturbation can block it [498, 499]. High intra-lumenal concentrations of lysosomotropic compounds may also cause rupture of lysosomal membrane as a result of excessive osmotic pressure, releasing lysosomal proteases that can initiate apoptosis [500]. Finally, lysosomotropic compounds have been shown to affect intracellular distribution of other drugs with similar chemical

properties [494, 501]. As such, phenothiazines can theoretically reduce the lysosomal sequesteration of certain anti-cancer drugs (e.g. daunorubicin), which may lead to increased therapeutic efficacy or exacerbated toxicity.

Cellular respiration and mitochondrial homeostasis

Phenothiazines have been shown to influence the activities of several metabolic enzymes that participate in glycolysis and the Kreb's cycle [502]. Thus, phenothiazines may disrupt mitochondrial energy production, although the concentrations of TFP (66 μM) needed to significantly inhibit respiration is well above that required for chemosensitization (10 μM) [503-505]. However, the interplay between phenothiazines and mitochondria is very complex (Figure 15). On the one hand, UVA-induced phenothiazine free radicals disrupt mitochondrial functions and cause cell death; on the other hand, phenothiazines have been shown to exhibit antioxidant activities and protect mitochondria from oxidative damage possibly through inhibition of mitochondrial permeability transition (mPT) [506-508]. Notably, the impact of phenothiazines on mitochondrial homeostasis and bioenergetics appears to be dose-dependent: at non-cytotoxic concentrations (<10 μM), phenothiazines behave predominantly as antioxidants, while at cytotoxic concentrations (>10 μM), phenothiazines promote mitochondrial swelling accompanied by dissipation of mitochondrial transmembrane potential, mPT and release of calcium [509]. Moreover, TFP potentiates DNA damage-induced ROS production and mitochondrial dysfunction in human NSCLC cells but antagonizes hydrogen peroxide-induced oxidative stress in rat pheochromocytoma PC12 cells, indicating that the mitochondrial effect of phenothiazines is likely to be context-dependent [504, 510]. The potential therapeutic benefits of harnessing both pro- and antioxidant properties of phenothiazines have been demonstrated in experimental models of photodynamic therapy and ischemia/oxidative injury, respectively [511-514].

Akt signaling

The PI-3K/Akt pathway has important pro-survival/anti-apoptotic functions, and as such is frequently hyper-activated in human tumors. Interestingly, several lines of evidence suggest that phenothiazines may modulate PI-3K/Akt signaling (Figure 15). In ovarian cancer cells, phenothiazines have been shown to block phosphorylation of Akt on both Thr-308 and Ser-473, which are required for full activation of Akt by upstream kinases [515, 516]. Phenothiazines have been reported to antagonize de-regulated mTOR activity in lung adenocarcinoma and to selectively induce apoptosis in PTEN-deficient tumors by blocking Akt-dependent export of FOXO1a into the cytosol [517, 518]. Moreover, phenothiazines are able to interfere the chaperone activity of Hsp70, which in turn causes down-regulation of its client protein Akt [519]. Taken together, these data indicate that phenothiazines may impact PI-3K/Akt/mTOR signaling on multiple levels.

Cell cycle progression

As CaM antagonists, phenothiazines are expected to negatively modulate G_1/S transition [520] (Figure 15). In line with this, TFP induces $p21^{Cip1/Waf1}$ expression via modulation of its transcriptional regulator Egr-1 in C6 and U87MG glioma cells [521, 522] as well as in U1810 NSCLC cells via a p53-independent mechanism (our unpublished data). Phenothiazines also influence mitosis (Figure 15). For instance, chlorpromazine

(CPZ) has been shown to be anti-mitotic, especially in combination with pentamidine [523-525]. The mitotic target of phenothiazines appear to be the Eg5 mitotic kinesin and its inhibition results in the formation of monopolar spindles, mitotic arrest and apoptosis [525, 526]. However, another report showed that inhibition of mitotic slippage by CPZ after paclitaxel treatment resulted in prolonged mitotic arrest and enhanced survival [527]. These conflicting results suggest that the outcome of phenothiazine-mediated inhibition of mitotic processes may be cell type- and/or context-dependent.

DNA repair

Evidences of phenothiazines as DNA repair modulators are well-documented (Figure 15). At the molecular level, studies comparing the effects of phenothiazines with non-phenothiazine CaM antagonists have implicated CaM antagonism as a likely mechanism. For instance, TFP and the naphthalene sulphonamide CaM antagonists W7 and W13 could inhibit DNA repair in CHO cells following bleomycin treatment while the much less potent W12 and TFP sulfoxide were largely ineffective [528]. In addition, TFP and the calcium chelator EGTA appear to interfere with the excision of UV-induced pyrimidine dimers by NER in normal human fibroblasts [529]. Another study found that intracellular calcium regulates a DNA repair signaling pathway in human mononuclear cells involving protein tyrosine kinase(s), CaM and calcineurin [530]. In response to IR, H2AX forms molecular complexes with CaM and several other calcium-regulated proteins in a dynamic and temporally distinct pattern, suggesting that Ca^{2+}/CaM signaling can modulate DNA damage-induced checkpoint and repair [531, 532]. Interestingly, the expression of CaM is up-regulated by low-dose IR in murine macrophages and forced over-expression of CaM results in enhanced H2AX phosphorylation without any changes in the extent of DNA damage incursion [533]. This mechanism may well account for the enhancement of DNA DSB repair fidelity observed after low-dose irradiation which is completely abolished by W7/W13, lending further support for a regulatory role of CaM in DNA repair [534]. Nevertheless, the assertion that phenothiazines and other CaM antagonists should act in identical manner (i.e. through CaM antagonism) has its own caveats. First, although CaM undoubtedly participates in certain aspects of the DDR, its relevance to cell survival under stress may be context-dependent, especially considering the special differences between human and rodents. Second, phenothiazines seem to target other cellular processes at concentrations where CaM antagonism is observed; therefore the *de facto* contribution of impaired Ca^{2+}/CaM signaling with regard to any observed cellular effects must be interpreted with caution [535]. This is supported by our own data showing that TFP-mediated augmentation of DDR signaling is not faithfully recapitulated by the CaM antagonists W7 or calmidazolium (CMZ), although all three compounds were used at concentrations corresponding to their respective IC_{50} for CaM antagonism (our unpublished data). Ultimately, the relevance (if any) of CaM antagonism in phenothiazine-mediated DNA repair modulation can only be defined through manipulation of CaM expression, for instance by siRNA.

Chromatin organization and gene expression

There is circumstantial evidence suggesting that chromatin may be a site of action for phenothiazines (Figure 15). First, ultrastructural studies in Ehrlich ascites carcinoma cells by electron microscopy clearly demonstrated their accumulation within the nuclear compartment, where immuno-positivity of phenothiazines was noted to be scattered in the nuclear matrix as well as in close association with electron-dense

chromatin [536]. Second, a variety of patient-derived cell types (both CNS and non-CNS) exhibited discernible alterations in chromatin structures following pimozide-based therapy, which is thought to act through similar mechanisms as phenothiazines, i.e., dopamine D_2 receptor antagonism [537]. Third, the D_2 receptor antagonist haloperidol was shown to induce chromatin remodeling in striatal neurons of both mice and rats [538]. Finally, *in silico* analysis by Connectivity Map (cmap, this thesis) indicated significant similarities in the gene expression signatures of human cancer cells treated with phenothiazines compared to those treated with several HDAC inhibitors (see Paper III). Consistent with the notion that phenothiazines may be *bona fide* chromatin-active compounds, a recent study identified fluphenazine (FPZ) as a candidate agent that could induce normalization of aberrant gene expression found in malignant neuroblastoma leading to loss of tumor cell viability [539]. Apart from their putative modulatory actions on chromatin, phenothiazines have also been shown to directly bind nucleic acids. The photodynamic DNA damaging potential of phenothiazines is well-established and is due to generation of free radical species, but their effect on nucleic acids in the absence of photo-activating UVA irradiation is less clear [540]. Although the planar tricyclic rings of phenothiazines can intercalate into the DNA double helix, studies on genotoxicity and mutagenicity have yielded conflicting results [541-544]. While phenothiazines have also been found to bind double-stranded RNA of both viral and human origins, it is not yet known whether phenothiazines can interact with miRNA or other types of non-coding RNA [545, 546].

1.8.3 Phenothiazines as potential anti-cancer therapeutics

Clinical experience

Although phenothiazines clearly possess anti-proliferative activities, these compounds have not been systematically evaluated as potential anti-cancer therapeutics [547, 548]. The major clinical use for phenothiazines is treatment of psychosis and management of chemotherapy-induced emesis [443, 445]. Interestingly, a number of studies on cancer incidence among patients with schizophrenia point to a possible protective effect of phenothiazines [549, 550]. There is also indirect and anecdotal evidence supporting a beneficial role of phenothiazines in cancer treatment, at least under certain conditions [551-553].

Pre-clinical evidences

Mono-therapy

In contrast to the relatively rare experience with patients, a wealth of studies conducted on human cell lines have clearly demonstrated that phenothiazines possess either cytotoxic or cytostatic potential, depending on the cell lines tested and/or the experimental context (Table 3). There is currently a lack of consensus as to why (and how) phenothiazines induce cell death in certain types of cells but not in others; the confusion stems partly from the fact that phenothiazines are clearly “dirty” drugs with a plethora of putative cellular targets (see below). Since mammalian proteins are subject to complex regulations, their availability and mode(s) of interaction with phenothiazines are not likely to be identical under all experimental conditions. Taking these factors into consideration, it is perhaps more informative to define the activities of phenothiazines as a function of any particular cellular context.

Table 3. Cytostatic and/or cytotoxic potential of phenothiazines.

Phenothiazine(s)	Tumor model/cell line	Effect	Reference
CPZ, FPZ, TFP	U1810, MDA-MB-231	cytostatic or cytotoxic	this thesis
CPZ	High-grade breast tumor cells	cytotoxic	490
PCZ	A549, H23, A427, ACC-LC-94, ACC-LC-319, SK-LC7	cytotoxic	518
TFP	A549	cytotoxic	554
TFP	MDA-MB-231	cytotoxic	555
TRDZ	MCF7, ZR75-1B, T47D, MDA-MB-231	cytostatic	556
CPZ, FPZ, PPZ, TFP, TRDZ	SH-SY5Y, C6, primary mouse neurons and glia	cytotoxic	557
CPZ, FPZ, TFP, TRDZ	HTB16, human peripheral blood leukocytes, L929, chick embryos	cytotoxic	558
CPZ, LVPZ, PMZ, TFP, TRDZ	Raji, Daudi, K562, BALL-1, HPB-ALL, MOLT4, CCRF-HSB2, normal lymphocytes	cytotoxic only in cancer cells	559
CPZ, TFP	HL60, HCT-8, MIA-PaCa, L1210, L5178Y	cytostatic only in cycling cells	474
TFP	SCC12B2, normal foreskin keratinocytes	cytostatic or cytotoxic	560
CPZ, TFP	V79	cytotoxic	561
TFP	C3H10T½	cytotoxic only in cycling cells	562

Abbreviations: CPZ, chlorpromazine ; FPZ, fluphenazine; LVPZ, levopromazine; PMZ, promethazine; PPZ, perphenazine; TFP, trifluoperazine; TRDZ, thioridazine.

Combination therapy – chemosensitization versus chemoprotection

There is an abundance of data demonstrating that phenothiazines are endowed with both chemosensitizing and chemoprotective activities (Tables 4, 5). Two general conclusions can be drawn from the available experimental evidence. First, phenothiazines are most active in combination with chemotherapeutic agents that induce DNA DSBs, while the effect of IR is not always potentiated. Second, cytotoxic agents that cause cellular injury without (markedly) damaging DNA, including heavy metals, non-steroidal anti-inflammatory drugs (in overdose) and microbial toxins, are generally antagonized by phenothiazines. With regards to their known targets, it is clear that phenothiazines should be able to affect cell viability both positively and negatively; the outcome depending on the overall result of the multitudes of individual targeted interactions. Yet this apparent dichotomy in activity is puzzling since exposure to either of the above-mentioned class of cytotoxic agents is associated with oxidative stress. One possibility is that phenothiazines can impede DNA repair while independently stabilizing mitochondria. An alternative but not mutually exclusive scenario is that phenothiazines may alter one or more cellular factors that *per se* are well tolerated by the targeted cells, but which becomes lethal only in the presence of DNA damage, a form of drug-induced contextual synthetic lethality.

Table 4. Chemosensitizing potential of phenothiazines in cell lines.

