Signaling pathways and novel genetic factors involved in modulation of cisplatin response in *C. elegans*

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To my family

‘Had my compatriot Marie Sklodowska-Curie never said to herself
“I don’t know”, she probably would have wound up teaching chemistry
at some private high school for young ladies from good families.’

‘Gdyby moja rodaczka Maria Skłodowska-Curie nie powiedziała sobie
“nie wiem”, zostałaby pewnie nauczycielką chemii na pensji dla
panienek z dobrych domów, i na tej ń skądinąd zacnej ń pracy
upłynęłoby jej życie.’

Wisława Szymborska, Nobel Lecture, December 7, 1996

Abstract

Cancer is a major cause of mortality throughout the world. Despite the effort of the scientific community, plenty remains to understand about the biology or genetics of cancer. In solid tumors often as many as 90-99% of all cells are non-dividing. Therefore, it is challenging to fully understand the therapeutic effect of the drug using *in vivo* models where cells grow rapidly. Cisplatin is one of the most widely used chemotherapeutics known to treat different types of solid tumors. However, because of its primary ability to bind DNA and influence proliferating cells, its role in the non-dividing cells is often neglected. Nevertheless, a growing body of evidence shows that cisplatin has different cytoplasmic targets and can kill enucleated cells. Therefore, using *C. elegans* as a post-mitotic model we aimed to learn more about signaling pathways and genetic factors involved in the modulation of chemotherapeutic response on non-dividing cells. This thesis builds on our previous work showing that inactivation of ASNA-1 increases cisplatin sensitivity in *C. elegans* and reveals a previously undescribed impact of cisplatin in post-mitotic cells. More specifically, we have discovered a new mechanism by which cisplatin-induced ROS generation inactivates the cisplatin response function of ASNA-1 via its oxidation, which in turn perturbs the targeting of a tail-anchored protein to the endoplasmic reticulum membrane. This allowed us to separate clinically relevant ASNA-1 function in cisplatin sensitivity from insulin signaling. Next, analysis of tissue and genetic requirements of ASNA-1 allowed us to separate protein functions even further with a focus on growth, reproduction, and cisplatin response. Lastly, we showed that the PMK-1–ATF-7-regulated immunity pathway is required for cisplatin resistance and identified immune effectors as necessary for this response. In summary, using genetic and molecular analyses in *C. elegans*, we identified signaling pathways and novel genetic factors involved in the modulation of cisplatin response in post-mitotic cells with clear implications for strategies to refine and improve cisplatin cancer therapy.

Keywords
C. elegans, cisplatin, cancer, stress, immunity, post-mitotic, tail-anchored proteins

Sammanfattning på svenska

Cancer är den andra vanligaste dödsorsaken i världen och trots vetenskapliga framsteg återstår mycket för att förstå cancerns biologi eller genetik. I solida tumörer kan 90-99% av alla celler vara icke-delande. Därför är det utmanande att fullt ut förstå den terapeutiska effekten av läkemedel med hjälp av *in vivo* - modeller där celldelningstakten är hög. Cisplatin är ett av de mest använda cellgifterna vid behandling av solida tumörer. På grund av cisplatins förmåga att binda till DNA och påverka prolifiererande celler försummas ofta dess roll i de icke-delande cellerna. En ökande mängd data pekar på att cisplatin har olika cytoplasmatiska mål. Därför använder vi masken *C. elegans* som en post-mitotisk modell för att förstå hur signalvägar och genetiska faktorer är involverade i moduleringen av cisplatins effekter på icke-delande celler. Denna avhandling baseras på våra tidigare fynd att inaktivering av ASNA-1 ökar cisplatinkänsligheten hos *C. elegans* och avslöjar en tidigare okänd effekt av cisplatin i post-mitotiska celler. Vi har upptäckt att cisplatininducerade fria syreradikaler inaktiverar ASNA-1 roll för cisplatinresistens via ASNA-1 oxidation och blockering av SEC-61β, ett svansförankrat protein, till endoplasmatiskt retikulum. Detta är kliniskt relevant eftersom vi då kan särskilja ASNA-1 roll för cisplatinresistens från dess roll vid inslinsignalering. Vidare har vi visat att den PMK-1-ATF-7-reglerade signalvägen för immunitet även krävs för cisplatinresistens och vi har identifierat involverade immuneffektorer.

Sammanfattningsvis har vi med hjälp av genetiska och molekylära analyser i *C. elegans*, identifierat signalvägar och nya genetiska faktorer som är involverade i modulering av cisplatinsvar i post-mitotiska celler. Det möjliggör strategier för att förfina och förbättra cancerbehandling med cisplatin.

