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Delineating the DNA damage response using systems biology approaches

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GENERAL DISCUSSION AND OUTLOOK

An unbiased approach for the study of the DNA damage response

Insults to our DNA originate from various endogenous and exogenous sources and induce a highly interconnected signaling response. This response includes a halt of cell cycle progression, attempts to repair the damage, and if damage is beyond repair the removal of damaged cells by apoptosis, senescence or differentiation [1; 2]. The DNA damage response (DDR) has been crucially linked to cancer formation and ageing, as well as cancer therapy [3; 4]. DNA damage-inducing cancer therapy using chemotherapeutic drugs or γ-irradiation is standard treatment regimen for various tumor types, but acquired or native resistance still constitutes a major hurdle for therapy success [5]. Unraveling DNA damage-induced signaling routes will help to elucidate the mechanisms, which underlie resistance to DNA damage-based cancer therapy, in order to better classify patient responses and introduce targeted therapeutic intervention [4; 6]. With some exceptions, most *in vitro* and *in vivo* studies indicate that not one gene alone at a time is responsible for therapy success or failure. Instead interactions between genes might be more successful biomarkers and therapeutic targets, as proven for the concept of synthetic lethality [4; 7].

Regulation of the abundance and activity of DNA repair and DDR signaling factors is often accountable for the resistance to DNA damage-induced cell killing. However, genotoxic stress evokes a plethora of cellular responses going far beyond the "core DDR" signaling cascade and often pathways, not directly related to the DDR will predict the cellular outcome of DNA damage accumulation [8; 9; 10]. DNA damage-induced cellular reactions are reflected in transcriptional changes, as well as changes in protein translation and stability; changes in the levels of small metabolites and changes in the interaction of signaling molecules and in the addition of posttranslational modifications to target molecules [9; 11]. The immense complexity of the cellular response to DNA damage necessitates the use of unbiased, high-throughput techniques for its examination.

In our studies we aim to identify mechanisms that underlie the resistance to genotoxic stress in different cell types, using a systems biology approach. We combine RNAi based knockdown screens, transcriptomics, phosphoproteomics and metabolomics analyses with the induction of DNA damage by the genotoxic drug cisplatin and integrate the obtained high-throughput dataset; retrieving highly enriched biological pathways and signaling networks. Next to DNA repair and DDR signaling networks we identify a number of developmental pathways such as Wnt/ β-Catenin-signaling, as well as changes in house keeping processes and metabolism, as crucial determinants of DNA damage-induced killing (Figure 1). On the basis of integrated signaling networks we identify molecules, which have crucial functions in executing the DNA damage-induced cellular responses, such as the kinase Csnk1a1 and the E3 ubiquitin ligase ARIH1, which mediate cisplatin-induced Wnt signaling or translation arrest, respectively.