Cell line	Phenothiazines	Chemo	Potentiation	Reference
A2780, A2780/CP8, A2780/CP30, A2780/CP70, OVCAR-3, OVCAR-4	TFP	cisplatin	yes (2/6 cell lines)	563
Hep-2	TFP	IR, H_2O_2	yes (IR); no (H_2O_2)	564
U1810, H23	TFP	bleomycin, cisplatin	yes	503, 504
SKOV3, A-253, normal human bone marrow cells	CPZ	bleomycin	yes	565
MCF7/TAM^R-1	CPZ	tamoxifen	yes	566
MCF7, MDA-MB-468	TFP	tamoxifen	yes	567
U87	PPZ	IR, TMZ, imatinib	yes (imatinib); additive (TMZ); antagonistic (IR)	568
H1299	FPZ-N2-chloroethane	TRAIL	yes	569
Sk-ChA-1	TFP	interferon-γ	yes	570
L1210	TFP, CPZ	bleomycin IR	yes (bleo) ; no (IR)	571, 572
P388/ADM, P388/VCR	TFP	doxorubicin vincristine	yes	458
CHO	TFP	bleomycin	yes	528
V79	CPZ	X-ray	yes	573

Abbreviations: 5-FdUrd, 5-fluorodeoxyuridine; ADM, adriamycin; TMZ, temozolomide; VCR, vincristine.

Rationales for combining phenothiazines with DNA damaging agents

Although lacking a precise molecular mechanism, the ability of phenothiazines to modulate DNA repair makes a convincing case for their inclusion in combinatorial chemotherapy along with DNA damaging agents. However, despite generally favorable outcomes in cell-based studies and pre-clinical animal models (Tables 4, 6), several early clinical trials conducted in the 80s and 90s have yielded disappointing results (Table 7), which led to a dramatic loss of confidence in phenothiazines as chemosensitizers. It is important to point out that the initial premise on which these clinical trials were conducted was not therapeutic DNA repair modulation but rather pharmacologic reversal of efflux pump-mediated drug resistance, as phenothiazines have been shown to enhance intracellular accumulation of chemotherapeutic agents that are substrates of pgp [458, 465-468]. Unfortunately, the majority of these clinical trials were single-armed studies enrolling relatively small numbers of patients who have invariably been heavily pre-treated and whose pgp expression status are not generally known, thereby precluding the possibility of comparing phenothiazine-containing regimen to conventional regimen. It is perhaps not surprising that phenothiazines failed to significantly improve the outcome of chemotherapy in these patients.

Table 5. Chemoprotective potential of phenothiazines in pre-clinical animal models.

Human/animal/cell line	Chemo	Phenothiazine	Toxicity combo v single	Reference
ddY mice	CDDP	CPZ	decreased acute nephrotoxicity	574
$B_6D_2F_1$/J mice, Fisher 344 rats	CDDP	PCZ	decreased nephrotoxicity	575
Swiss albino mice	DOX	CPZ	decreased toxicity	576
Syrian hamsters	BLM	TFP	decreased acute inflammation and lymphocytes in BAL fluid	577
Syrian hamsters	DMBA	CPZ	decreased tumor formation	578
SKH-1 mice (hairless)	UV	CPZ	decreased tumor formation	579
Sprague-Dawley rats	NNM	TFP	decreased tumor formation	580
Albino mice	APAP	TFP	decreased toxicity	581
ddY mice	APAP	TFP	decreased hepatic toxicity	582
CF-1 mice	Cd	CPZ, TFP	decreased testicular damage	583
n.a.	*V. vulnificus* cytolysin	TFP	increased survival	584

Abbreviations: APAP, acetaminophen; BAL, bronchoaveolar lavage; BLM, bleomycin; C. albicans, Candida albicans; Cd, cadmium; CDDP, cisplatin; C. neoformans, Cryptococcus neoformans; DMBA, 9,10-dimethyl-1,2-benzanthrene; DOX, doxorubicin; MB, methylene blue; NM, nitrogen mustard; NNM, N-nitrosomorpholine; VBL, vinblastine; V. vulnificus, Vibrio vulnificus; n.a., not available.

Our understanding on the molecular intricacies of DNA damage signaling and repair has greatly improved since the days when phenothiazines were first suggested as modulators of DNA repair. Conceptual and methodological advances now allow detailed characterization of the various DDR signaling pathways and sub-modules that may be sensitive to perturbation by phenothiazines. This may enable identification of cellular context as well as biomarkers that predict sensitivity to phenothiazines, allowing more rigorous patient selection for future trials. Given that phenothiazines were generally well-tolerated, further investigation into the clinical utility of phenothiazine-based chemosensitizers is clearly warranted.

Table 6. Phenothiazines as chemosensitizers in pre-clinical animal models.

Tumor type	Recipient	Chemo	Pheno-thiazine	Tumor growth/survival combo v single	Reference
B16 melanoma	C57BL6 mice	BLM	CPZ	decreased growth	585
B16a-Pt melanoma	C56BL6 mice	CDDP	TFP	no difference	586
Ehrlich ascites	BALB/c mice	MMC	CPZ	decreased growth	542
$P388D_1$ leukemia	CDF_1 mice	DOX	CPZ	increased growth	587
sarcoma-180 ascites	ddN mice	CTX	PCZ, PMZ	decreased growth	588
L1210 leukemia	BALB/c x DBA mice	BCNU	CPZ, PCZ, TFP, FPZ	increased survival	589
fibrosarcoma	Swiss mice	X-ray	CPZ, PMZ, PPZ, TPZ	decreased growth	590, 591
Fortner's melanoma	Syrian hamsters	X-ray	CPZ, 7-OH CPZ	decreased growth	592

Abbreviations: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CTX, cyclophosphamide; MMC, mitomycin C; PCZ, prochlorperazine; PZ, promazine; TPZ, trimeprazine

Table 7. Clinical trials of phenothiazines as chemosensitizers.

Study type	Tumor type	Chemo	Pheno-thiazine	Response single v combo	Reference
phase II	breast cancer	VBL	TFP	7% vs 6%	466
phase II	breast cancer	DOX	TFP	45%	465
phase II	renal cell carcinoma	DV	TFP	0%	467
phase II	pancreatic cancer	EPI	TFP	13%	468
phase I/II	various	DOX	TFP	19%	593
phase I	various	DOX	PCZ	27%	464
phase II	glioma	BLM	TFP	0%	594
phase I	various	BLM	TFP	23%	595
phase II	malignant melanoma	BDV	CPZ	22%	596
phase II	malignant melanoma	MeCCNU	CPZ[a]	11% vs 12%	597
phase I	various	CV	PCZ [b]	0%	463
phase II	squamous cell carcinoma of the H&N	IR	CPZ [c]	31% vs 59%	598
retrospective	various	5-FU	various	no difference	599

Abbreviations. 5-FU, 5-fluorouracil; BDV, BCNU+decarbazine+vincristine; CV, carboplatin+VP16; CsA, cyclosporine A; DP, dipyridamole; DV, doxorubicin+vinblastine; EPI, epirubicin; H&N, head and neck; MeCCNU, 1-(2-Chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea; MMC, mitomycin C; MTD, maximum tolerated dose; RT, radiotherapy
[a] CPZ + caffeine; [b] PCZ + dipyridamole + cyclosporine A; [c] CPZ + bleomycin + mitomycin C + nicotinamide + dicoumarol

2 AIMS

The overall aim of this thesis was to evaluate the feasibility of using phenothiazines to enhance the sensitivity of human tumor cells to DNA damaging chemotherapeutic agents. The specific aims of each project were:

- To determine whether TFP suppresses DNA repair in general and DNA DSB repair in particular in bleomycin-treated NSCLC cells (**Paper I**).

- To elucidate the downstream apoptotic pathways that account for phenothiazine-mediated chemosensitization in relation to the DNA damage response in NSCLC cells (**Paper II**).

- To investigate in detail the impact of phenothiazines on DNA damage-induced chromatin-proximal signaling events and its potential importance to chemosensitization in tumor versus normal cells (**Paper III**).

- To uncover putative molecular determinants that predict responsiveness to phenothiazine-mediated chemosensitization which can be tested experimentally (**Paper III**).

- To characterize the transcriptional response of platinum-refractory NSCLC cells and to identify putative mechanisms of drug resistance as well as cellular processes that are targeted by TFP to impart chemosensitivity (**Paper IV**).

3 MATERIALS AND METHODS

As illustrated in Figure 16, a variety of molecular biological techniques were used in the current thesis work. This section provides basic information on the assays and the rationale for which they were chosen.

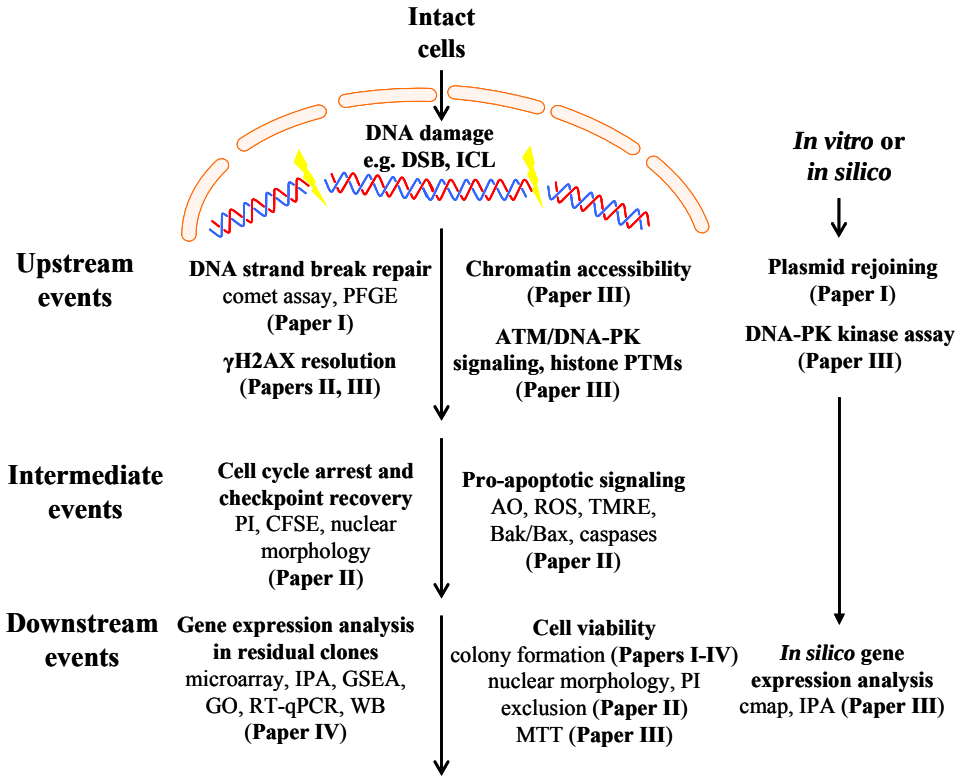

Figure 16. An overview of the experimental setup.

3.1 Cell lines

The human NSCLC cell line U1810 was used as the primary cell model system to study the effects of phenothiazines on chemo- and radiosensitivity [600]. U1810 cells, which do not express functional p53, were reported to be highly resistant to apoptosis induction by IR and cisplatin [389]. Where indicated, experimental data obtained for U1810 cells were verified in additional cell lines, including A549, H125, H23 (NSCLC), MDA-MB-231, MCF-7, T47D, BT474 (breast cancer), A2780, SKOV3 (ovarian cancer), WI-38 human normal lung fibroblasts, hTERT-RPE1 immortalized human retinal epithelial cells (non-cancerous). Of these, A549, MCF7, A2780, WI-38 and hTERT-RPE1 express wild-type p53 while the remaining cell lines all harbor p53 mutations.

3.2 DNA damaging agents

Bleomycin, an antibiotic compound isolated from *Streptomyces verticillus* [601], was used as a model substance to directly induce DNA DSBs. Where indicated, calicheamicin (an enediyne antibiotic derived from *Micromonospora echinospora*) and IR (Co^{60} source) were used as additional direct-acting DNA DSB inducers. The relative

efficiency of with which these three agents generate DNA DSBs are not identical, with calicheamcin being the most proficient and IR being the least proficient [602, 603]. Moreover, the chemical and structural complexities of the resultant DNA DSBs also differ, with IR and bleomycin causing more complex DNA DSBs than calicheamicin [604-607]. Thus, the cellular responses to IR, bleomycin and calicheamicin are not expected to be identical. In addition, cisplatin was used because ICLs generated by platinum compounds can be converted into DNA DSBs during DNA replication and/or ICL repair [608, 609]. As a comparison, several indirect-acting DNA damaging agents (gemcitabine, etoposide, aphidicolin) as well as a non-DNA damaging agent (staurosporine) were also tested.

3.3 Assessment of cytotoxicity

3.3.1 Short-term assay

3.3.1.1 MTT assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is a cell-permeable compound that is reduced by mitochondrial metabolic enzymes into a purple colored formazan precipitate. The formazan crystals can be solubilized in SDS/HCl-containing buffer and conveniently quantified by any spectrophotometer equipped with a 595 nm filter. In **Paper III**, the MTT assay was used to determine whether cell viability after DNA damaging treatment. A drawback for this assay is that it cannot easily distinguish terminally (or even transiently) arrested cells from actively cycling cells. This may explain why phenothiazine appeared to be weaker chemosensitizers in the MTT assay than in the long-term colony formation assay.