Streszenie po polsku

Rak jest główną przyczyną śmiertelności na całym świecie. W guzach litych często aż 90-99% wszystkich komórek stanowią komórki niedzielące się. Dlatego też wyzwaniem jest pełne zrozumienie działania leku przy użyciu modeli *in vivo*, w których komórki szybko się namnażają. Cisplatyna jest jednym z najczęściej stosowanych chemioterapeutyków znanych z leczenia różnych typów nowotworów. Jednak ze względu na jej zdolność do wiązania DNA i wpływania na proliferujące komórki, jej rola w niedzielących się (post-mitotycznych) komórkach jest często pomijana. Niemniej jednak coraz więcej dowodów wskazuje, że cisplatyna ma różne cele poza DNA, może zabijać komórki pozbawione jąder i wpływać na post-mitotyczne organy takie jak nerki czy neurony. Dlatego, używając *Caenorhabditis elegans* jako modelu post-mitotycznego, chcieliśmy dowiedzieć się więcej o szlakach sygnałowych i czynnikach genetycznych zaangażowanych w modulację odpowiedzi chemioterapeutycznej na niedzielących się komórkach. Bazą do tej tezy były wcześniejsze badania pokazujące, że inaktywacja genu *asna-1* zwiększa wrażliwość na cisplatynę u *C. elegans*. Ta teza ujawnia wcześniej nieopisany wpływ cisplatyny na komórki postmitotyczne. Dokładniej, odkryliśmy nowy mechanizm, w którym cisplatyna powoduje wytwarzanie reaktywnych form tlenu co prowadzi to zmiany funkcji białka ASNA-1 poprzez jego utlenianie i w konsekwencji zakłóca wkładanie specjalnej klasy białek do błony siateczki śródplazmatycznej. To odkrycie pozwoliło nam oddzielić dwie ważne funkcje białka ASNA-1 na tą która ma role w odpowiedzi na cisplatynę oraz tą która odpowiada za kontrolowanie sekrecji insuliny w komórkach. Następnie analiza pojedynczych mutacji aminokwasów w ASNA-1 pozwoliła nam jeszcze bardziej rozdzielić funkcje ASNA-1, z naciskiem na wzrost, reprodukcję i odpowiedź na cisplatynę. Na koniec wykazaliśmy istotną role szlaku odporności wrodzonej na powstawanie oporności na chemioterapie oraz zidentyfikowaliśmy efektory immunologiczne niezbędne do tej odpowiedzi. Podsumowując, wykorzystując analizy genetyczne i molekularne u *C. elegans*, zidentyfikowaliśmy szlaki sygnałowe i czynniki genetyczne zaangażowane w kontrole odpowiedzi na cisplatynę w komórkach niedzielących się, z wyraźnymi zastosowaniami prowadzącymi do udoskonalania i ulepszania terapii przeciwnowotworowej.

List of papers

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Raj D***, Billing O*, Podraza-Farhanieh A*, Kraish B, Hemmingsson O, Kao G, Naredi P.
*Equal contribution

Alternative redox forms of ASNA-1 separate insulin signaling from tail-anchored protein targeting and cisplatin resistance in *C. elegans*

Scientific Reports 2021; 11(1): 8678.

II. **Raj D**, Podraza-Farhanieh A, Kao G, Naredi P.

Analysis of tissue and genetic requirements of ASNA-1 for growth, reproduction and cisplatin response in *C. elegans*

Manuscript

III. **Raj D**, Kraish B, Martikainen J, Kao G, Naredi P.

The innate immune system promotes cisplatin chemoresistance in post-mitotic *C. elegans* via activation of the p38/MAPK pathway

Manuscript under revision

Publications not included in the thesis:

Podraza-Farhanieh A, Natarajan B, **Raj D**, Kao G, Naredi P.

ENPL-1, the *C. elegans* homolog of GRP94, promotes insulin secretion via regulation of proinsulin processing and maturation

Development 2020; 147(20): dev190082

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Abbreviations

ASNA	Arsenite-translocating ATPase family
CSC	Cancer stem cell
DAMP	Damage-associated molecular pattern
DIC	Differential interference contrast
DTC	Distal tip cell
EMC	ER membrane complex
EndoH	Endoglycosidase H
ER	Endoplasmic reticulum
FDA	Food and Drug Administration
GET	Guided entry of tail-anchored proteins
GFP	Green fluorescent protein
H_2DCFDA	2,7- dichlorodihydrofluorescein diacetate
IIS	Insulin/IGF-1 signaling
mtDNA	Mitochondrial DNA
NGM	Nematode growth media
MAPK	Mitogen-Activated Protein Kinase
PRP	Pattern recognition receptor
qPCR	Quantitative polymerase chain reaction
ROS	Reactive oxidative species
SND	SRP-independent targeting
SQS	Squalene synthase
SRP	Signal recognition particle
TAP	Tail-anchored protein
TF	Transcription factor
TMD	Transmembrane domain
TRC40	Transmembrane domain recognition complex 40 kDa
TPR	Tetratricopeptide repeat
Ubl	Ubiquitin-like

Introduction

Cancer

Cancer is one of the greatest challenges to global health nowadays. It is the second leading cause of death in the world, accounting for an estimated 9.5 million deaths in 2018 (1) and it continues to be a worldwide killer despite the enormous amount of research and quick progress in drug development. It is estimated that by 2030 the cancer-related deaths will rise to 13 million (1).

Cancer results from the breakdown of the regulatory mechanisms in proliferation, differentiation, and survival that oversee normal cell behavior. This regulation is lost in cancer cells that grow and divide uncontrollably and ultimately it leads to the spreading of cancer cells throughout the body and interferes with the functions of normal tissues and organs. However, there is a growing body of evidence showing that solid tumor contains both types of cells: dividing and non-dividing. This theory is supported by experiments showing that the doubling time of many tumor types is much longer than seen *in vitro* or even in the animal model (2). Experiments have also shown that the mean labeling index measured by radiolabeled imaging is often very low in the tumor (3) indicating that only a very small amount of tumor cells are dividing with a mitotic index often lower than 1% (4).

Models of cancer development

Cancer results from abnormal proliferation of different types of cells in the body giving rise to more than one hundred different types of cancer varying substantially in behavior and response to treatment. There are currently two models of cancer development: (i) clonal evolution and (ii) development of cancer stem cell (CSC).

The clonal model was first proposed by Peter Nowell in 1976 and suggests that initial DNA damage in the cell will lead to benign tumor growth and over time with the accumulation of heritable genetic and epigenetic changes this will lead to a transformation of normal cells into a lineage of malignant cells (5,6). The single-cell mutational analysis has provided sufficient examples of the subclonal segregation of mutations (7,8).

The cancer stem cell model suggests that cancer growth and progression are driven by a small subpopulation of cancer stem cells (CSCs) (9,10). The ability

of CSCs to self-renew by mitotic division with at least one progeny cell maintaining ability to multiply provides successful survival and proliferation of CSCs and creates the subpopulation of cells within a tumor responsible for the progression of malignancy (11). Consistent with this model, some malignancies including germ cell cancer (12) and leukemias (13,14) has been known to contain neoplastic cells that differentiate into post-mitotic byproducts and later hierarchically organized cancers.