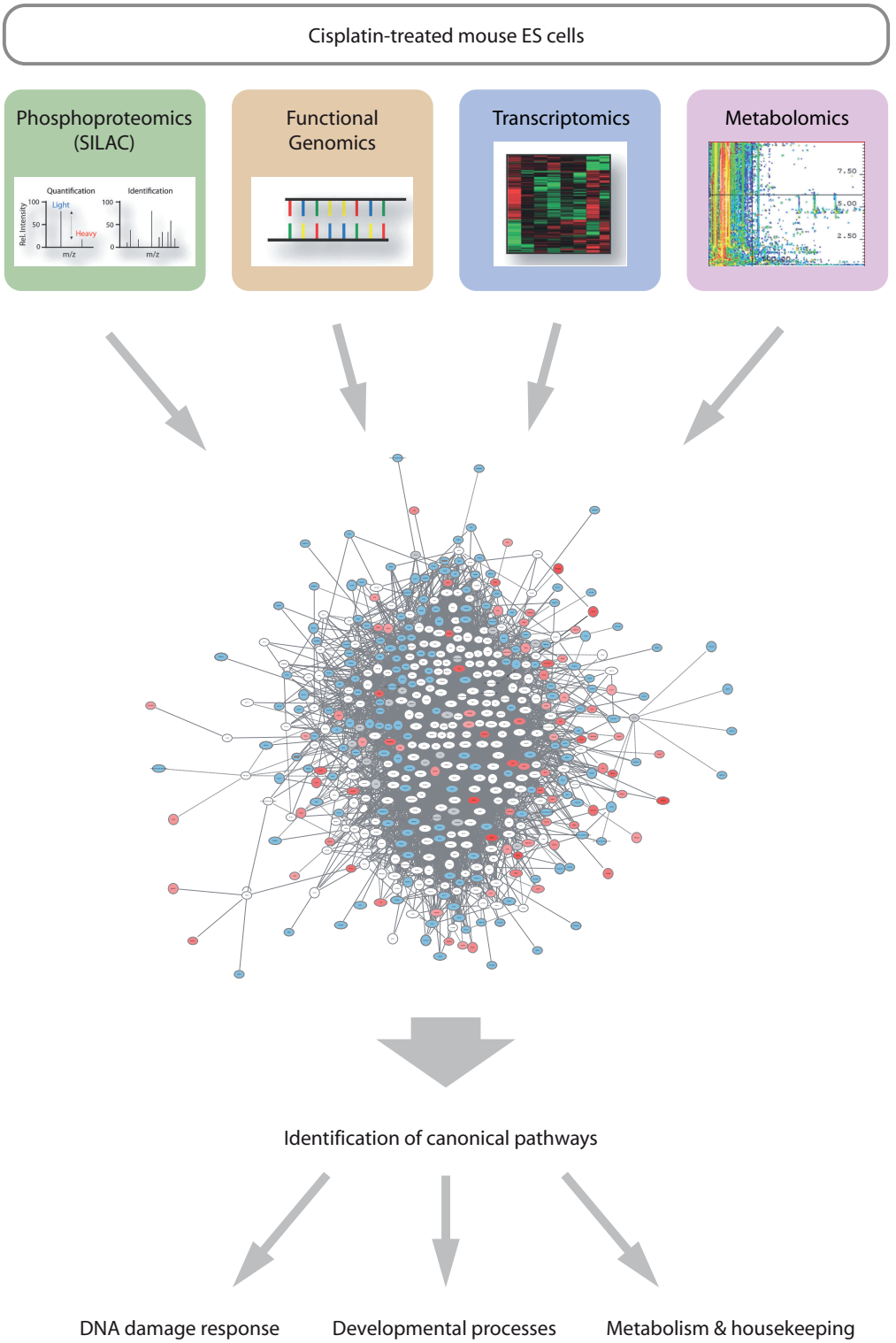

Figure 1 Systems approaches to study the DDR signaling network. Integration of high-throughput datasets reveals pertubations of the DDR network which correlate to changes in DDR pathways, developmental processes and metabolism and housekeeping functions.

Deciphering the DNA damage response from ES cells to cancer cells

Embryonic stem (ES) cells are defined by pluripotency determining their potential to differentiate into all three germ layers. Cultured in the presence of feeders and correct accessory factors, they maintain an indefinite proliferative potential [12], without the requirement for silencing key tumor suppressors (p53, p16, hTERT), which is often necessary for the expansion of cell lines under culture conditions. Therefore ES cells can serve as a good model system to study a comparably naïve DDR.

Interestingly, ES cell-specific features, which underlie successful embryonic development, also resemble cancer cell characteristics. Both cell types share a high proliferative rate, lack of contact inhibition, high telomerase activity, overlapping gene expression patterns, including the expression of certain oncogenes such as Myc or Klf4, as well as a lack of a G1/ S checkpoint in response to DNA damage [13; 14; 15].

However, in contrast to cancer cells, ES cells can not tolerate a high mutational burden. In order to maintain fidelity of the lineage, ES cells show a very robust onset of apoptosis after DNA damage induction and can furthermore induce differentiation to remove cells with corrupted genetic material from the pluripotency pool [14; 16]. Additionally ES cells upregulate certain repair pathways such as base excision repair and use high fidelity homologous recombination (HR) based double strand break (DSB) repair pathways rather than the error prone non-homologous end joining pathway [14].