3.3.1.2 Propidium iodide exclusion

The plasma membrane of healthy mammalian cells has very limited permeability for propidium iodide (PI). Damage to the plasma membrane, which may be caused by mechanical force, disruption of membrane lipid organization (fluidization) or cell death, will typically lead to increases in the permeability of PI that can be conveniently detected by flow cytometry. This technique was used in **Paper II** as an alternative to the MTT assay to study DNA damage-induced cell death in the short-term. A major drawback for PI exclusion assay is that it cannot readily distinguish late stage apoptosis from necrosis because both are associated with loss of membrane integrity. Therefore, PI exclusion is useful for measuring cell viability but should not be used to study cell death modes.

3.3.2 Long-term assay

The colony formation assay provides a measure of the ability of single cells to produce colonies over time, typically 1-2 weeks [610]. A colony is defined as an aggregate of at least 50 cells that are all progenies of the original single cell. It is the method of choice for assessing the long-term cytotoxic effects of a given drug treatment. This is because cells can die with very different kinetics (hours versus days) and by multiple modes (e.g. apoptosis, necrosis, mitotic catastrophe) [221, 611]. Cells may also become senescent and lose clonogenic potential without actually dying [303]. To obtain accurate results, it is imperative that single cells are plated homogeneously over the entire surface area of the culture dish. If necessary, culture dishes can be coated with biomolecules (e.g.

fibronectin) to enhance the attachment of adherent cells. Anchorage-independent growth can be achieved by suspending non-adherent cells in soft agar. The colony formation assay can be performed in two ways. In one, cells are plated first as single cells, allowed to attach (usually overnight) and then exposed to the indicated drug(s) for a pre-determined amount of time. Thereafter, the drug is removed by washing cells gently but thoroughly with sterile PBS and fresh culture medium is added. In the other, cells are grown as monolayer at the desired confluency and exposed to the drug(s) for a pre-determined amount of time. Immediately after drug treatment, cells are harvested, washed and then plated as single cells for colony formation. Both of these approaches have their pros and cons. The first one necessitates the use of drug(s) at low doses due to the small number of sparsely seeded cells but allows the drug(s) to be rapidly removed making it particularly suitable for studying the effects of pulse treatment. Therefore, this approach was adopted throughout the current thesis work (**Papers I-IV**). Conversely, the second approach permits the use of drug(s) at much higher doses but as plating cells after treatment takes some time to perform, it is more suited for assessing the effects of prolonged drug treatment. At the end of the experiment, colonies are fixed and stained with crystal violet for visualization.

3.4 Analysis of DNA damage induction AND DNA repair

3.4.1 Electrophoresis-based assays

Single cell gel electrophoresis (aka comet assay) and pulsed-field gel electrophoresis (PFGE) are two commonly used methods for detecting the physical presence of DNA strand breaks [612, 613]. They are based on similar principles and both measure the extent of migration of unwound DNA when it is subjected to an electric field, with fragmented DNA migrating further than intact DNA. For comet assay, the pH of lysis (and electrophoresis) buffer determines the type of DNA damage that can be unmasked; neutral pH allows detection of only DNA DSBs while alkaline pH enables detection of DNA SSBs as well as DSBs. In **Paper I**, the alkaline comet assay was used to study the general effect of TFP on DNA strand break repair and PFGE was then conducted to specifically address its impact on DNA DSB repair. One major difference between comet assay and PFGE is the directionality of the electric field during electrophoresis, being constant and uni-directional in comet assay whereas in PFGE the voltage is periodically switched among three directions (along the central axis as well as at 120 degree angles on either side). Another significant difference is that the former is used to quantify DNA damage at the level of individual cells whereas the latter measures DNA damage in a population of cells.

While comet assay and PFGE are suitable for the assessment of DNA repair in intact cells, plasmid-based assays are useful for studying DNA repair *in vitro*. To this end, the plasmid is first digested with a restriction endonuclease to produce dsDNA ends of the desired configuration. In **Paper I**, the plasmid pBR322 was used as a DNA substrate for *Pst*I and *Pvu*II, which generate 3'-staggering and blunt ends, respectively. The linearized plasmid substrate was then purified and added to a reaction mixture optimized for *in vitro* end joining, which contains nuclear extract, nucleotides (dNTPs) and ATP. After the completion of reaction, proteins were digested with proteinase K and the recovered plasmid is separate by standard agarose gel electrophoresis. The material on the gel was transferred onto a nitrocellulose membrane, hybridized to a radio-labeled complementary DNA probe and then visualized using autoradiography.

Alternatively, visualization of rejoined and non-rejoined plasmid can be achieved by direct staining of the gel with a DNA dye (e.g. ethidium bromide, SYBR Green).

3.4.2 Antibody-based assays

Phosphorylation of histone H2AX on serine-139 (called γH2AX) is one of the earliest molecular events that take place in response to the induction of DNA DSBs [614]. The PI-3K-related protein kinases (PIKKs) DNA-PK, ATM and ATR are all capable of phosphorylating H2AX [615-619]; their relative contribution to γH2AX formation depends on complex and not yet fully defined parameters such as the cell cycle stage, the type of DNA DSB (direct versus enzymatically processed repair intermediates) and the relative expression levels of individual PIKKs. In general, DNA-PK, ATM and ATR can functionally compensate for each other with regard to H2AX phosphorylation [617, 619, 620]. Therefore, specific inhibitors of each of these PIKKs need to be included in the assay to query which of the PIKKs are primarily responsible for DSB-induced H2AX phosphorylation in a given cell line. The extent of H2AX phosphorylation is thought to provide a good estimate of the cellular level of unrepaired DNA DSBs [621]. Consequently, detection of γH2AX with phospho-specific antibodies (immunoblotting, immunofluorescence and flow cytometry) was carried out in **Papers II** and **III** to study the formation and resolution of DNA DSBs in tumor cells. On a cautionary note, however, it should be pointed out that formation of γH2AX can also be triggered by non-DSB type aberrant DNA structures and during mitosis independent of DNA damage [622-624]. Furthermore, apoptotic DNA fragmentation results in high levels of γH2AX in a manner that's dependent on c-Jun N-terminal kinase (JNK) or DNA-PK [625, 626]. To distinguish exogenous drug-elicited DSBs from apoptosis-associated DSBs, dosage of DSB-inducing agents must be carefully titrated to ensure that apoptosis is not triggered within the duration of the experiment.

DNA-PK is an important regulator of NHEJ in mammalian cells. Activation of DNA-PK is associated with multiple autophosphorylation events [627]. In **Paper III**, activation of DNA-PK kinase activity in intact cells was indirectly determined by its autophosphorylation status at serine-2056 using phospho-specific antibodies.

ATM is a key regulator of DDR by promoting DNA DSB repair, chromatin remodeling and pro-survival signaling [11, 628]. Activation of ATM is accompanied by its autophosphorylation on serine-1981 [30], which was conveniently detected by immunoblotting using phospho-specific antibodies in **Paper III.** Similar procedures was used to determine the phosphorylation status of ATM substrates such as H2AX (serine-139), KAP-1 (serine-824), SMC1 (serine-966), NBS1 (serine-343) and Chk2 (threonine-68) [54, 57, 58, 616, 629-632].

3.4.3 *In vitro* kinase assays

The central importance of DNA-PK kinase activity for NHEJ has prompted the development of techniques that enable quantification of DNA-PK kinase activity *in vitro* [153]. As outlined in **Paper III**, the kinase activity of purified and endogenous DNA-PK were directly monitored by the SignaTECT® DNA-PK kinase assay, which measures the incorporation of radio-labeled phosphate into an optimized substrate peptide in the presence of activating calf thymus DNA (Molecular Probes). These results were later verified using a complementary assay whereby DNA-PK kinase activity was determined indirectly with the EasyLite® luminescence ATP detection

system, which measures the amount of ATP remaining after DNA-dependent kinase activation (i.e. ATP consumption) (PerkinElmer).

3.5 Assessment of chromatin accessibility

The tight packing of chromatin potentially limits interaction between DNA and non-chromatin DNA-binding proteins. The timely operation of various chromosome transactions depends on local as well as global modulation of the chromatin structure leading to increased or decreased access for certain regulatory factors. In **Paper III**, acridine orange (AO) was used as a probe to monitor chromatin accessibility. AO is a cell-permeable fluorescent cationic dye that binds DNA as well as RNA and also accumulates in acidic organelles such as endosomes and lysosomes. To ensure that only DNA-associated AO fluorescence is measured, we conducted the assays on RNase A-treated cell nuclei [633, 634]. In this manner, changes in chromatin accessibility would be reflected by changes in AO retention, which can be readily quantified by flow cytometry.

3.6 Analysis of cell cycle progression

Two general techniques can be used to monitor cell cycle progression. In one, single cells in suspension are sequentially fixed with cold 70% ethanol (overnight), washed free of ethanol, permeabilized and then labeled with DNA-binding dye (e.g. PI) [635]. Since PI stains both DNA and RNA, it is important to remove RNA prior to or during the labeling process by an RNase, typically RNase A. As long as the concentration of PI is not limiting, its accumulation within cells is proportional to the DNA content, thereby allowing the different cell cycle phases to be distinguished. PI staining was used in **Papers II** and **IV** to analyze cell cycle distribution after DNA damage. However, PI staining does not provide a measure on how fast or slow a population (or sub-population) of cells undergo division. To obtain such information, cells need to be loaded with a tracking dye such as carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) [636]. CFDA-SE is non-fluorescent and highly cell-permeable. Following its uptake, proteolytic processing by cellular esterases generates a cleaved form called carboxyfluorescein succinimidyl ester (CFSE) which is fluorescent but much less cell-permeable. CFSE is stably retained within cells by forming covalent linkages to intracellular biomolecules. Each time a pre-labeled cell divides, its associated CFSE fluorescence is halved. Therefore, CFSE labeling was used in **Paper II** to assess cell division/mitotic activity after DNA damage. This gives an indication on the reversibility of checkpoint arrest.

3.7 Assessment of apoptosis and mitotic fidelity

Apoptosis and mitosis are both associated with distinct changes in the appearance of chromatin. These morphological changes in the appearance of chromatin can be conveniently traced by a DNA-binding dye, such as 4',6-diamidino-2-phenylindole (DAPI), PI and Hoechst 33342. In particular, condensation of chromatin during both apoptosis and mitosis leads to the accumulation of large amounts of dye molecules in a relatively small space, causing the chromatin to fluoresce intensely bright. This increase in fluorescence signal along with the distinct morphology of the chromatin is very useful for the identification of apoptotic and mitotic cells using a fluorescent microscope.

During early apoptosis, the chromatin becomes markedly condensed and moves towards the periphery of the nucleus (marginalization). This is followed by large scale DNA fragmentation via caspase-dependent and caspase-independent mechanisms [268, 637]. The cytoplasm and nucleus may subsequently fragment into small membrane-enclosed vesicles containing remnants of cellular components and highly condensed DNA. These vesicles, called apoptotic bodies, are normally rapidly cleared by professional phagocytes *in vivo*. For the purposes of quantifying apoptosis in **Paper II**, cells whose chromatin exhibited clear-cut signs of fragmentation as well as cells which have fragmented into apoptotic bodies were tallied. Cells with marginalized chromatin were not counted as apoptotic.

From late prophase through to metaphase, sister chromatids become attached to mitotic spindles and align themselves at the metaphase plate. During anaphase and telophase, the sister chromatids are pulled towards the opposite poles of the spindle and segregate into two daughter cells. If a chromatid fails to properly attach to the mitotic spindle at its centromere, it might lag behind during anaphase due to a lack of pulling force. A lagging chromosome is most often excluded from the reformed nucleus of either daughter cells and becomes instead enclosed in a small cytoplasmic vesicle called a micronucleus [638, 639]. Similarly, a chromosome that has two centromeres attached to microtubules emanating from opposite spindle poles or sister chromatids that are fused at their ends usually fail to completely segregate during anaphase, forming "string-like" connections (anaphase bridges) between the two separated daughter cells [640]. For the purpose of assessing mitotic fidelity in **Paper II**, abnormal mitosis was defined as mitotic events that are associated with highly irregular metaphase (mitotic catastrophe), lagging chromosomes or anaphase bridges. Interphase cells containing one or more micronuclei were also counted as abnormal mitosis.

3.8 Analysis of pro-apoptotic signaling

3.8.1 Oxidative stress

Intracellular ROS damages cellular components and macromolecules and its increased production leads to a condition called oxidative stress. In **Paper II**, 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA) was used to gauge ROS production in live cells. CM-H_2DCFDA diffuses passively into cells, where its acetate groups are cleaved by intracellular esterases and becomes trapped. Subsequent oxidation generates a green fluorescent product that was readily detected by flow cytometry.