Cisplatin

Cisplatin is a platinum-based chemotherapeutic drug widely used for cancer treatment. It was first synthesized in 1844 by M. Peyrone but become popular in 1960s when B. Rosenberg created much interest in the possible use of cisplatin in cancer therapy (15). It was the first FDA-approved platinum compound for cancer treatment and it is still commonly used in the clinic. Cisplatin is used to combat different types of malignancies including sarcomas and carcinomas with relatively high treatment efficiency (16,17).

Mode of action

Cisplatin is composed of a doubly charged platinum ion surrounded by four ligands: amine ligands on the left and chloride ligands on the right, which allow forming of bonds with DNA. Nevertheless, for the interaction to occur, cisplatin has to be activated by series of aquation reactions, which involve the replacement of chloride ligands with molecules of water (18). Activated cisplatin can efficiently bind to different molecules including nucleic acids, amino acids, peptides, and proteins (18).

It is accepted that the major mechanism of cisplatin action is binding of platinum to DNA and forming intra- and inter-stranded crosslinks. This leads to cell cycle arrest and eventually apoptosis in fast proliferating cells (17,18). However, only 1-10% of intracellular cisplatin can be found in the nucleus, which in consequence will lead to DNA damage in proliferating cells (19–21). Other studies have suggested that cisplatin could have other cellular targets beyond nuclear DNA: mitochondria, endoplasmic reticulum, or cytoplasm (22). Revealing the cellular pathways influenced by cisplatin could provide important information for the design of new cancer treatment strategies targeting slow-cycling or non-dividing cells.

Cisplatin cellular targets and binding sites

Solid tumors contain both rapidly dividing and non-dividing cells whereas non-dividing cells can account for as much as 90-99% of the tumor mass (23). Moreover, cisplatin exposure leads to damage of post-mitotic cells and results in toxicity in tissues like sensory neurons, kidneys, and ear (24–26). This led to speculation that cisplatin must have mechanism of action other than targeting of DNA and alternative effects of cisplatin might play a role in good therapeutic response.

A broad body of evidence shows that mitochondrial DNA (mtDNA) is one of the cisplatin cellular targets and indeed the number of adducts between cisplatin in mtDNA is higher than in nuclear DNA (27–29). Cisplatin treatment also leads to serious mtDNA damage which results in delayed tumor growth (30). Another key cisplatin interactor is the endoplasmic reticulum (ER). It has been shown that cisplatin can kill enucleated cells through activation of ER-specific caspase-12 (19,31). Moreover, cisplatin can also strongly influence cytoskeleton (32,33), and binds to proteins such as hemoglobin, transferrin, and metallothionein (34–36).

This broad list of cellular targets and in consequence pathways influenced by cisplatin might provide us with some vital clues for designing novel cancer treatment strategies and finding new potential targets for cisplatin-based chemotherapy. The effect of cisplatin in the context of the whole body is still weakly studied and global omics studies can give a better understanding of the non-DNA binding sites and action of cisplatin in non-dividing cells.

Mechanism of resistance

Despite the high drug activity in the treatment of many solid tumors, the drug resistance of tumor cells has decreased the clinical utility of cisplatin (37). The initial response to cisplatin treatment is often quenched and other non-platinum-based therapies need to be used. There are two forms of resistance, innate resistance developed without any previous drug exposure and acquired resistance as an effect of drug exposure (37). The cisplatin resistance in dividing cells occurs via increased DNA repair, altered cellular accumulation and/or increased drug inactivation. The mechanism of resistance depends on the severity of resistance and those three mechanisms are not mutually exclusive (38–41). Studies unraveling the mechanism of cisplatin resistance have huge potential to generate better responses in the clinic.

Caenorhabditis elegans

C. elegans is a free-living nematode first studied by Victor Nigon and Ellsworth Dougherty but re-introduced to the world of science as model organism by Sydney Brenner (42). Nematodes exist in two forms: as self-fertilizing hermaphrodites and cross-fertilizing males. They are characterized by a short generation cycle of ~3.5 days which involves an embryonic stage, four larval stages, and an adult stage (Figure 1a). Adult *C. elegans* worms do not undergo any somatic cell divisions. A wild-type hermaphrodite produces approximately 300 progeny in its short life cycle of 2-3 weeks under favorable conditions. Hermaphrodites and males contain a fixed number of 959 and 1031 somatic cells respectively and the size of the worm oscillates around 1 mm when reaching adulthood. *C. elegans* was also the first multicellular organism with a fully sequenced genome (43).

Straightforward and well-established forward and reverse genetics approaches have allowed for comprehensive analysis of genetic pathways and protein function in *C. elegans* (44). Notably, nematodes homologs have been identified for many (60-80%) of human genes and numerous biological processes are conserved between humans and *C. elegans*. These features make worms a perfect model for studies of genes and pathways involved in various diseases, including neurodegeneration and cancer. The nematode is also a popular model organism thanks to the availability of excellent tools in molecular genetics and cell biology which allows us to model and understand processes relevant for humans. Recent development in CRISPR/Cas9 gene-editing techniques allow for precision modified alleles in *C. elegans* (45–48), in particular insertions or deletions of specific sequences, modification of single bases and even replacement of worm genes with their human orthologs. Modeling diseases in simple invertebrates has also the advantage of dissecting complex molecular pathways giving a valuable insight into disease mechanism.

There is a growing body of research showing that genes and pathways involved in cancer development and progression are highly conserved in *C. elegans*. The advantage of modeling these pathways in nematodes comes from the fact that gene families involved contain fewer members consequently reducing likelihood of the genetic redundancy. For example, pRb and p53 tumor suppressors, where each contains three members in the mammalian family whereas *C. elegans* contains only a single member of each family, LIN-35/pRb and CEP-1/p53, correspondingly (49,50).