We use mouse ES cells as a model system to study a relatively naïve DDR and further extend our findings to cancer cell lines with variable genetic backgrounds, to identify both common and exclusive mechanisms of DNA damage resistance.

Cisplatin – an alkylating drug commonly used in cancer therapy

We use the alkylating drug cisplatin, as standard genotoxic treatment for our high-throughput experiments and further validate cisplatin based observations using other genotoxic and non-genotoxic compounds, as well as UV- and γ–irradiation.

Cisplatin belongs to the family of DNA crosslink-inducing chemotherapeutics, which were the first chemotherapeutic drugs in clinical application and still form the largest family, used for the treatment of various solid tumors, e.g. ovarian, non-small-cell lung, head and neck, bladder, colorectal and testicular cancer. Although platinum drugs are seen as comparably successful chemotherapeutic agents, acquired (common in ovarian cancer) or native (common in colorectal and lung cancer) resistance still impairs successful patient cure [17]. Furthermore, high toxicity to healthy tissues, including neuro- and renal as well as gastrial toxicity narrows the therapeutic window [17; 18; 19].

Various cisplatin resistance mechanisms have been described, including modifications in the cellular uptake, the repair of cisplatin-induced DNA lesions, modulation of cisplatin-induced apoptotic responses, processes counteracting cisplatin-induced non-genotoxic stresses, as well as cisplatin response-independent survival pathways, which might compensate cisplatin-induced killing [17; 19].

Cisplatin: mechanism of action

Diffusion and active transport mediated by copper transporters lead to uptake of cisplatin into the cytoplasm. There, the originally inert molecule loses its chloro-ligands in an aquation reaction and becomes positively charged, now being able to interact with nucleophilic molecules [17; 20]. The most significant of these interactions is the one with DNA; with a number of different types of adducts being formed. Those include monoadducts, intrastrand crosslinks and interstrand crosslinks, as well as secondary DSBs resulting from the collapse of replication forks that encounter the crosslinks [18; 20].

Intrastrand crosslinks; extremely toxic lesions, which affect both transcription and replication by hindering the crucial separation of the DNA strands, are the most commonly formed DNA:cisplatin adducts [18]. Cisplatin-induced intrastrand crosslinks can be sensed by different damage recognition proteins, such as high-mobility group box protein 1 (HMGB1), TATA box-binding protein (TBP) and human upstream binding factor (UBF) [20]. Interestingly, in our siRNA screens we find that silencing of UBF strongly sensitizes ES cells and H1299 lung cancer cells to cisplatin-induced killing **(Chapter III & VI).**

Next to directly acting on DNA, cisplatin also induces non-genotoxic stresses. Amongst those non-genotoxic actions are the induction of oxidative stress by a shift in the cellular redox balance through binding to nucleophilic molecules and endoplasmatic reticulum (ER) stress, which has been shown to kill enucleated cells [17; 21]. In our metabolomics studies we detect an increase in sulfur-containing compounds, including both the oxidized and reduced form of glutathione, as well as the amino acid methionine **(Chapter V)**. Those compounds can directly bind cisplatin and block its access to DNA and may furthermore act as reducing agents to relieve secondary, cisplatin-induced oxidative stresses [22; 23]. A number of metabolic pathways affected by cisplatin can have pro- or antioxidant function, including for example enhanced levels of metabolic enzymes, such as spermine oxidase, which can produce H_2O_2 while oxidizing spermine [24]. However, overall metabolic profiling argues against a general increase in oxidation, at the studied timepoints in ES cells **(Chapter V)**. In line with this, a reactive oxygen species (ROS) detection assay does not sense an increase in ROS formation after treatment with cisplatin concentrations used in our studies in ES cells **(Chapter V).**

Furthermore, in ES cells and different cancer cell lines, we do not detect any canonical markers of the ER stress response, such as an increase in the phosphorylation of eif2α or elevated levels of CHOP (data not shown). Taken together, these data suggest that cisplatin-induced effects in our studies are, to a large extent, due to its genotoxic properties.