3.8.2 Caspase activation and caspase activity

Flow cytometry-based assays

Enzymatic activation of caspases, which is usually required for apoptosis, can be detected by several techniques. There are at least two flow cytometry-based methods using different probes. In one, the probe, known as fluorescent labeled inhibitor of caspases (FLICA), is comprised of a fluorochrome (e.g. FAM) conjugated to a high affinity peptide substrate sequence that is linked to a fluoromethyl ketone (FMK) moiety. Binding of the peptide substrate to the active site of an activated caspase causes an irreversible covalent coupling of FMK to the active site cysteine residue, thereby labeling it with fluorescence. In the other, the probe is an antibody that specifically

recognizes the active form of a caspase. This antibody can be conjugated to a fluorochrome (e.g. PE, FITC) to facilitate direct detection of activated caspases in fixed cells. Both FLICA and active caspase-specific antibodies were used in the current thesis (**Papers II** and **III**).

Immunoblotting

Full activation of pro-caspases requires proteolytic cleavage. In turn, active caspases cleave a variety of substrates including PARP [247]. In **Paper II**, these apoptosis-associated events were detected by immunoblotting with commercially available antibodies. Some of these antibodies recognize specifically the cleavage products (caspase-9) while others recognize the non-cleaved as well as the cleaved forms of proteins (caspase-8, PARP).

3.8.3 Organelle dysfunction

Vacuolation

Cytoplasmic vacuolation is a frequently observed phenomenon in cells in response to a variety of stressful stimuli, such as pathogens, toxins and drugs [641]. It is generally believed that vacuolation represents an adaptive response that cells mount to limit damage incursion. However, uncontrolled and irreversible vacuolation can cause cell death. In **Paper II**, two methods for measuring vacuolation in live cells were used. The first is direct detection by light microscopy, where vacuoles appear as discrete small membrane-enclosed translucent vesicles. The second approach is indirect detection by flow cytometry. Collision of the incident laser light with granular structures (e.g. vacuoles) within a cell causes it to deflect sideways at wide angles (side scatter) instead of passing straight through or deflect forward at low angles (forward scatter). Therefore, the extent of side scattering of a cell is roughly proportional to its granularity.

Mitochondrial dysfunction

Loss of mitochondrial function is an important step in apoptosis execution [249]. In healthy cells, mitochondria maintain a negative electric potential across its inner membrane, which is mediated by extrusion of protons from the electron transport chain into the intermembrane space. This electrochemical gradient generates a proton motive force (PMF) that is used by the mitochondrial F_0F_1 ATP synthase to drive ATP synthesis. In response to pro-apoptotic stimuli, the mitochondrial transmembrane potential ($\Delta\Psi_m$) may increase initially as a result of an uncoupling of electron transport from ATP synthesis. If the harmful stimulus cannot be removed, persistent pro-apoptotic signaling eventually leads to the activation of Bak and Bax which triggers MOMP [642]. In turn, MOMP releases a number of apoptogenic factors (e.g. cytochrome c, Smac/Diablo) from the intermembrane space into the cytosol where they promote the activation of caspases [249, 263]. MOMP can also cause dissipation of $\Delta\Psi_m$ (depolarization) especially in conjunction with active caspases, which can cleave components of the electron transport chain [642, 643]. In **Paper II**, tetramethylrhodamine ethyl ester (TMRE) was used to gauge $\Delta\Psi_m$ in live cells. TMRE is a cell-permeable cationic fluorescent dye whose extent of accumulation in mitochondria is determined by $\Delta\Psi_m$. Therefore, mitochondrial hyper-polarization and depolarization will cause increased and decreased accumulation of TMRE, respectively. In addition, the

activation of Bak/Bax was assessed in fixed cells using conformation-sensitive antibodies that specifically recognize the active forms of Bak/Bax. For this purpose it is important to use a non-denaturing permeabilizing agent (e.g. digitonin) because some common detergents (e.g. Triton X-100) themselves can elicit conformational changes in Bak/Bax. Both of these events were recorded by flow cytometry.

Lysosomal dysfunction

Lysosomes are the primary organelles involved in the degradation of cellular waste material. The acidic intraluminal pH of lysosomes is maintained by the proton extruding activity of vacuolar ATPases. This is essential for lysosome functions because most of its complement of proteolytic enzymes attain optimal activities at low pH [644]. Compounds that are weak bases tend to accumulate in acidic organelles (lysosomotropism) where they become protonated [494, 501]. This frequently leads to a decrease in their membrane permeability due to the acquisition of an extra positive charge, which causes such compounds to be retained within the lumen [494]. In **Paper II**, two fluorescent lysosomotropic probes, acridine orange (AO) and LysoTracker Green, were used to study lysosomal function [645]. AO and LysoTracker Green accumulate in lysosomes as well as other acidic organelles such as late endosomes. Their fluorescence correlates with the pH as well as the size of the cellular acidic compartment. Staining with AO and LysoTracker Green was analyzed by flow cytometry and immunofluorescence microscopy, respectively.

3.9 Assessment of gene expression

3.9.1 DNA microarray

DNA microarray is a convenient method for simultaneous measurement of cellular gene expression. A gene chip typically contains >10,000 immobilized probes each of which can recognize a complementary region unique to a transcribed RNA molecule. In **Paper IV**, the Affymetrix® whole transcript GeneChip® Human Gene 1.0 ST Arrays platform, which contains probes for 28,869 genes, was used. RNA was harvested from cells following different treatments, i.e. mock (DMSO), TFP alone, cisplatin alone and cisplatin+TFP. The RNA was reverse transcribed into cDNA and labeled with a fluorescent dye. The labeled cDNA samples were then added to the gene chips allowing complementary sequences to hybridize to the probes. Finally, the gene chips were scanned. The fluorescence signals for each probe was corrected for background and normalized with the median intensity across all chips.

3.9.2 Real time quantitative PCR

Real time quantitative polymerase chain reaction (RT-qPCR) is a technique that allows simultaneous amplification and quantification of a cDNA molecule. The procedure is similar to conventional reverse transcriptase PCR except that the amplified DNA is detected in real time as the reaction progresses. RT-qPCR can be conveniently used to measure mRNA levels in cells or tissues. To this end, mRNA is reverse transcribed into cDNA and then amplified in the presence of primers specific for the gene of interest, a DNA-dependent DNA polymerase (usually Taq polymerase), dNTPs and a fluorescent DNA-binding reporter dye (e.g. SYBR Green) in a PCR reaction mixture. Subsequent thermal cycling is similar to conventional PCR. Comparative expression of the gene of

interest in relation to a reference housekeeping gene (e.g. GAPDH, TBP, 18S) can be calculated with the $2^{-\Delta\Delta Ct}$ method. In **Paper IV**, RT-qPCR was used to measure the mRNA expression levels of selected DNA repair genes in residual clones that survived different treatments (i.e. mock, TFP alone, cisplatin alone, cisplatin+TFP). GAPDH was chosen as the reference gene to correct for unequal loading.

3.9.3 Data mining and bioinformatics analysis

Connectivity map

The Connectivity Map (cmap) is a bioinformatics database developed by the Broad institute that collects microarray data generated from human cell lines treated with a vast array of small molecule compounds (perturbagens) [480]. An instance is defined as the unique gene expression signature induced by a given perturbagen at a particular occasion. Because microarray data for certain perturbagens have been deposited more than once, there are currently 6100 instances corresponding to approximately 1000 perturbagens in the cmap database (Build 01). The fundamental concept behind cmap is that perturbagens with similar mechanism(s) of action have a higher probability of eliciting similar gene expression signatures than perturbagens whose mechanisms of action are dissimilar. The utility of cmap for uncovering mechanism(s) of action of novel anti-cancer drugs and in the rational design of drugs that target signaling pathways specifically de-regulated in tumors have been clearly demonstrated by a number of recent publications [516, 518, 646-648]. Specifically, cmap allows users to compare the gene expression signature induced by a perturbagen of interest (query) to all other instances [480, 649]. The degree of gene expression concordance between the query and any given instance is provided by the up/down score. The up score shows the extent of similarity in terms of absolute enrichment and directionality of change for the up-regulated genes between the query and the instance, while the down score does the same with down-regulated genes. A connectivity score is also provided for each instance. This score, which is between 1 and -1, is used to rank all instances with regard to their relative similarities to the query. It is also possible to compute a mean connectivity score and a permutation p-value for all instances corresponding to same perturbagen. This enables the comparison between the query and perturbagen taking into considerations possible confounding factors such as batch, cell line or lab. In **Papers III** and **IV**, cmap was used as a hypothesis generating tool to identify putative cellular targets of phenothiazines.

3.9.4 Hierarchical clustering and principal component analysis

The goal of a cluster analysis is to assign a set of objects or observations into groups such that objects/observations within a cluster are more similar to each other than those in other clusters. Hierarchical clustering is a form of cluster analysis which seeks to order clusters in a hierarchy. In **Paper IV**, hierarchical clustering was performed on the complete Affymetix gene expression datasets encompassing all four treatment conditions, i.e. mock (DMSO), TFP alone, cisplatin alone and cisplatin+TFP. This analysis enabled the comparison of gene expression as a function of treatment.

A large set of observations may be composed of multiple variables, some of which are likely to be redundant because they are correlated with one another. Principal component analysis (PCA) is a mathematical procedure that can be used to remove such redundancy by transforming the original set of variables into a new set of artificial

variables called principal components. By definition, the first component has the largest possible variance and accounts for most of the variance in the original variables. Although the number of principal components is always less than or equal to the number of original variables, in practice, two or three principal components are usually calculated. This is because of two reasons. One, a maximum of three dimensions can be depicted graphically. Two, the first three principal components usually account for the majority of the observed variance. In **Paper IV**, PCA analysis was performed (in three dimensions) on the complete Affymetrix gene expression datasets encompassing all four treatment conditions, i.e. mock (DMSO), TFP alone, cisplatin alone and cisplatin+TFP. This analysis permitted the visualization of gene expression similarities as a function of treatment.

3.9.5 Ingenuity pathway analysis

The Ingenuity pathway analysis (IPA) software (www.ingenuity.com) is a systems biology tool that can be used to analyze and model complex biological data (e.g. gene expression). IPA uses the Ingenuity Knowledge Base, which contains a vast amount of manually curated annotations on biological, chemical and functional relationships between genes, proteins, cells, tissues, drugs and diseases. The IPA core analysis can be conveniently used to identify which signaling pathways, molecular networks and biological processes are most significantly altered in a dataset, usually a list of up- and down-regulated genes. It can also be used to build custom networks from hubs specified by the user. IPA provides probable biological contexts for the observed changes in gene expression and predicts their possible downstream effects. This information is useful for generating testable hypothesis as well as for experimental design. In **Paper III**, publicly available gene expression data was used to perform *in silico* IPA analysis. In **Paper IV**, expression data generated from to the aforementioned microarray experiments were uploaded into IPA. These analyses enabled the identification of candidate cellular and biological processes that are preferentially modulated by phenothiazines (*in silico*, **Paper III**), cisplatin alone (**Paper IV**) or cisplatin+TFP (**Paper IV**), respectively.

3.9.6 Gene ontology terms

The Gene Ontology (GO) project is a collaborative effort aimed at standardizing the nomenclature and description of gene products (i.e. annotations). GO terms are used to systematically organize all available information on gene products into a multi-tier matrix comprising of three main categories (biological processes, cellular components, molecular functions) and numerous progressively narrower sub-categories. In **Paper IV**, the GeneTrail software was used to identify sub-categories of biological processes showing enrichment of genes that were found to be differentially expressed by microarray analysis. This facilitated the identification of candidate biological processes that are preferentially targeted by cisplatin alone or cisplatin+TFP.

3.9.7 Gene set enrichment analysis

A DNA microarray experiment typically identifies a large number of differentially regulated genes. Frequently, a few genes showing the greatest magnitude of expression changes are selected as candidates for further analysis. However, this approach does not take into consideration potential interactions between sets of genes within a given signaling pathway. Moreover, as alterations in cellular processes often affect sets of

genes acting in concert, it is usually more informative to analyze the behavior of gene sets rather than single genes. Gene set enrichment analysis (GSEA) is a statistical method that can be used to evaluate gene expression data at the level of gene sets [650]. The gene sets themselves are defined based on *a priori* knowledge, e.g. database annotations, and usually corresponds to cellular processes. The goal of GSEA is to determine whether members of a given gene set show a high degree of concordance in terms of expression changes. If the answer is yes, then that particular gene set, and by extension the cellular process it regulates, is considered to be enriched. In **Paper IV**, the GeneTrail software was used to perform GSEA on genes that were found to be differentially expressed in the microarray analysis. This allowed the identification of cellular processes that are putatively regulated by cisplatin alone or cisplatin+TFP.