As mentioned above, somatic cells in adult *C. elegans* animals are purely post-mitotic. Therefore, taking advantage of the simplicity of this *in vivo* model, in this thesis *C. elegans* adult animals were used to uncover the response of post-mitotic cells to the widely used chemotherapeutic drug cisplatin.

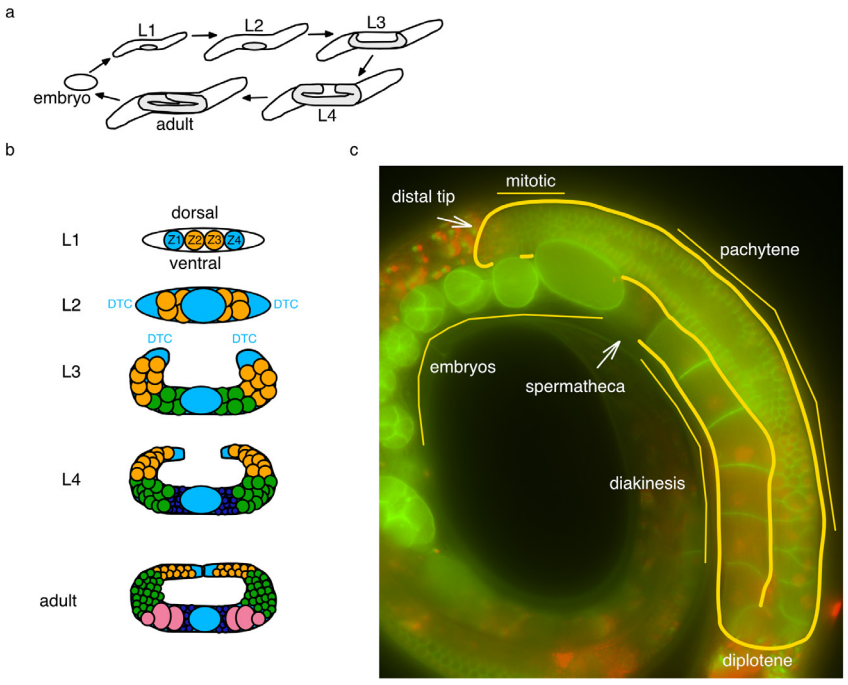

Figure 1 C. elegans life cycle and germline development. *(a) The nematode life cycle is characterized embryonic state, four larval stages (L1-L4), and an adult stage, in which all somatic cells are post-mitotic. At each stage, the developing gonad is indicated in grey. (b) A drawing of germline development within the gonad (underrepresented cell counts from L2 onwards). Gonadogenesis begins at L1 stage with four gonad precursors: Z2 and Z3, the germline progenitors colored in orange, in between the somatic Z1 and Z4 (pale blue), which will divide and form distal tip cells (DTCs) and somatic gonad cells. Central oval represents multiple cells, sheath cells were omitted in the drawing for clarity. Germ cells color code: meiotic cells in green, mitotic cells in orange, sperm in dark blue, and oocytes in pink. Adapted from* (51)*. (c) Fluorescence image of C. elegans adult hermaphrodite gonad with visualization of plasma membrane in green and chromatin in red. U-shaped gonad arm is outlined in yellow. Stages of meiotic Prophase I (pachytene, diplotene and diakiesis) are marked in the gonad.*

Hermaphrodite gonadogenesis

Gonadogenesis of *C. elegans* is a highly regulated and determined process taking place during post-embryonic development. The process begins in L1 larvae with four gonad precursor cells: two primordial germ cells (Z2 and Z3) inserted between two somatic gonad precursors (Z1 and Z4) and surrounded by a basal lamina (Figure 1b). The proliferation of Z2 and Z3 begins mid-L1 and gives rise to germ cells within the gonad. Z1 and Z4 also begin to proliferate

giving rise to two distal tip cells (DTCs) on each side of the gonad and ten cells that form the hermaphrodite somatic gonad primordium (52). DTCs on each side of hermaphrodite gonads play two important roles. One, as a migratory driver that gives rise to the U-shape of each gonad arm. Second, a signaling role to promote the proliferative germ cell fate (53). Ten cells, which give rise to somatic gonad primordium, will further create the somatic gonadal sheath cells, the spermatheca, and the uterus of hermaphrodite.

At the L4 stage the gonad of hermaphrodite starts to look like the gonad of an adult. In the U-shaped gonadal tube capped with DTC, germ cells divide mitotically when located closer to DTC, while further located cells enter mitosis. Many more cells enter the meiotic prophase than become oocytes in the process of oogenesis. Germ cells progress from pachytene to diplotene followed by oocyte differentiation (Figure 1c). The proximal 4 or 5 oocytes are in diakinesis and the most proximal oocyte bordering the spermatheca undergoes meiotic maturation then is pushed through the spermatheca and fertilized (54). Therefore, oocyte maturation occurs only in the presence of sperm and fertilization initiates zygotic development and marks the end of the meiosis I.

p38 MAPK cascade

The p38 mitogen-activated protein kinase (MAPK) signaling cascade plays a vital role in the cell. Its ability to integrate external stimuli and elaborate suitable responses makes the pathway a key player in stress response as well as numerous other cellular responses. Other stimuli like inflammatory cytokines or non-stress activators also play a role in p38 MAPK activation.

The typical activation of MAPK cascade is based on a sequence of phosphorylation reactions where MAP kinase kinase kinases (MAPKKKs or MAP3Ks) activate MAP kinase kinases (MAPKKs or MAP2Ks) which in turn activate MAPKs like p38 (Figure 2). Most known MAPKK activating p38 are MKK3 and MKK6 (55,56). Four isoforms of p38 (α, β, γ, and δ) have been identified and they share a high degree of homology. Nevertheless, p38α is the founding member of the family with essential function in embryonic development (57,58). Extensive research in recent years has shown roles for p38α in tumor cells where it can both, facilitate and interfere with tumor development.