DNA repair-related cisplatin resistance mechanisms

The variety of primary and secondary lesions induced by cisplatin requires essentially all DNA repair pathways for their removal, including Fanconi anemia (FA), nucleotide excision repair (NER) and HR. Furthermore, damage bypass by translesion synthesis (TLS) can be an important determinant of survival after cisplatin treatment [18; 20].

Indeed *in vivo* cisplatin sensitivity is often related to lack of DNA repair factors, such as the endonuclease Ercc1, which associates with XPF to incise the cisplatin damage and is linked to cisplatin resistance in different in vitro systems and tumor types [25; 26]. HR factors such as BRCA1 and BRCA2 are additional determinants of cisplatin sensitivity. Mutations in those genes are common for high grade serous ovarian cancers, a tumor type, which initially responds well to platinum based therapy [4; 27].

Pathway analysis on functional genomics, transcriptomics and phosphoproteomics datasets of cisplatin treated ES cells predicts a strong enrichment of DNA repair-related processes, mainly associated with DSB repair **(Chapter III-VI)**. Indeed, our RNAi screens determine the HR repair factors BRCA1, BRCA2 and the replication stress and HR-related protein Tonsl [28; 29] as crucial determinants of survival after DNA damage in ES cells and different cancer cell lines **(Chapter III , IV & VI).** Moreover, we identify a number of other DNA repair factors, such as the E3 ligases SHPRH (involved in postreplication repair), Rfwd3 (involved in RPA-mediated repair of single strand breaks) or Pirh2 (involved in damage bypass by TLS) [30; 31; 32] as genes, whose function is required to resist cisplatin-induced killing and hence represent potential targets for inhibition **(Chapter IV).**

It is interesting to note is that the high proliferation rate of both ES cells and many cancer cell lines has particular consequences for the repair pathways of choice. Many primary lesions are transcribed into double strand breaks when encountering a replication fork and the predominant cell cycle stage also determines the accessibility for certain repair mechanisms such as FA and HR, which are functionally restricted to the S and G2 stages of the cell cycle [18].

p53 as a central hub in the ES cell DDR

The transcription factor p53 is a crucial DDR signaling hub, determining the outcome of DNA damage by regulating plethoric responses ranging from apoptosis induction, over DNA repair and differentiation to metabolic processes [16; 33; 34]. Although p53 was previously thought to act mainly as a tumor suppressor, a number of recent studies implicated that, dependent on the cellular context, p53 proficient cells had a survival advantage, since p53-mediated cell cycle arrest prevented aberrant mitosis and subsequent mitotic catastrophe [35; 36; 37].

In contrast to previous reports [38], we observe (nuclear) accumulation of active p53 in cisplatin-treated ES cells **(Chapter III).** Moreover, transient siRNA-mediated-, as well as stable shRNA-mediated knockdown and knockout of p53 in ES cells strongly protects against killing induced by cisplatin, as well as other genotoxic drugs including the topoisomerase inhibitors doxorubicin and etoposide or the DNA crosslinker mitomycin C and UV-irradiation **(Chapter III & IV).** To a lesser extent, knockdown of p53 also provides a survival advantage against the oxidative stress-induced by the glutathione depletor DEM **(Chapter IV).**

The crucial function of p53 as a DDR signaling hub in ES cells is further reflected by the central role p53 holds in many of the identified signaling networks, including

DDR-related networks such as the one centered on ATM signaling, but also the RAR- or Wnt/β-Catenin signaling networks, which reflect developmental pathways **(Chapter III)**. Genes, whose expression is differentially regulated by cisplatin, comprise a great number of p53 target genes, reflecting the whole spectrum of p53-mediated responses **(Chapter III, V).** Interestingly we can confirm a role of p53 in regulating differentiation by controling the levels of the transcription factor Nanog **(Chapter III)** [16].