4 RESULTS AND DISCUSSION

4.1 Paper I

The antipsychotic drug trifluoperazine inhibits DNA repair and sensitizes non small cell lung carcinoma cells to DNA double-strand break induced cell death

The primary aim of this study was to determine the impact of TFP on DNA repair in general and DSB repair in particular. A previous study by Chafouleas et al showed that both TFP and the naphthalene sulfonamide CaM antagonist W13 significantly suppressed the ability of CHO cells to recover from bleomycin-induced potentially lethal damage [528]. They found that bleomycin-induced DNA damage caused relaxation of supercoiled DNA in nucleoids prepared from CHO cells which reduced its migration during sucrose gradient centrifugation as a result of decreased buoyant density. Subsequent incubation of cells in drug-free culture medium allowed DNA repair and restored DNA supercoiling, but this effect was largely abolished in the presence of W13, suggesting that CaM antagonists might inhibit DNA repair. However, this study did not assess the effect of TFP on restoration of repair-associated DNA supercoiling. Therefore, we decided to investigate the impact of TFP on DNA repair by directly measuring DNA strand break induction and resolution.

We used a colony formation assay to verify that TFP could indeed impart bleomycin sensitivity in the human NSCLC cell line U1810. In this case, a non-toxic concentration of TFP (10 μM) was given concurrently with bleomycin as a one hour pulse treatment. Since TFP was not present during recovery, the results indicated that rapid inhibition of DNA repair by TFP during the one hour when cells were exposed to bleomycin might be enough to markedly suppress clonogenic potential. Consistent with this notion, alkaline comet assay showed that bleomycin-treated U1810 cells accumulated significantly higher levels of DNA strand breaks in the presence of TFP. Notably, when TFP was present also during recovery, differences in the level of DNA strand breaks became even more pronounced. Since the cytotoxicity of bleomycin is thought to be mostly dependent on its ability to generate DNA DSBs, we then employed PFGE to specifically address the effect of TFP on DSB repair. In line with our alkaline comet assay data, we found that TFP significantly impeded the repair of DNA DSBs. Unlike DNA SSBs, however, TFP did not affect the initial level of DNA DSBs immediately after bleomycin. These data suggest that TFP might interfere with SSB as well as DSB repair. The difference in the initial levels of DNA SSB and DSB could be explained by the fact that SSB repair likely proceed with faster kinetics than DSB repair because the latter may require enzymatic end processing before ligation can occur [604, 607]. Consequently, TFP preferentially inhibited SSB repair at early time points while its effects on DSB repair only became apparent at later time points. Significantly, TFP-mediated inhibition of DSB repair was observed long after TFP itself was removed from the culture medium. These data suggest two non-mutually exclusive possibilities. One, intracellular TFP might be able to remain active in cells following its uptake for prolonged periods of time, or two, TFP might perturb an early response to DNA damage that is not easily reversed. In the latter case, one can envision a scenario whereby TFP might bind DNA and alter the chromatin response to DNA damage. Regardless, the inhibitory effect TFP exerted on DNA DSB rejoining indicated a possible block in NHEJ since mammalian cells deficient in Ku-dependent NHEJ are known to be hypersensitive to bleomycin [651-653]. In line with this, TFP (100 μM)

significantly reduced the ability of crude U1810 nuclear extract to support end-to-end joining of restriction endonuclease-linearized plasmids *in vitro*. Importantly, TFP did not affect plasmid cleavage by bleomycin, suggesting that its effect on the accumulation of DNA strand breaks was a result of repair inhibition and not enhanced DNA damage induction. Collectively, our data clearly demonstrated that TFP could inhibit DNA strand break repair and provided a plausible mechanism to account for its chemosensitizing activity.

4.2 Paper II

Chemosensitization by phenothiazines in human lung cancer cells: impaired resolution of γH2AX and increased oxidative stress elicit apoptosis associated with lysosomal expansion and intense vacuolation

The primary aim of this paper was to determine the consequences of TFP-mediated DNA repair modulation on cell fate after DNA damaging treatment. We conducted our studies on two human NSCLC cell lines, the chemo-/radioresistant U1810 and the inherently more chemo-/radiosensitive H23. This enabled us to explore whether TFP may differentially affect tumor cells based on their relative chemo-/radiosensitivities. Although bleomycin was used the primary DNA DSB inducing agent, we also tested TFP in combination with cisplatin, a drug commonly used in the clinical regimen for NSCLC. Moreover, we extended our work on TFP by analyzing whether its chemosensitizing potential might be shared with other structurally related phenothiazine compounds (e.g. FPZ, triflupromazine, TFPZ). The scheduling of TFP was also modified so that TFP was given 1 h before DNA damaging treatment and was allowed to remain for up to 24 h afterwards, even though the length of exposure to bleomycin or cisplatin was still limited to one hour. This adjustment in scheduling was done in order to ascertain whether the chemosensitizing (and DNA repair inhibitory) activity of TFP can be improved by increasing the length of exposure. Indeed, the modified schedule imparted stronger bleomycin sensitization than the previous one, albeit the gain was relatively modest.

Using colony formation assay, we first confirmed that TFP was active in combination with either bleomycin or cisplatin against both NSCLC cell lines. As our previous work implicated NHEJ and possibly DNA-PK as a putative target of TFP [503, 654], we included in the analyses a commercially available DNA-PK kinase inhibitor, NU7026 [432]. Our data showed that at an equivalent concentration (10 μM) TFP performed better than NU7026 in terms of chemosensitization in U1810 cells. Importantly, we found that both FPZ and TFPZ imparted platinum sensitivity in NSCLC cells, indicating that chemosensitization was a property shared amongst phenothiazines with different side chain structures. Moreover, both TFP and TFPZ impaired cellular recovery from bleomycin exposure as their presence significantly delayed the resolution of γH2AX, a surrogate marker of unrepaired DNA DSBs [655]. TFP also impeded γH2AX resolution after cisplatin treatment. The patterns of γH2AX formation and clearance in U1810 cells was consistent with the notion that bleomycin rapidly induced DNA DSBs independent of the cell cycle phase whereas cisplatin-induced γH2AX peaked at 6-12 h post-treatment, which coincided with S phase accumulation. TFP enhanced the arrest of bleomycin-treated U1810 cells in G_2-M and cisplatin-treated cells in S phase. In the absence of TFP, bleomycin-induced initial G2-M arrest lasted for about 12 h, after

which cells began to divide again. After exposure to cisplatin, the initial S phase arrest began at somewhere between 6-12 h post-treatment and lasted until 18 h post-treatment, after which cells entered G_2-M. TFP significantly delayed the resumption of cell cycle progression in U1810 cells following the initial checkpoint arrest. These cells could still recover, albeit more slowly, as evidenced by the decrease in CFSE fluorescence after 24 h. Over the next 72 h, the proliferation rates of cells treated with mock, TFP alone or bleomycin alone were comparable, suggesting that bleomycin at the dose used (2.5 μg/ml) had no lasting effect on cell cycle competency. In striking contrast, U1810 cells co-treated with bleomycin and TFP (or TFPZ) showed a delayed drop in proliferation, with a subset of cells displaying signs consistent with secondary arrest. Notably, the frequency of abnormal mitosis increased significantly among TFP co-treated cells, which might be a cause for the delayed activation of checkpoint mechanisms. In agreement with this notion, we observed that TFP co-treatment was associated with a delayed activation of the G_2 checkpoint effectors $p21^{Cip1/Waf1}$ and p38MAPK in U1810 cells.

In addition to its impact on mitotic fidelity, TFP co-treatment led to marked increases in apoptosis. This effect was time-dependent and became apparent 48 and 72 h after exposure to bleomycin and cisplatin, respectively. Consequently, it appears that DNA damage-induced apoptosis in U1810 cells was linked to cell cycle progression after initial checkpoint adaptation. Apoptosis in these cells was mediated by multiple caspases, including the initiator caspases-8 and -9 as well as the effector caspase-3. Notably, increased caspase-3 activation after TFP co-treatment was mostly observed in U1810 cells with 4n DNA content, suggesting that mitotic defects and/or secondary checkpoint arrest might trigger apoptosis. At approximately 18 h post bleomycin treatment when TFP co-treated cells were still locked in G_2-M arrest, a subset apparently underwent LMP, as shown by their diminished accumulation of AO. This was not observed in U1810 cells exposed to bleomycin alone, which had just begun to recommence cell cycle progression following the initial checkpoint arrest. Since LMP occurred prior to the onset of apoptosis, it is tempting to speculate that it might contribute to caspase activation [255]. However, this early wave of LMP after TFP co-treatment was followed by a progressive vacuolation phase that was readily discernible by light microscopy as well as flow cytometry (i.e. increased side scattering). Over time, TFP co-treated U1810 cells retained significantly higher amounts of AO, which accumulates preferentially in acidic organelles such as endosomes or lysosomes. In line with this, TFP co-treated cells fluoresced more brightly when labeled with LysoTracker Green, indicating an expansion of the lysosomal compartment. Notably, abrogation of this process by bafilomycin A1, an inhibitor of vacuolar ATPases, markedly reduced caspase-3 activation in these cells, suggesting that early LMP might trigger compensatory lysosomal expansion which ultimately leads to caspase activation.

We found that TFP co-treatment was associated with an early induction of oxidative stress whose onset at 12-16 h post bleomycin treatment slightly preceded that of LMP. Therefore, it is possible that LMP was caused by increased oxidative damage to the lysosomal membrane. In this case, the most likely site of ROS production was mitochondria, which were markedly hyper-polarized in TFP co-treated U1810 cells. Moreover, mitochondrial depolarization occurred coincidently with the induction of oxidative stress and both became progressively more severe over time with similar kinetics. Enhanced conformation-driven activation of Bak/Bax was observed in TFP co-treated cells within the same time frame as caspase activation, suggesting that oxidative stress and LMP might cooperate to trigger the intrinsic apoptotic pathway. In line with this, the antioxidant compound N-acetylcysteine (NAC) was able to partially

rescue both the short-tem viability and the long-term clonogenicity of TFP co-treated cells. Collectively, these data support a scenario whereby inhibition of DNA DSB repair by TFP leads to oxidative stress and organelle dysfunction which is exacerbated upon checkpoint adaptation leading to caspase-mediated apoptosis.

4.3 Paper III:

Identification of phenothiazines as putative regulators of the chromatin response to DNA damage provides a rationale for context-dependent chemosensitization

The primary aim of this paper was to uncover the molecular mechanism(s) by which TFP and related phenothiazines affect DNA repair. Our previous works showed that TFP could antagonize the repair of DNA SSBs, DSBs as well as ICLs. Moreover, our data indicated that the effect TFP exerted on DNA repair was very rapid and was not easily reversed upon drug wash-out. Therefore, we hypothesized that TFP might modulate an early component of the DDR, such as damage-induced chromatin remodeling, rather than directly inhibiting a repair enzyme.

A compound's mechanism of action is likely to be reflected by changes in the pattern of gene expression in treated cells. This is simply because any extraneous stimulus that disturbs cellular homeostasis would elicit a reaction that attempts to restore status quo. We downloaded two publicly available gene signatures (i.e. up- and down-regulated genes) associated with phenothiazine treatment and used them as queries to search for similarities against 6100 unique gene expression signatures corresponding to >1000 small molecule compounds that were deposited in the cmap database (build 01) [480, 656]. The signature taken from Lamb et al was derived from cell lines after short-term drug exposure (4 h), while the signature taken from Choi et al was derived from deceased patients that have presumably been exposed to the drugs for longer time. Interestingly, our analysis revealed gene expression similarities between phenothiazines and several HDAC inhibitors, such as trichostatin A and vorinostat. To confirm this finding, we reciprocated the analysis using two publicly available gene signatures associated with HDAC treatment as queries. The signature taken from Glaser et al was a composite derived from cell lines after 24 h drug exposure. By contrast, the signature taken from Ellis et al was agent-specific and derived from patients suffering from cutaneous T-cell lymphoma who were undergoing treatment with panobinostat [657, 658]. Despite these differences, both query signatures were able to identify a number of phenothiazines, including thioridazine (TRDZ) and TFP. Thus, phenothiazines and HDAC inhibitors appear to induce similar gene expression changes in cells and tissues suggesting that phenothiazines might be chromatin-active compounds. In line with this, exposure of U1810 cells to 10 μM TFP or the HDAC inhibitor valproic acid (VPA, 5 mM) for 24 h resulted in increased nuclear DNA intercalation by AO, indicating that TFP might enhance chromatin accessibility. However, when the length of exposure was shortened to 5 h, TFP treatment had only a modest effect on AO intercalation. While this short-term exposure to TFP was sufficient to enhance bleomycin-induced caspase-3 activation, it did not cause any discernible changes in the acetylation status of histone H4. These data suggest that TFP treatment might alter chromatin compaction without affecting histone acetylation.