In breast cancer high p38 MAPK phosphorylation levels positively correlated with reduced breast tumorigenesis in Wip1-knockout mice and inhibition of p38 MAPK abolished this effect (59). On the contrary, some inhibitors of p38 MAPK reduced tumor growth (60,61). The dual role of p38α was shown in colon cancer where p38α-deficient intestinal epithelial cells are more inclined to

colon tumorigenesis but downregulation of p38α in colon cancer led to reduction of tumor bulk in mice (62) and reduced tumor growth in human colon cancer xenografts in mice (63). Another evidence for a role of p38α in tumor progression comes from studies showing an increase in phosphorylation in human lung tumor samples (64).

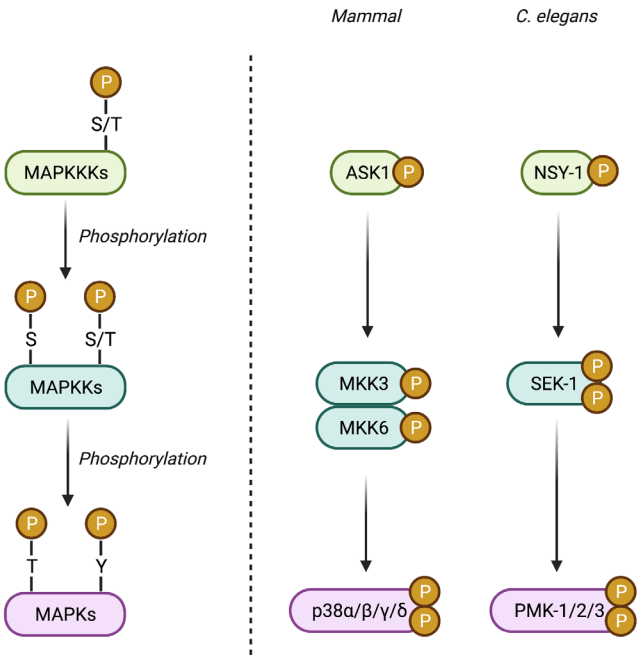

Figure 2. Simplified homologous p38 pathways in mammals and Caenorhabditis elegans. *In all MAPK cascades, MAPKs are activated by MAPKK- catalyzed phosphorylation of Tyr and Thr residues. MAPKKs are also activated by MAPKKKs-catalyzed phosphorylation of Ser/Thr residues. Adapted from* (65)*. Created with BioRender.com*

Dormancy

Interestingly, the p38 MAPK cascade also plays a role in the dormancy of cancer cells, which maybe is the reason for the resistance of many tumor types to chemotherapy. This survival mechanism of dormant cells often depends on sufficient p38 MAPK activity to induce growth arrest without activating apoptosis. During dormancy of T-cell acute lymphoblastic leukemia, the p38 activity is maintained at high level in order to keep cells quiescent but uncommitted to full differentiation (66). It is also true for squamous carcinoma cells, where activation of p38 promoted the survival of dormant tumor cells (67), as well as for

prostate cancer stem-like cells in the bone, where activation of p38 induced dormancy (68).

Chemotherapy

Based on recent discoveries, a mechanism was proposed where p38α can mediate an anti-neoplastic effect of some chemotherapeutic drugs. Cisplatin was able to induce p38α-driven apoptosis in a colon cancer cell line (69), rituximab induced p38α-driven apoptosis which contributed to the generation of the antileukemic effects (70), and inhibition of p38α reversed the growth-inhibitory effect of STI571 (imatinib mesylate) on leukemic cells (71). Additionally, inhibition of p38α in the mouse model had an additive effect to cisplatin in reducing the size of breast tumors (60). However, more *in vivo* studies confirming the p38α-mediated chemotherapeutic effect need to be performed to validate the results obtained in the cell lines.

Immune response

Regulation of the immune response by the p38 MAPK pathway has attracted much attention in the context of carcinogenesis. Tumor progression can be strongly modulated by immune cells secreting cytokines and chemokines and the p38 MAPK pathway regulates the production of many interleukins and cytokines. However, the details of the relationship between p38-mediated immune response and tumor development have not been characterized. Nevertheless, also in that case the p38 MAPK pathway can act as a double-edged sword. Inhibition of p38α results in enhanced inflammation-associated development of hepatocellular carcinoma in a mouse model (72) whereas other reports contradict the pro-inflammatory or pro-tumorigenic roles of p38 (73,74).

p38 MAPK pathway in *C. elegans*

Presence in *C. elegans* of the p38 MAPK pathway homologs (Figure 2) allows exploiting the power of genetics and to characterize this pathway *in vivo*. The *C. elegans* genome encodes three homologues for p38 MAPK: PMK-1 (64% identity to p38α), PMK-2 (56% identity), and PMK-3 (42% identity). As also seen in mammals, the *C. elegans* pathway is triggered by a variety of cellular responses and also plays a role in infection responses and environmental stress (75–78).

Innate immunity

The first investigation of the pathways involved in the innate immune response led to an association of the p38 MAPK signaling cascade in that process. The conserved pathway is composed of NSY-1-SEK-1-PMK-1 (77) (in mammals: ASK1-MKK3/6-p38) with TIR-1 (SARM in mammals) working upstream of the pathway (Figure 3) (79–81).

The TIR-1-NSY-1-SEK-1-PMK-1 pathway regulates innate immunity in the intestine (75) and epidermis (82,83). In the intestine, PMK-1 phosphorylation activates ATF-7, a member of basic-region leucine zipper (bZIP) transcription factor family orthologous to mammalian ATF2, which in turn regulates the expression of innate immune genes (76).

Oxidative stress

As mentioned, various environmental signals activate the conserved p38 MAPK pathway, including reactive oxygen species (ROS) (78). The canonical MAPK cascade consists of MAP3K-SEK-1-PMK-1, as in the case of the innate immunity pathway. However, the downstream effectors of the p38 pathway differ among stress responses, and instead of the ATF-7 transcription factor, the oxidative stress response triggers phosphorylation of the SKN-1 (human homolog of NRF2) transcription factor and its accumulation in the nucleus (Figure 3) (78). High levels of nuclear SKN-1 lead to the expression of phase II detoxification genes (78,84,85).