Moreover, we find an enrichment of metabolic enzymes (e.g. nucleotide or amino acid metabolic enzymes) amongst the p53 target genes, including for example the proline catabolic enzymes Prodh and Aldh4a1, as well as the arginine catabolic enzyme GATM **(Chapter V)**. Prodh, which is a key player in proline breakdown has itself been implicated as a tumor suppressor [39].

The protein stability and activity of p53 is highly regulated by posttranslational modifications, most prominently by ubiquitination [34]. Indeed, many of the (de)-ubiquitinases that affect cisplatin-induced killing have been previously shown to directly or indirectly modulate p53 stability or activity, including for example the E3 ligase Rfwd3 or the deubiquitinase USP7 (HAUSP) **(Chapter IV)** [40; 41].

p53-independent mechanisms of sensitization

Quite interestingly however, despite the overwhelming role of p53 in DNA damage-induced apoptosis in mouse ES cells a number of p53-independent processes prove to be important for survival after DNA damage. Despite its inclusion into the Wnt signaling network, we find that genotoxic stress-induced enhancement of the Wnt signaling pathway is, in fact, p53-independent. The same holds true for the cisplatin-induced accumulation of ES cells in the S/G2-phase of the cell cycle **(Chapter III)**. Although cell cycle-related p53 target genes such as p21 or GADD45 are induced after cisplatin treatment, knockdown of p53 does not affect cell cycle arrest and silencing of p21 itself fails to modulate ES cell survival after cisplatin treatment **(Chapter III)**.

Based on phosphoproteomics studies we find indications for a role of MAP-kinase (MAPK)-signaling pathways in the cisplatin response of ES cells **(Chapter III)** [42]. Cisplatin has been shown to evoke a number of MAPK signaling responses, which can be both pro- and antiapoptotic, depending on the cellular context and p53 status [43; 44]. p38/MAPK signaling can mediate cisplatin-induced apoptotic responses [19], but has also been implicated to be important for checkpoint signaling in p53-deficient cells, counteracting mitotic catastrophe [37].

However, none of the MAPKs themselves are identified as a modulator of DNA damage-induced killing in ES cell functional genomics screens **(Chapter III)**. Nevertheless, the dual specific phosphatase DUSP15, which sensitizes ES cells and all tested cancer cell lines might act via p38 signaling, in a cell type specific manner (**Chapter III & VI)**. DUSP15 knockdown affects p38-mediated apoptosis in p53-wildtype cancer cells (potentially involving an activation of p53, which is described as a downstream target of p38 signaling pathways [44]). In contrast, another mechanism of action is likely in the p53-deficient and caspase-3-deficient cancer cell lines H1299 and MCF7 respectively [45; 46].

Next to DUSP15 our siRNA screens identify a number of other genes whose knockdown suffices to sensitize tumor cell lines with varying p53 status, including the protein kinase D subunit PRKCM, the E3 ubiquitin ligase ARIH1 and the ribosomal protein Rpl7l1 [47; 48; 49].

In contrast to the drastic effect observed in ES cells, knockdown of p53 itself does not affect survival in any of the tested cancer cells lines, with the exception of the human liver cancer cell line HepG2 **(Chapter VI).**

Developmental pathways modulate the ES cell response to DNA damage

Pathway analyses on our high-throughput datasets of cisplatin treated ES cells, indicates the involvement of developmental pathways, as modulators of DNA damage-induced cell killing in ES cells. These pathways include, Notch-, Hedgehog, TGF β/ BMP and Wnt/β-Catenin **(Chapter III & IV)**. After further validation experiments special emphasis can be attributed to the Wnt/ β-Catenin pathway in regulating ES cell survival in the presence of genotoxic stress **(Chapter III)**.