We tested the hypothesis that TFP may interfere with chromatin-proximal DDR signaling. Notably, when U1810 cells were exposed to bleomycin in the presence of TFP, significantly increased DNA-PKcs autophosphorylation (S2056) was observed. Similarly, TFP enhanced the DNA damage-induced phosphorylation of several chromatin-proximal ATM substrates, such as H2AX (S139), NBS1 (S343), KAP-1 (S824), SMC1 (S966) and to a lesser extent ATM itself (S1981). By contrast, phosphorylation of the ATM substrate Chk2 (T68), which does not accumulate at DNA DSB sites and whose function is not thought to involve DNA damage-associated chromatin remodeling, was not affected by TFP. To establish the generality of our findings, we compared the impact of TFP on bleomycin-induced DDR signaling in two tumor (U1810, MDA-MB-231) and two non-transformed (hTERT-RPE1, WI-38) cell lines. We found that TFP augmented DNA-PK/ATM signaling in both tumor cell lines as well as in the immortalized hTERT-RPE1 cells. However, this effect was not observed in the non-immortalized WI-38 cells. These data suggest that the process of immortalization, which represents the first step towards malignant transformation, might induce chromatin re-organization in a manner that renders cells more susceptible to perturbations by TFP. Several structurally related phenothiazines also augmented bleomycin-induced DDR signaling in U1810 cells. Furthermore, TFP enhanced DNA-PK/ATM signaling in response to calicheamicin and cisplatin. These results raise the interesting prospect that such combinations might be used to potentiate the therapeutic efficacy of platinum and calicheamicin in lung cancer and AML (in the context of gemtuzumab ozogamicin), respectively [659, 660]. On the other hand, TFP did not augment DNA-PK/ATM signaling in response to IR, etoposide or aphidicolin nor did it increase their cytotoxicity. Therefore, our data suggest that DNA damaging agents which are thought to produce similar types of lesions might in fact trigger very different DDR responses.

Given that TFP delayed cell cycle recovery after DNA damaging treatment, we next assessed whether TFP might affect the resolution of DDR signaling. To this end, U1810 cells were pulse-treated with bleomycin in the presence of TFP and then allowed to recover either in drug-free medium or medium containing TFP for various lengths of time. Notably, TFP co-treated cells had consistently higher levels of phosphorylated DNA-PKcs, KAP-1 and H2AX than counterparts that were treated with bleomycin alone. This difference was evident for at least 18 h after removal of bleomycin, indicating that TFP co-treatment was associated with prolonged DDR signaling. Moreover, TFP largely abrogated the DNA damage-induced up-regulation in WRN and FANCD2 in U1810 cells over time. This response was specific since TFP did not overtly affect the expression of several other DNA repair proteins (e.g. Ku70, Ku80, XRCC4, DNA Ligase IV, Rad51, PARP-1), suggesting that TFP might also interfere with transcriptional response to DNA damage. Regardless of the mechanism, TFP-mediated augmentation of DNA-PK autophosphorylation was observed over a wide range of bleomycin concentrations and was recapitulated in all of the tumor cell lines that we tested (e.g. H23, H125, MCF7, MDA-MB-231, T47D, BT474, SKOV3). Once again, TFP did not affect the level of DNA-PK autophosphorylation in the non-cancerous hTERT-RPE1 and WI-38 cells. These data indicated that TFP may selectively inhibit DNA DSB repair in tumor cells without affecting the response of normal cells to chemotherapy. In line with this notion, TFP delayed the re-acetylation of H3K56 in U1810 and MDA-MB-231 cells after exposure to bleomycin and cisplatin, respectively, suggesting that chromatin reassembly which normally accompany DNA repair was compromised in the presence of TFP.

As not all clinically used chemotherapeutic agents act directly on chromatin to induce DNA damage, we next examined the ability of TFP to perturb DNA-PK/ATM signaling in response to the indirect-acting DNA damaging agents etoposide, gemcitabine and aphidicolin. We found that these agents induced DNA-PK autophosphorylation poorly unless used at very high concentrations. Furthermore, TFP did not affect the phosphorylation of DNA-PK or KAP-1 even after prolonged exposure to these indirect-acting DNA damaging agents. This was in stark contrast to the rapid and overt augmentation of DNA-PK/ATM signaling observed when TFP was used in combination with the direct-acting DNA damaging agents bleomycin, calicheamicin and cisplatin. A possible interpretation of this data is that direct-acting DNA damaging agents may activate DNA-PK/ATM signaling in a TFP-sensitive manner that is different from DNA-PK/ATM activation in response to indirect-acting DNA damaging agents. To determine the functional relevance of this differential modulation of DDR signaling by TFP, we compared the chemosensitizing potential of TFP in a panel of tumor and non-cancerous cell lines treated with direct-acting or indirect-acting DNA damaging agents. Three general conclusions could be drawn from this analysis. First, TFP significant sensitized all p53-deficient tumor cells to at least one direct-acting DNA damaging agent, while it had no such effect in p53 wild-type tumor or non-cancerous cells. Second, none of the test cell lines were sensitized by TFP to any of the indirect-acting DNA damaging agents, regardless of the p53 status. Third, TFP might confer protection against the non-DNA damaging agent staurosporine as well as the indirect-acting DNA damaging agent aphidicolin. There were two apparent exceptions to this generalization. One, TFP was able to sensitize MCF7 cells to direct-acting DNA damaging agents, although these cells expression wild-type p53. Two, TFP did not confer radiosensitization in any of the cell lines despite IR being a direct-acting DNA damaging agent. With respect to the function of p53 in MCF7 cells, a study recently reported subtle defects in the DNA damage-induced p53 transcriptional response of MCF7, including the inability to maintain $p21^{Cip1/Waf1}$ expression. It is therefore possible that the wild-type p53 in MCF7 cells is deficient in some aspects of DDR signaling which might explain why MCF7 cells were responsive to TFP-mediated chemosensitization. The second discrepancy is more puzzling because bleomycin is considered to be a radiomimetic [604]. We found that TFP did not affect IR-induced DNA-PK/ATM signaling but the reason for this is not clear at the moment. Taken together, our data suggest the combination of two parameters determines whether a particular cell line would respond to TFP-mediated chemosensitization; the optimal context appears to be a direct-acting DNA damaging agent that elicits TFP-amplifiable DNA-PK/ATM signaling in a tumor cell line with deficient p53 function.

To further explore the consequence of DDR hyper-activation, we utilized specific small molecule inhibitors to forcibly abolish DNA-PK/ATM signaling in TFP co-treated U1810 cells. To our surprise, suppression of DNA-PK or ATM activity by NU7026 and KU55993, respectively, resulted in further chemosensitization, suggesting that the hyper-activation of DNA-PK/ATM signaling was likely an adaptive response to TFP co-treatment. Consistent with this notion, at concentrations below 100 μM TFP *per se* had no effect on DNA-PK kinase activity *in vitro*. Similarly, augmentation of bleomycin-induced DNA-PKcs autophosphorylation by TFP did not appear to involve a direct effect of TFP on cellular protein phosphatases. Moreover, saponin-permeabilization of U1810 and MDA-MB-231 cells did not affect TFP-mediated chemosensitization, indicating that the membrane perturbing activity of TFP could not account for its ability to confer chemosensitization. Collectively, our data suggest that the most likely cause of TFP-mediated chemosensitization is DNA repair inhibition as a result of disturbed chromatin responses. We propose that cancer types for which

bleomycin, cisplatin or calicheamicin-containing regimen is routinely used, such as NSCLC, breast cancer, ovarian cancer, melanoma, glioblastoma and AML, should be considered for future clinical trials to evaluate the clinical efficacy of phenothiazine-based chemosensitizers.

4.4 Paper IV

Gene expression analysis reveals DNA repair pathway modulation as a potential mechanism for phenothiazine-mediated long term cisplatin sensitization in NSCLC

Platinum doublets, usually in combination with gemcitabine, are commonly used for the treatment of NSCLC but their therapeutic efficacies are limited by intrinsic or acquired drug resistance [661]. The primary aim of this paper was to explore possible mechanism(s) of platinum resistance in NSCLC and ascertain whether TFP could confer long term chemosensitization by antagonizing such processes. To this end, we pulse-treated NSCLC U1810 cells with a therapeutically relevant dose of cisplatin (10 μM) either alone or in combination with 10 μM TFP and then allowed them to form colonies. In the absence of TFP, U1810 cells were relatively insensitive to treatment with cisplatin at this concentration (<20% suppression of clonogenic capacity). Notably, while TFP alone did not affect clonogenic capacity (<5% suppression of clonogenic capacity), it significantly increased the sensitivity of U1810 cells to cisplatin. After nine days, the surviving colonies (residual clones) were harvested from which RNA was extracted. The RNA samples were used to obtain gene expression profiles of the residual clones in response to each treatment, i.e. mock, TFP alone, cisplatin alone, cisplatin/TFP.

We found that the highest number of differentially regulated genes (1141 up, 1290 down) was observed in residual clones that survived cisplatin treatment, followed by residual clones that survived cisplatin/TFP co-treatment (882 up, 491 down) and finally residual clones that survived TFP treatment (139 up, 351 down). Analysis of the gene expression data by PCA and hierarchical clustering indicated that the gene expression profile of residual clones that survived cisplatin/TFP co-treatment showed features intermediate to the gene expression profiles induced by either TFP or cisplatin. In line with this, functional analysis by GSEA showed that while cisplatin treatment led to the enrichment in residual clones of genes involved DNA-dependent DNA replication/replication initiation and DNA metabolic processes, no such enrichment of was found in residual clones that survived cisplatin/TFP co-treatment. These data suggest that TFP may fundamentally affect the ability of cells to modulate certain cellular signaling pathways in response to cisplatin. Intriguingly, we have shown in Paper III using a publicly available gene expression data set [480] that short-term exposure of MCF7 cells to phenothiazines resulted in down-regulation of a number of genes implicated in mRNA metabolism. Therefore, it seems plausible that TFP might broadly counteract the transcriptional response to cisplatin by modulating the mRNA synthesis and translation. Interestingly, when we performed IPA analysis on genes that were up-regulated in residual clones that survived cisplatin treatment, the top scored networks were found to contain components of several DNA repair pathways, including NER

(TFIIH), MMR (MSH2), FA-ICL repair (FANCD2, FANCI), HRR (MRE11A) and NHEJ (MRE11A, PRKDC), suggesting that survival from cisplatin may require parallel de-regulation of multiple repair modules. Consistent with this notion, Nojima et al reported that tolerance to cross-linking agents (e.g. cisplatin) in avian DT40 cells is mediated by multiple DNA repair pathways, including translesion synthesis (TLS), FA-ICL repair, HRR and to a lesser extent NER [416]. Notably, the top scored networks of genes up-regulated residual clones that survived cisplatin/TFP co-treatment did not contain any of the above-mentioned DNA repair genes, suggesting that TFP-mediated chemosensitization might be related to disruption of cisplatin-induced compensatory up-regulation of tumor DNA repair capacity.

To validate our findings from gene expression analysis, we determined the mRNA and protein expression for a number of DNA repair genes identified as up-regulated in cisplatin-resistant residual clones. Some of these (PRKDC, FANCI, FANCD2, MRE11A) were found in the top scored networks while others (RRM1, RRM2, RAD51) were not but have nevertheless been implicated in the clinical responsiveness of platinum-based therapy. Our preliminary data demonstrated that DNA-PKcs (encoded by PRKDC) also increased in protein expression. The other five candidates showed discrepant mRNA-protein expression and a possible explanation is that a significant proportion of mRNAs in cisplatin-surviving residual clones may not be translated. A recent report showed that the development of treatment-induced platinum resistance in ovarian carcinoma is accompanied by an increased activity of the DNA-PK/Akt pathway [662]. Similarly, we have previously shown that acquired resistance to radiotherapy in cervical carcinoma was associated with increased expression of all three components of the DNA-PK complex in residual tumors [361]. Therefore, it seems that enhanced DNA repair capacity is a major contributor of acquired platinum resistance although it might not necessarily be involved in the maintenance of intrinsic platinum resistance. When U1810 residual clones were replated and subjected to a second round of cisplatin treatment, we found that residual clones which survived the first round of cisplatin treatment were also resistant in response to a second round of cisplatin treatment. However, the relative platinum sensitivity of parental U1810 cells and cisplatin-surviving residual clones were similar (70% versus 73%), suggesting that while U1810 cells were intrinsically resistant to cisplatin, residual clones that survived the first round of cisplatin treatment did not acquire additional resistance. These data might explain the paucity of over-expressed DNA repair proteins in residual clones that survived cisplatin treatment. However, it is also possible that a single round of cisplatin treatment was not sufficient to elicit acquired resistance. We found that CDK12 was up-regulated in cisplatin-surviving residual clones. A CDK12/cyclin K complex was recently shown to promote RNA polymerase II-dependent transcription of many genes involved in the maintenance of genomic stability, such as ATR, FANCD2 and FANCI [663], all of which were concurrently up-regulated in cisplatin-resistant residual clones. It is therefore conceivable that de-regulation of DNA repair proteins other than or in addition to the ones tested might progressively induce the development of acquired platinum resistance upon repeated (two or more) drug exposure. More studies are needed to determine the functional relevance of de-regulated expression of DNA repair genes with regard to intrinsic as well as acquired platinum resistance.