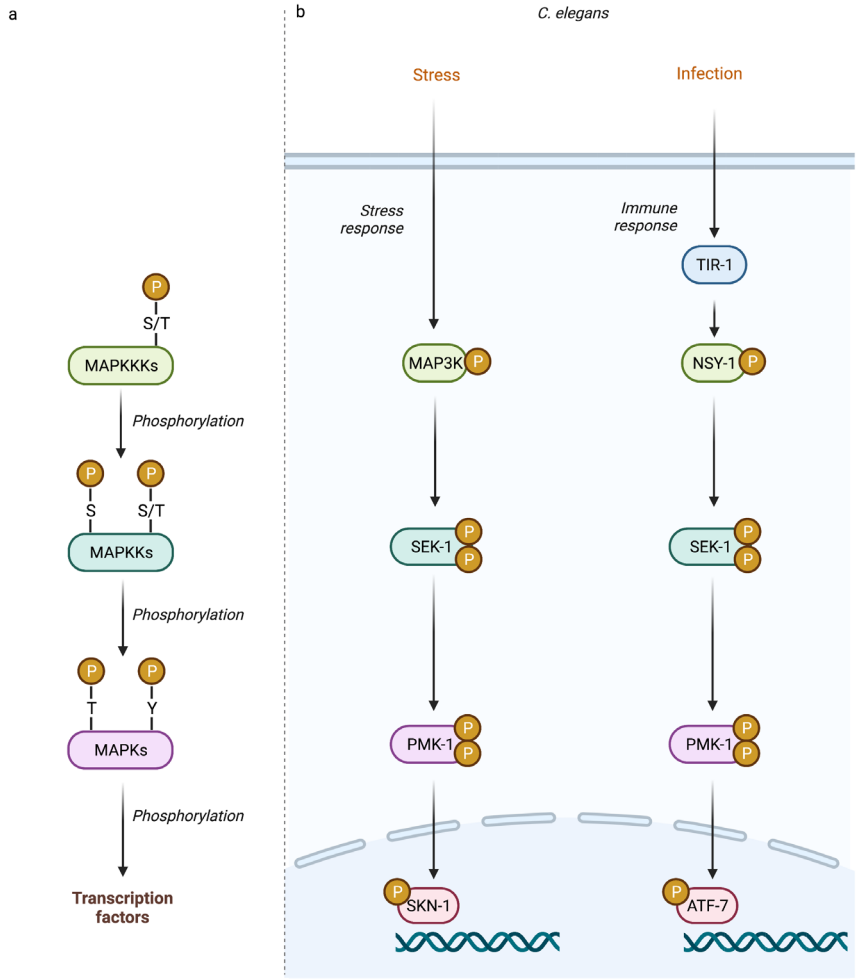

Figure 3. C. elegans p38 pathway activation in stress and immune response. *(a) Graphical representation of p38 cascade activation. (b) Differences between C. elegans p38 MAPK pathway in stress and immune response. Created with BioRender.com*

Targeting pathways of tail-anchored proteins

Tail anchored (TA) proteins

Tail-anchored proteins (TAPs) belong to a special class of polypeptides with N-terminal functional cytosolic region, followed by a single transmembrane domain (TMD) which allows for insertion into the lipid bilayer and short C-terminal luminal region no longer than 30 residues (86). Broad bioinformatic analysis across species has established that TAPs are required in multiply essential functions like membrane fusion, protein translocation, apoptosis regulation, and enzyme catalysis (87–90).

The membrane targeting sequence is placed at the C-terminal TMD region of the protein and it emerges from the ribosome only after termination of translation. Due to TA protein structure with a very short C-terminal region TAPs are destined for insertion into the multiple target membranes of peroxisomes, mitochondria, chloroplast, cytoplasm, or ER by post-translational pathways (91,92). The targeting destination is encoded by hydrophobicity and helical content of TMD (Figure 4) (88,93,94).

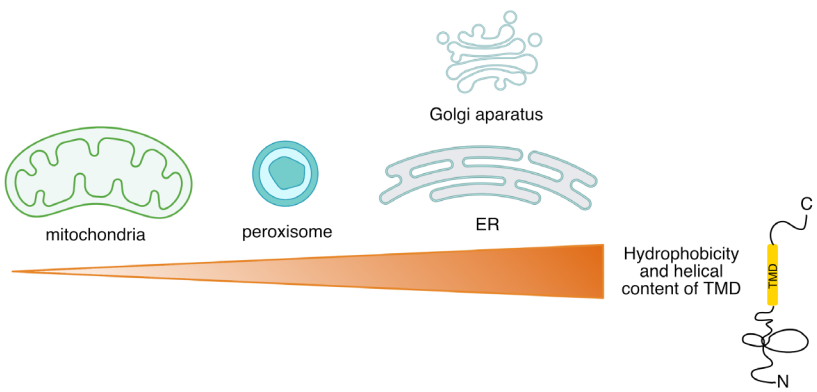

Figure 4. Simplified summary of TA protein destination based on the TMD hydrophobicity. *Insertion into the organelle is strongly influenced by the hydrophobicity and helical content of the TMD. Proteins with less hydrophobic TMD will be targeted into the outer membrane of mitochondria, moderate TMD hydrophobicity will cause peroxisomal targeting, while TAP with strong hydrophobic TMD will be targeted into the ER membrane and later into Golgi apparatus and plasma membrane if needed. Created with BioRender.com*

Insertion of TAPs into the ER membrane

As mentioned above, TAPs can be inserted into multiple membranes however, the ER membrane is a major destination for newly sensitized TA proteins. There are two main insertion pathways into the ER (i) unassisted for substrates with weak hydrophobic TMD and (ii) chaperone-mediated for energy-required TAPs insertion.