Wnt signaling crucially controls many cellular functions ranging from developmental processes and regulation of self renewal and differentiation capacity of ES cells and adult stem cells, to cancer progression and cancer stem cell biology [50; 51]. Integration of different omics datasets into a combined Wnt/ β-Catenin signaling network enables us to identify key molecules within the pathway to be regulated on transcriptional and posttranslational level, including the upregulation of mRNA of different Wnt ligands, as well as downregulation of negative regulators such as the Tcf transcription factors Tcf7L1 and Tcf7L2, and the kinases Gsk3β and Csnk1a1 [50; 51; 52] **(Chapter III)**.

Testing the hypothesis made by the integrated Wnt/ β-Catenin signaling network using a Tcf/Lef-responsive Luciferase reporter system, we confirm an increase in Wnt signaling after treatment with different genotoxic compounds, but not by non-genotoxic stresses. We furthermore show that enhanced Wnt/ β-Catenin signaling protects ES cells against cisplatin-induced apoptosis. Examining the effect of the knockdown of different molecules within the integrated Wnt/ β-Catenin signaling network on survival and Wnt reporter response we identify the kinase Csnk1a1 as a key player in the cisplatin-mediated Wnt response. Csnk1a1, which is downregulated on the transcriptional level upon cisplatin treatment, is implied as a strong negative regulator of Wnt signaling and Csnk1a1 silencing counteracts the cisplatin-induced apoptotic response in a p53-independent fashion. Csnk1a1 has been previously identified as a tumor suppressor in melanoma and colon cancer [52; 53]. Going beyond its role as a tumor suppressor, we can further link Csnk1a1-mediated effects on Wnt signaling to the response to genotoxic stresses.

This role of the Wnt signaling pathway in mediating chemoresistance may be particularly interesting in the context of cancer stem cells. Cancer stem cells or tumor initiating cells have been implicated in tumor recurrence and are believed to be more resistant to chemotherapy and γ-radiation than the bulk of the tumor. Different mechanisms have been suggested for this (chemo/radio) resistance phenotype

of cancer stem cells, including upregulation of ATP-binding drug transporters, upregulation of DNA damage signaling and repair, as well as involvement of survival pathways such as PI3K Akt signaling, but also developmental pathways such as Wnt signaling [54; 55; 56; 57]. Our findings of Wnt-mediated survival of ES cells after DNA damage provide an interesting link for the therapy responses of cancer stem cells, which share common features with ES cells [15].

Changes in housekeeping functions after DNA damage

Cellular housekeeping functions (e.g. DNA replication, transcription and translation) have to be adjusted under conditions of stress, such as hypoxia, starvation or genotoxic stress, generally including a repression of normal cellular processes and an increase in stress specific programs [58; 59; 60].

Many of our results indicate that cisplatin induces clear changes in housekeeping functions. These include rearrangements in energy, nucleotide and aminoacid metabolism, translational block and, although this evidence is more indirect, a transcriptional block **(Chapter IV & V).** The transcription factor siRNA library contains a number of siRNA targeting genes, which had been associated with transcription, including the transcriptional repressor DMAP1, whose knockdown sensitized ES cells and cancer cells to cisplatin **(Chapter III & VI)**. While DMAP1 has been shown to repress transcription, via recruitment of DMNT1 it has been also implicated in chromatin rearrangements, which are necessary for the recruitment of DNA repair factors such as ATM [61; 62]. Both mechanisms might be responsible for the sensitization induced by knockdown of DMAP1.

The E3 ligase ARIH1 induces repression of translation by modulating 4ehp cap-binding

Under conditions of stress, cap-dependent mRNA translation is repressed, often by the modulation of abundance or negative inhibition of the cap binding protein eukaryotic translation initiation factor eIF4E [59; 63]. In different cases it has been shown that this general translation block favors the translation of IRES containing transcripts, which has for example been described for p53 [64]. However, those repressive mechanisms are frequently disabled in cancer cells and the increase in translation contributes to the enhanced growth requirements of tumors [63].