In agreement with our findings that TFP may antagonize the adaptive transcriptional response to cisplatin treatment, residual clones that survived an initial round of cispatin/TFP co-treatment exhibited greater relative platinum sensitivity than cisplatin-

surviving residual clones when re-exposed to cisplatin for a second treatment round (60% versus 73%). Moreover, we found that U1810 cells exposed to an initial round of cisplatin/TFP co-treatment had reduced clonogenic capacity in the absence of a second round of treatment, suggesting that TFP may induce a long-lasting state of cellular stress. We reasoned that this effect might be reflected in their pattern of gene expression. Indeed, residual clones that survived cisplatin/TFP co-treatment showed a preferential up-regulation of stress-inducible genes involved in chromatin remodeling (e.g. ASF1A, ASF1B, KAT2A/GCN5) and replication (e.g. MCM2, MCM5, MCM6), suggesting that TFP may interfere with chromatin-related processes that normally operate in response to cisplatin-induced DNA damage. In addition, these residual clones contained increased transcripts of genes implicated in ribosome/RNA metabolism (e.g. RAE1, RPL32, RPL27L, SSB, UPF3B, NPM3). Since CPZ treatment was previously shown to trigger a rapid shutdown of translation in yeast and mammalian cells, it is plausible that this late increase in the expression of genes involved in RNA biosynthesis may represent a compensatory response to an earlier translational stress [485]. As mentioned earlier, the top scored networks of differentially regulated genes in residual clones that survived cisplatin/TFP co-treatment contained few DNA repair components, although it is not clear whether this effect was caused by the disturbance in RNA metabolism. Notably, the mRNA and protein expression of PRKDC were also elevated in residual clones that survived cisplatin/TFP co-treatment, albeit not to the same extent as in cisplatin-surviving residual clones. Moreover, the transcript levels of several predicted interaction partners of PRKDC were also elevated in residual clones that survived cisplatin/TFP co-treatment, including KAT2A (GCN5) and USF1. DNA-PK has been shown to phosphorylate the protein products of these two genes, which resulted in transcriptional repression and activation, respectively [664, 665]. It is therefore possible that the transcriptional response of cisplatin-surviving residual clones might be partly driven by the up-regulation of DNA-PK. Interestingly, we found that TFP augmented DNA-PKcs autophosphorylation in response to cisplatin. It remains to be determined whether TFP could also modulate DNA-PK-mediated phosphorylation of GCN5 or USF1 and whether this might contribute to the increased platinum sensitivity of TFP co-treated cells.

In summary, we present evidence that global genomic profiling of residual clones that survived cisplatin treatment can be used in tandem with pathway analysis to identify putative mechanism(s) driving platinum resistance. Collectively, our data showed that concurrently transcriptional de-regulation of multiple DNA repair genes in cisplatin-survivng NSCLC residual clones might facilitate the development of acquired resistance upon additional drug exposure. Finally, we found that alterations in the cisplatin-induced adaptive transcriptional responses by TFP might account for its ability to confer platinum sensitization. Our data raise the possibility that TFP may be useful for sensitizing intrinsically platinum-resistant tumors as well as for circumventing the development of acquired platinum resistance in initially sensitive tumors.

5 CONCLUSIONS & FUTURE PERSPECTIVE

5.1 Our main conclusions

In **Paper I**, we demonstrate that TFP-mediated augmentation of bleomycin sensitivity in human NSCLC cells is associated with its ability to inhibit DNA strand break repair *in vitro* and *in situ*. In **Paper II**, we show that TFP and related phenothiazines impair γH2AX resolution in human NSCLC cells leading to prolonged checkpoint arrest and delayed check proliferative recovery after exposure to bleomycin or cisplatin. We further demonstrate that defective DNA DSB repair in TFP co-treated cells is associated with abnormal mitosis, secondary arrest and caspase-mediated apoptosis initiating from the G_2-M phase. We provide evidence that severe oxidative stress in conjunction with extensive lysosomal/mitochondrial dysfunction elicit caspase-3 activation and loss of cell viability. In **Paper III**, we provide evidence based on *in silico* predictions that phenothiazines are putative chromatin-active agents. Our analyses indicate that TFP selectively sensitize cancer cells to direct-acting DNA damaging agents. We show that TFP disrupts DDR signaling and its resolution by inducing hyper-activation of DNA-PK and ATM preferentially in cancer cells. We demonstrate that TFP-induced hyper-activation of DNA damage-induced DDR signaling represents an adaptive response that can be targeted with DNA-PK or ATM inhibitors to further enhance tumor killing. Finally, in **Paper IV**, we demonstrate that TFP profoundly alters the cisplatin-induced transcriptional survival responses leading to sustained suppression of clonogenicity. Based on these data, a tentative model for phenothiazine-mediated chemosensitization is constructed (Figure 17).

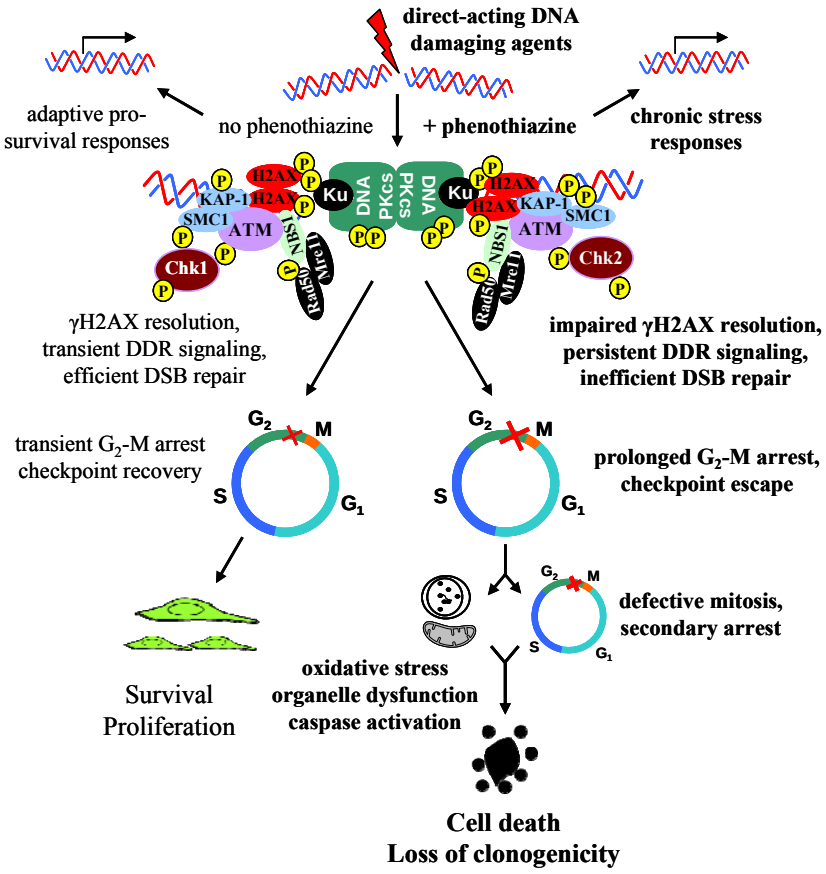

Figure 17. A tentative model of phenothiazine-mediated chemosensitization based on results presented in this thesis.

5.2 Unresolved questions and future perspectives

5.2.1 Intracellular localization of phenothiazines

The pharmacologic activity of a compound is determined by its localization. The chance for it to interact with multiple targets increases if the compound accumulates in different subcellular compartments. Conversely, confinement to a single location increases the likelihood that it would interact with high specificity with a single target. The precise cellular localization of phenothiazines is not known. Ultrastructural studies using electron microscopy have revealed diffuse phenothiazine immunopositivity associated with many subcellular structures, including chromatin, nuclear matrix, cytosol, ER, ribosomes and mitochondria [536]. Moreover, phenothiazines have also been shown to partition into model membranes whose lipid composition resembles that of the plasma membrane. Consequently, phenothiazines have many potential interaction partners throughout the cell. However, it is not known whether phenothiazines would accumulate at the different sites with the same relative ratio or if there might be a preference for one or more cellular compartment. In this thesis we have shown that TFP can modulate DNA damage-induced DNA-PK/ATM signaling, suggesting that it can localize to chromatin, but whether TFP localized elsewhere in the cell contribute significantly to chemosensitization is still an open question. Conjugating phenothiazines with a fluorescence tag may help resolve this issue if the tag does not appreciably alter the chemical and pharmacological properties of its host molecule. This can be nevertheless be tricky due to the low molecular weight (e.g. TFP, MW 480) of phenothiazines because and the relatively large sizes of most conventional fluorescent tags (e.g. FITC, MW 389). Furthermore, most clinically used phenothiazine anti-psychotics lack an amine functional group that needed for chemical conjugation, which requires the use of specialized derivatives (e.g. 10-[2-(aminooxy)ethyl]-10H-phenothiazine) whose activities must first be rigorous tested and confirmed to resemble that of traditional phenothiazines. As an alternative strategy, it is possible to radioactively label phenothiazines. However, it is not possible to detect radiolabeled phenothiazines *in situ* and fractionation is a labor-intensive and crude way of separating subcellular compartments which is prone to cross-contamination. A third strategy is to generate antibodies that specifically recognize phenothiazines. In this respect, it is important that the antibodies should bind to its target in the context of free phenothiazines as well as phenothiazines that exist in complex with other biomolecules. Although this does not permit real time tracking, it would allow detection of phenothiazines *in situ*. Regardless of the strategy, elucidating the subcellular localization(s) of phenothiazines may go a long way to help defining their molecular targets.

5.2.2 Analysis of TFP-interacting partners

Given that phenothiazines are likely to have multiple cellular targets, which of these are most relevant for chemosensitization? Our data suggest one or more chromatin-localized proteins. To answer this question fully, however, we need to first establish the identities of all the proteins that bind to phenothiazines (i.e. the interactome). To this end, two different approaches may be applied. In one, phenothiazines with suitable amine group would be conjugated onto a sepharose column. Total cell lysates or fractions thereof can then be passed through the column. After extensive washing, any proteins that were bound to the immobilized phenothiazine can be eluted by disrupting the binding with high concentrations of salt and their identities determined by mass spectrometry. Alternatively, if a high affinity monoclonal antibody can be raised

against a particular phenothiazine, it may be used to immunoprecipate phenothiazine-binding proteins. A potential pitfall here is that the antibody may not be able to recognize phenothiazine-binding protein that have deep substrate binding pockets or that the interactions are weak and/or transient. Once the phenothiazine-interactome is established, one can then systematically study the most likely candidates and their relative contribution to chemosensitization. This issue needs to be urgently addressed before structure-activity relationship (SAR) which is required for the development of a clinically optimized compound that can be used for chemotherapy sensitizing purposes.

5.2.3 TFP in combination chemotherapy

Drug combinations

A survey amongst published reports showed that phenothiazines were always active in combination with bleomycin in tumor cells whose p53 status were either null (C6 rat glioma [666]) or mutant (L1210 mouse leukemia [571, 572, 667], SKOV3 human ovarian cancer [565]). The chemosensitizing activity of phenothiazines in p53 wild-type cells was more ambiguous, with CPZ shown to potentiate bleomycin in B16 mouse melanoma [585] while TFP was largely ineffective in EMT6 mouse breast cancer [668]. These data corroborate our own results which showed that TFP conferred bleomycin sensitization in p53-deficient (U1810, H23, MDA-MB-231) but not p53 wild-type (A549, hTERT-RPE1) cells. As mentioned previously, MCF7 cells exhibited a subtle defect in p53 signaling despite being wild-type for p53, which might explain its responsiveness to TFP-mediated chemosensitization [669]. The proficiency of wild-type p53 in B16 melanoma cells have not been thoroughly assessed, although it is tempting to speculate that it may also be functionally sub-optimal. Importantly, we and others have shown that phenothiazines did not increase bleomycin sensitivity in non-cancerous cells (Paper III) [670]. Taken together, the available data suggest that bleomycin should be an excellent candidate chemotherapeutic agent for use in combination with phenothiazine-based chemosensitizers.

The few studies that examined the efficacy of phenothiazines as cisplatin sensitizers yielded results that were not entirely consistent with our own data. Onoda et al found that TFP did not affect the growth of p53 wild-type B16 melanoma cells [586], which was in line with our findings that TFP lacked effect in A549, A2780 and hTERT-RPE1 cells. On the contrary, Perez et al reported that TFP did impart cisplatin sensitization in A2780 parental cells as well as two of its platinum-resistant sub-clones [563]. Moreover, they showed that TFP was ineffective against OVCAR-3 which is mutant for p53, while our data (Paper III) showed that TFP sensitized all of the three p53-mutant cell lines that we have tested so far (U1810, H125, MDA-MB-231). The reason for this discrepancy is not clear. As Perez et al did not provide an outline of their drug treatment scheme it is not possible to determine whether TFP scheduling could be the cause of these contrasting results. More studies are needed to fully establish the utility of phenothiazines as cisplatin sensitizers. Notably, there is a substantial amount of data supporting a potential protective effect of phenothiazines on cisplatin-induced nephrotoxicity and ototoxicity, two major dose-limiting side effects of cisplatin encountered in the clinical setting [574, 575]. Therefore, phenothiazines might be useful for increasing the therapeutic indices of platinum-based regimens.