Unassisted insertion

Mammalian cytochrome b5 (b5) is one of the best understood examples of the TAP destined for unassisted insertion into the ER membrane. Analysis showed that none of the membrane or cytosolic proteins are necessary for the insertion of b5 TA protein into the ER membrane (95). The substitution of TMD between b5 and synaptobrevin 2 (Syb2), a known TAP inserted into the ER by a chaperone, allowed for an exchange of insertion pathway. The b5 was inserted into the ER phospholipid bilayer by assisted pathway while Syb2 was inserted in an unassisted manner (96). This revealed not only the importance of TMD hydrophobicity but allowed researchers to understand more about the mechanism of chaperone-mediated pathways.

Chaperone-mediated insertion

Comprehensive research over the years has characterized three chaperone-mediated pathways (i) SRP-iNDependent targeting (SND), (ii) ER membrane complex (EMC), (iii) Guided Entry of Tail-anchored proteins (GET).

SND forms an alternative targeting pathway and was first characterized in yeast (97). Sequence alignment described human TMEM208/SND2 as a homolog to yeast Snd2 (97,98) and subsequent studies its function in post-translational ER targeting of TAPs (99–102).

EMC is a big transmembrane protein complex first characterized in yeast (103) followed by characterization in mammals (104). Initially, research also in yeast showed its role in co-translational protein insertion (105). However, evidence followed separating its role in TAP insertion based on the cell-free system and squalene synthase (SQS) substrate insertion analysis (106). Authors postulated that the inability of TAP insertion in GET-dependent will engage the EMC pathway (106).

The GET pathway is a well-studied and very conserved pathway that targets relatively hydrophobic TAPs to the ER membrane. Initial identification of the GET pathway role in TAP insertion in yeast (107) was soon followed by identification of its homolog pathway in mammalian cells (108). Components of the yeast GET pathways have orthologues or functional homologs in mammalian cells, yeast, as well as in *C. elegans* (Table 1). Much of the work to understand

the mechanism of GET-dependent TAPs insertion has been done in the yeast model system. Therefore, this pathway with a focus on the yeast system will be more broadly discussed in the following section than its mammalian homolog.

Table 1. Components of the GET pathway with homologs in yeast, mammals, and C. elegans.

Function	Protein		
	Human	**Yeast**	***C. elegans***
Upstream chaperone	SGTA	Sgt2	SGT-1
Scaffolding complex	TRC35	Get4	CEE-1
	UBL4A[2]	Get5	
	BAG6[1]		ZK688.5
Cytosolic ATPase	ASNA1 (TRC40)	Get3	ASNA-1
Membrane receptors	WRB	Get1	WRB-1
	CAML[1, 2] (functional Get2 equivalent)	Get2	

[1]The two instances, in which yeast and mammalian homologs do not exist.

[2]The two instances, in which *C. elegans* and mammalian or yeast homologs do not exist.

Guided Entry of Tail-anchored proteins (GET) pathway

GET-dependent insertion is one of the best characterized pathways facilitating post-translational TAP-insertion with Get3 (in yeast) or TRC40/ASNA1 (in mammalian cells) playing a central role in the pathway in bringing client proteins in the cytosol and releasing them to the ER membrane for insertion. A simplified model for a TAPs handling and insertion by the yeast GET pathway is shown in Figure 5.

GET pathway steps might be divided into two parts: (i) pre-targeting steps where TAP is captured by Sgt2 and transferred to Get3, and (ii) membrane-associated steps where Get3-TAP complex is targeted to the Get1/2 complex at the ER, TAP is released and inserted followed by Get3 recycle (109).

Get3, an ATPase which exists as a functional homodimer, is a central player of the GET pathway. It is composed of two identical subunits linked by tightly coordinated zinc ion, each subunit with two coupled regions: an ATPase part and an α-helical region involved in TAP binding. Get3 can adopt different conformations in the TAP targeting cycle ranging from 'closed', through 'loose' to 'open'. The interactions with different ligands dictate the conformational changes of Get3 to coordinate TAP binding and release cycle. Get3 does not associate with the ribosome. Therefore, the pre-targeting complex is formed to avoid protein aggregation and shield the TAP (103). This allows for capturing and handing of TAP to Get3.

First, Sgt2 captures the protein via interactions with its methionine-rich hydrophobic domain (Figure 5, step 1). It has been recently shown that cytosolic Hsp70 (Ssa1) can more efficiently bind to TAPs than Sgt2 (110) and interacts with the tetratricopeptide repeat (TPR) domain of Sgt2 (111). This raises the possibility that at least for some of the TAPs, Hsp70 is the first player of the GET cascade (112,113).

Next, a pre-targeting complex is formed to transfer the TAP to Get3 (112). It is a heterotetrametric complex with two copies of dimerizing protein Get5, which contains ubiquitin-like (Ubl) domain, and two copies of the protein Get4. Sgt2 interacts via its N-terminal domain with the Ubl domain of Get5 in the pre-targeting complex (114–116). The Get4/5 binds to ATP-bound Get3 in a 'closed' state (117) and brings Sgt2-TAP complex into proximity to facilitate protected transfer (118,119) (Figure 5, step 2). Once the transfer is completed, the Get3-TAP complex dissociates from Get4/5, due to ATP hydrolysis and TAP binding (120), and changes its conformation from 'closed' to 'loose'. ATP hydrolysis is a necessary process before Get3 can release its cargo (121–123).

The third step is membrane targeting and occurs after the TAP is loaded onto Get3 (Figure 5, step3). The Get3-TAP complex is targeted onto the ER transmembrane complex Get1/2 (121–123). In the current membrane targeting model membrane insertase, Get1/2 is a heterotetramer, which forms upon Get3 binding (124). Combining cryo-electron microscopy reconstructions and native mass spectrometry of *Saccharomyces cerevisiae* Get1/Get2/Get3 and human WRB/CAML/TRC40 complexes McDowell *et at*. (2020) elegantly showed that Get2/CAML as a heterodimer captures Get3/TRC40-TAP complex in a 'loose' state. The handover of the Get3/TRC40-TAP complex from Get2/CAML to Get1/WRB activates the opening of Get3/TRC40 and the formation of the heterotetrametric membrane complex (Get1/Get2 in yeast or WRB/CAML in humans). Afterwards, the C-terminus of TAP interacts with the Get1/WRB, which allows for release and membrane insertion of TAP (124) (Figure 5, step 4).