In our siRNA screens we identify the E3 ubiquitin ligase ARIH1, whose knockdown sensitizes ES cells and cancer cells to different genotoxic stresses, independent of their p53 or caspase-3 status **(Chapter IV & VI).** ARIH1 protein levels accumulate after genotoxic stress, leading to enhanced interaction with 4ehp, a homologue of eIF4E that is not able to lead to ribosome recruitment and therefore acts as a negative regulator of cap-dependent translation [65]. We find that ARIH1 mediates the genotoxic stress-induced cap binding of 4ehp, inducing a translation block. In the absence of ARIH1, the cisplatin-induced translation block is attenuated leading to enhanced killing. Interestingly, this suggests that interfering with ARIH1 function may represent a manner to sensitize

cancer cells to therapy. It will be important to further unravel i) the mechanism of ARIH1 protein induction and ribosomal localization after DNA damage, ii) the manner in which ARIH1 regulates the cap binding of 4ehp, and iii) whether other, non-4ehp-mediated roles of ARIH1 are important in this respect **(Chapter IV)**.

Cisplatin-induced metabolic changes

Metabolomics studies are the newest development in the omics field with the promise to provide novel (potentially non-invasive) biomarkers for disease stage and therapy response [66]. However, variability of metabolomics profiles due to measurement settings and difficulties to detect certain metabolite groups, restrict the amount of qualitative and quantitative information [66; 67].

To identify metabolic networks affected by cisplatin we combine metabolomics with transcriptomics and integration with bioinformatics analysis tools **(Chapter V).** This allows identification of cisplatin responsive metabolic pathways, many of which have been previously linked to cancer formation and therapy response. Amongst those, we identify changes in proline metabolism. The aminoacid proline has been shown to counteract oxidative stress and its breakdown can yield electrons, which can be used both for proapoptotic ROS formation or prosurvival energy production [39]. Interestingly, enzymes functioning in proline synthesis, Pycr2 and breakdown pathways such as the p53 target genes, Prodh and Adh4a1 show elevated mRNA levels after cisplatin treatment. Knockdown of either Pycr2 or Prodh1 sensitizes ES cells to cisplatin treatment arguing against a proapoptotic role of proline metabolism (data not shown) **(Chapter V).**

CONCLUSIONS AND FUTURE PERSPECTIVES

In the presented studies we use systems integration of high-throughput datasets acquired in ES cells treated with the genotoxic drug cisplatin, to discover resistance mechanisms to genotoxic stress. The use of a systems approach and integration of different omics datasets into signaling networks in many cases allows us to narrow down large scale datasets and get to a testable hypothesis, finally leading to the identification of crucial signaling molecules in DDR signaling cascades.

We detect a number of genes, whose product protects both ES and cancer cells to cisplatin and other types of genotoxic stress. The fact, that knockdown of these genes sensitizes cancer cells of varying genetic backgrounds and cisplatin sensitivities suggests that these may represent interesting candidate drug targets.

Further research will be required to clarify the mechanism of action of ARIH1-mediated translation arrest, DNA damage-induced modulation of ARIH1 protein levels and subcellular localization, as well as the (p38-independent) mechanism of action of DUSP15, to potentially exploit inhibition of the targets of those genes for therapeutic intervention.

Our datasets shed light on the DDR in stem and cancer cells, and provide cisplatin response factors that might in future be translated into biomarkers for therapy response. Knockdown of Csnk1a1 enhanced cell survival in cisplatin-treated cells, via an upregulation of Wnt signaling, implicating a potential function of Csnk1a1 as a biomarker for therapy response. Studies of Csnk1a1 expression levels in tumors exposed to DNA damage inducing therapy can further advance this line of research.

Furthermore, modulation of the levels of small metabolites in response to DNA damage might serve as biomarkers, the feasibility of which will have to be tested by checking levels of identified metabolites such as 4-Methylcytidine in chemotherapy-treated patient samples.

We combine high-throughput datasets using pathway analysis and by identification of integrated signaling networks. Future (smaller scale) studies of the DDR in ES cells, might further allow to use mathematical modeling for describing the significance of specific aspects of DNA damage-induced cellular responses (such as DNA repair, Wnt signaling, translation arrest) for the sensitivity to genotoxic stress.

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