Based on our works, it appears that TFP has little or no effect in combination with chemotherapeutic drugs which do not directly damage DNA, such as etoposide or

doxorubicin. There is no consensus on this subject in the literature where seemingly conflicting reports have been published. Nevertheless, we discovered upon closer scrutiny that phenothiazines selectively re-sensitized a variety of doxorubicin-resistant cancer cells *in vitro* as well as *in vivo* but had no effect in any of the parental cell lines except MCG101-AA, a chemically-induced mouse sarcoma whose relative sensitivity to anthracyclines was not known [469, 587, 671-676]. Strikingly, while adding TFP to doxorubicin-based regimen failed to demonstrate any extra benefit amongst unselected patient populations [465, 467, 468], in a phase I/II trial, the combination of doxorubicin and TFP produced clinical responses in 7 of 36 patients (19%) with acquired drug resistance (previous response followed by relapse) but in none of the 21 patients with intrinsic drug resistance (no previous response) [593]. Similarly, TFP was found to cross-sensitize doxorubicin-resistant sub-clones but not parental cells to etoposide [572, 677, 678]. Several potential mechanisms have been proposed, including but limited to enhanced drug retention, increased induction of DNA damage and down-regulation of pgp expression. On a cautionary note, however, phenothiazine-mediated sensitization to anthracyclines was also observed in non-cancerous V79-379A hamster lung fibroblasts, LLC-PK1/MDR1 porcine kidney cells and primary human kidney cells that express pgp [470, 670, 679]. In addition, prolonged exposure of doxorubicin-sensitive parental L1210 cells to TFP in combination with low doses of doxorubicin was associated with compensatory up-regulation of pgp expression and development of cross resistance to other drugs (e.g. etoposide, amsacrine, vincristine) [680]. Moreover, when mice bearing MDR P388 leukemia were treated with drugs that are substrates of pgp, such as vincristine, taxol or the TFP-related compound trans-flupenthixol, it led to metastasis, faster tumor progression and decreased survival [681]. There are also uncertainties as to what impact phenothiazines might have on the acute toxicity of anthracyclines, with one study showing a protective effect in C57/BL6 mice and another study showing exacerbated toxicity in ddY mice [673, 682]. Collectively, these data suggest that phenothiazines might be useful as resistance modifiers in the second line for a subset of patients whose tumors show acquired resistance to anthracyclines or etoposide. Due to the high risk of collateral damage to normal tissues and inadvertent exacerbation of disease progression, we propose that any use of phenothiazines as general sensitizers for doxorubicin should be avoided.

Tumor types

The works described in this thesis provide a partial answer to this issue. Thus, we demonstrated that TFP is an effective chemosensitizer in tumor cell lines with deficient p53 functions but it has little or no effect in p53-proficient cells. Notably, the particular cause of p53 deficiency did not seem to impact on TFP responsiveness, be it non-sense mutation (U1810), missense mutations (e.g. H23, MDA-MB-231) or even subtle functional deficiency of the wild type protein (MCF7) [669]. These data suggest that a fully functional p53-dependent transcriptional program might be required to counteract the actions of TFP. This is unlikely to involve p53-mediated checkpoint maintenance, given that cell cycle arrest was longer in TFP co-treated U1810 and MDA-MB-231 cells. Rather, TFP may be selectively active when cells are unable to activate p53-mediated processes that promote DNA repair, survival or checkpoint recovery [683-685]. Regardless of the exact mechanism, our results indicate that p53-mutant tumors should be considered for phenothiazine-mediated chemosensitization. Tumors that are wild type for p53 but which are functionally deficient due to over-expression of its negative regulators (e.g. MDM2) may also be sensitive to TFP. Additional experiments are needed to test this hypothesis.

In this thesis, we showed that TFP and structurally related phenothiazines are potent sensitizers of direct-acting DNA damaging agents in multiple cell lines derived from different solid tumors, including NSCLC, breast cancer and ovarian cancer. Are there any tumor types that may be responsive to phenothiazine-based chemosensitization? Because phenothiazines have a demonstrable affinity for melanin, we propose that malignant melanomas can be a good candidate for testing the efficacy of phenothiazine as chemosensitizers. There are three main arguments for choosing melanomas. First, CPZ clearly accumulated in rodent melanocytes *in vivo* [686, 687]; depending on the experimental system, CPZ was either cytotoxic *per se* or conferred sensitization towards chemotherapy and radiation [585, 592, 688-694]. Second, p53 mutations are very common in human melanomas [695, 696]. Finally, bleomycin and cisplatin are both routinely used in melanoma treatment as part of multi-drug chemotherapy regimens (CVD, cisplatin-vinblastine-dacarbazine; BOLD, bleomycin-vincristine-lomustine-dacarbazine) and their toxicity profiles are known in these settings. Moreover, previous clinical trials have demonstrated that phenothiazines cand be given safely in combination with bleomycin [594]. Since our analyses showed that TFP significantly sensitized p53-deficient tumor cells towards bleomycin and cisplatin, it might be worthwhile to evaluate the efficacy of such combination chemotherapy in human melanomas.

Another group of patients that might benefit from TFP-mediated chemosensitization are those that have CNS tumors. This is due to two properties of phenothiazines. One, phenothiazines are amphiphilic at physiologic pH and this allows them to freely cross the BBB. Two, phenothiazines could potentially disrupt the barrier function of the BBB by virtue of its ability to inhibit ABC transporters, which are highly expressed in endothelial cells. This phenomenon has been demonstrated in mice and rats where TFP enhanced the CNS accumulation of etoposide and ivermectin but not vinblastine [460-462]. Neither bleomycin nor cisplatin is particularly active against gliomas and it is not known whether phenothiazines could increase their CNS penetration. In this regard, both electrochemotherapy (ECT) and direct intracranial injection have been tested for their abilities to enhance bleomycin accumulation in the CNS. These studies concluded that high dose bleomycin can be given safely. Exposure of human and rat glioma cell lines to diverse phenothiazines *in vitro* resulted in either growth arrest or cell death [521, 557, 697] and there is also some evidence for phenothiazine-mediated sensitization towards bleomycin and nitrosoureas [666, 698]. By contrast, the effect of phenothiazines in combination with temozolomide or IR, which are the mainstay of treatment for glioma, was additive and sub-additive, respectively [568]. These data are consistent with our own results showing that TFP preferentially sensitized tumor cells to direct-acting DNA damaging agents. The particular combination of bleomycin and TFP has been tested in a phase II clinical trial previously and was not associated with any objective responses [594]. However, that study was conducted in patients with high grade gliomas who had been heavily pre-treated, making it uncertain that they would have responded to any type of treatment. Moreover, the study was single-armed making it impossible to assess any potential advantage from the combination treatment. We therefore propose that phenothiazines should be re-evaluated for their efficacy as chemosensitizers in a more selected patient population whose tumors are of lower grade and harbor defined p53 mutations. In light of our findings that TFP could enhance the cytotoxicity of calicheamicin and the ICL-inducing agent cisplatin, it is tempting to speculate that phenothiazines may be useful in combination with gemtuzumab ozogamicin (GO, an antibody-conjugate of calicheamicin) or melphalan (an ICL-inducing agent) for the treatment of AML and multiple myeloma, respectively.

Scheduling of TFP

To obtain a thorough overview on the temporal patterns of TFP-mediated chemosensitization, we expanded our TFP scheduling schemes and tested various combinations of pre- and/or post-incubation for their ability to augment caspase-3 activation in U1810 cells after a pulse treatment with bleomycin or cisplatin. We found that when the duration of TFP post-incubation was kept constant, cells that were also pre-incubated or received TFP concurrently with a DNA damaging agent had significantly higher levels of active caspase-3 than counterparts that were only post-incubated. Similarly, when TFP pre-incubation was applied, caspase-3 activation increased with the lengths of post-incubation. These data suggest that TFP-mediated chemosensitization has two temporal components. The early component is likely to be related to its ability to perturb DDR signaling. In line with this, we showed that if cells were first exposed to bleomycin and then post-incubated with TFP upon bleomycin removal, TFP could no longer augment DNA-PKcs autophosphorylation. Our data is also corroborated by the findings of Kwok and Twentyman, who reported that if TFP was given after bleomycin exposure, it had no effect on the survival of EMT6 cells [699]. It is not yet clear which process or processes might be involved in the late component of TFP-mediated chemosensitization. One possibility is that it could be related to inhibition of mitotic kinesins by TFP [525, 526]. Collectively, the available data suggest that in order to maximum efficacy, TFP needs be given prior to or concurrently with a direct-acting DNA damaging agent and should remain for at least as long as the latter is present. Future studies with animal models will be required to test the validity of this hypothesis.

Concentration of TFP *in vivo*

The minimal concentration of TFP that produces a discernible effect on DDR signaling in bleomycin-treated U1810 cells was 5 μM (our unpublished data), while 10 μM was required to significantly suppress their clonogenic potential. Are these concentrations achievable *in vivo*? There are no simple answers to this question. Previous studies have established the maximum tolerated doses (MTD) of TFP and prochloperazine (PCZ) as 60 mg/d (orally 15 mg four time per day) and 180 mg/m^2 (intravenously 2 h infusion), respectively [464, 593]. Based on these schedules, the maximum plasma level of TFP was approximately 130 ng/ml, which corresponds to about 0.32 μM (MW 407.5) [593]. The peak plasma levels of PCZ varied greatly between patients (91-3215 ng/ml), which corresponds to a range of 0.24-8.6 μM (MW 373.9) [464]. Hence, the plasma concentrations of TFP are well below the 10 μM needed for chemosensitization *in vitro* while for PCZ it might be achievable in some patients. However, phenothiazines generally have very large volumes of distribution (e.g. 350.1 ± 183.8 L/m^2 for PCZ) due to their high lipophilicity [464]. Moreover, phenothiazines may exhibit tissue tropism. An example of this is CPZ, which has affinity for melanin. In hamsters and mice bearing transplantable melanomas, CPZ showed >100 fold greater accumulation in melanoma than in muscle or blood [686]. Therefore, the plasma level of phenothiazines might not adequately reflect their concentration in tissues. It is possible that concentrations of phenothiazines sufficiently high to confer therapeutic chemosensitization can be achieved in at least some tumors. More studies are required to determine if this is the case.

5.3 A unified view of phenothiazine-mediated chemosensitization

Due to a large number of potential target sites and molecular interaction partners, it is rather difficult to obtain a unified view of phenothiazine-mediated chemosensitization. To this end, we propose a tentative ranking system where each known target site or interaction partner acts as an independent molecular checkpoint and the sum total of all interactions determine whether phenothiazine-mediated chemosensitization is likely to be observed or not (Table 8). This analysis suggests that colon cancer Caco-2 cells are likely to be sensitized to cisplatin by phenothiazines. Additional experiments are needed to verify the validity of this prediction.

Table 8. A tentative ranking system for predicting phenothiazine-mediated chemosensitization.

Cell line		U1810	U1810	U1810	MB-231	A2780	Caco-2
Treatment		BLM	VP16	STS	CDDP	CDDP	CDDP
p53 [a]	mutant 3p	3	3	3	3		3
	wild-type 0p					0	
Type of DNA lesion	DSB 2p	2					
	ICL 2p				2	2	2
	SSB 0.5p	0.5	0.5		0.5	0.5	0.5
	oxidative damage 0.5p	0.5					
	replication lesions 0.5p		0.5		0.5	0.5	0.5
	none 0p			0			
Mode of damage induction	direct 2p	2			2	2	2
	indirect 0.5p		0.5				
	none 0p			0			
Transport by efflux pumps	yes 1p [b]						1
	no 0p	0	0	0	0	0	
Expression of efflux pumps	yes 1p						1
	no 0p	0	0	0	0	0	
Total		**8**	5	0	**8.5**	4.5	**10.5**
Sensitization		**yes**	no	no	**yes**	no	n.d.

Abbreviations: STS, staurosporine; MB-231, MDA-MB-231; n.d., not determined.

[a] functional proficiency or deficiency; [b] The point is given on if the cell also expresses ATP-dependent efflux pumps.

5.4 CONCLUDING REMARKS

Phenothiazines have demonstrable cytotoxic and/or chemosensitizing activities against a wide range of human tumor cells. Their apparent failure to recapitulate these effects in a series of earlier clinical trials is likely due a scarcity of knowledge about the molecular determinants of phenothiazine-mediated chemosensitization. As described in this thesis work, such parameters are beginning to be elucidated and this will allow a more refined definition of patients who are most likely to benefit from phenothiazine-containing regimen. Moreover, the ability of phenothiazines to allviate disease-related anxiety, suppress chemotherapy-induced emesis and potentially limit collateral damage to normal tissues can all be harnessed to improve the quality of life of cancer patients. It is time give this class of "old" drugs a second chance what show us what they are really capable of.

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