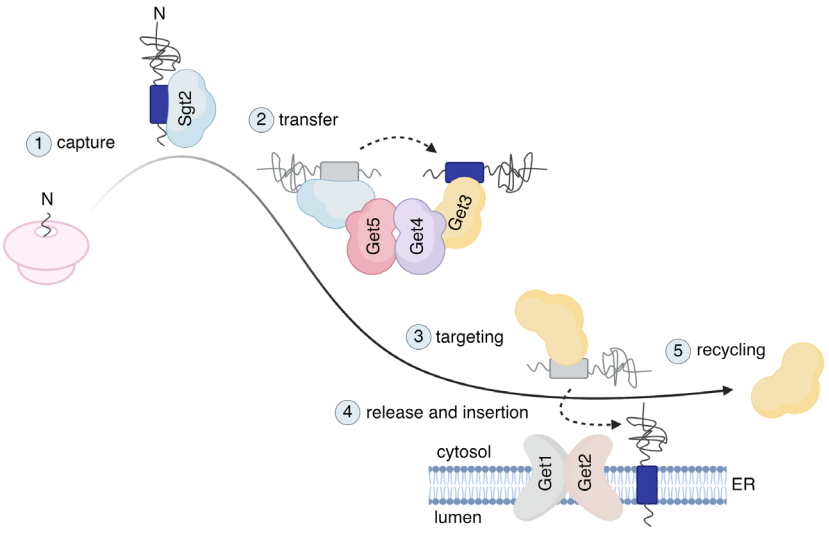

Figure 5. GET pathway overview. *A simplified model of TAP insertion into the ER membrane by the yeast GET pathway. See text for details. Adapted from* (109). *Created with BioRender.com*

Afterward, Get3 in the 'open' state disassociates from the Get1/2 complex and is recycled to the cytosol, ready for another round of substrate loading (Figure 5, step 5).

The GET-dependent TAP insertion in humans differs slightly from the yeast pathway. The main difference is the scaffolding complex which in humans contains three proteins: TRC35 (Get4 homolog), UBL4A (Get5 homolog), and a unique subunit, BAG6 (125) (Table 1). In contrast to the yeast complex, TRC35 and UBL4A are present in a monomeric state and instead of directly interacting with each other, they bind to the C-terminal motif of BAG6 (126,127). A TAP that fails to engage with TRC40 instead interacts with BAG6 and is send for degradation (128).

As mentioned above, the *C. elegans* homolog of Get3 exists and particular components of the GET pathway have homologs in the worm (Table 1). However, depside fast progress in defining the molecular details of TAP insertion, this pathway has yet not been characterized in *C. elegans* leaving the important questions about tail-anchored protein biogenesis by the GET pathway in worms still unanswered.

ASNA-1 and its homologs

A redox-regulated dual function protein Get3

The embryonic lethality phenotype of mice lacking TRC40 (129) made it impossible to study the consequences of the knockdown on the organismal level. Interestingly, yeast knockdown of Get3 did not reproduce the lethality phenotype (130,131) but caused several other phenotypes like copper, hygromycin and H_2O_2 sensitivity, heat sensitivity, and lack of growth in iron-limited media (107,130). This raised the important question: are all of those phenotypes a consequence of TAP insertion deficiency or there is another role of Get3/TRC40 in maintaining metal homeostasis and oxidative stress?

Get3 contains a pair of highly conserved cysteines coordinated by zinc in positions 285 and 288 (C285/C288), which are essential for dimer formation and growth (131). The presence of this motif resembles the oxidation-sensitive zinc-binding motif located in *Hsp33*. *Escherichia coli* heat shock protein *Hsp33* is an ATP-dependent and redox-regulated chaperone, which inhibits oxidative stress mediated protein aggregation and increases bacterial stress resistance by zinc release and disulfide bond formation (132). Similarly, Get3 has been found to act as an ATP-independent general chaperone *in vitro* with a role in protection against aggregation of unfolding proteins (133). Similarities between *E. coli* heat shock protein *Hsp33* and Get3 as well as the discovery of the holdase function of Get3 fueled further research. Voth *et al*. (2014) elegantly showed that Get3 functions as an ATP-independent chaperone under oxidative stress conditions. Incubation of Get3 with oxidative stress generator H_2O_2 reversibly switched protein function from TAP-binding into general chaperone holdase function in ATP-depleted condition (134). Moreover, the change in the function was coupled with massive conformational rearrangements and showed that Get3 in tetrameric or higher oligomeric form drives the holdase function of the protein (134).

These results indicate that Get3 is a redox-sensitive dual function protein: under non-stress conditions, it plays role in TAPs targeting while under ATP-depleted oxidative stress conditions it changes shape and acts as a holdase chaperone to prevent protein aggregation (135). Unfortunately, little is known about the precise activation mechanism of these rearrangements in yeast and mammals and future research should focus on answering these questions. This extensive research although shed a light on possible Get3 function separation based on the oxidative state of the protein.

Mammalian Asna1/TRC40 and its functions

ASNA1, often referred to as Transmembrane domain recognition complex 40 kDa (TRC40), is an essential cytosolic ATPase evolutionarily linked to the bacterial arsenite transport factor ArsA (136) and suggests that it is rather a bacterial paralogue rather than orthologue (137). The mammalian homolog shares only 27% identity with *E. coli* ArsA (129) and although both are involved in metal resistance, mammalian ASNA1 has acquired additional functions and became an interesting and clinically relevant target.

The mammalian homolog ASNA1 plays an essential role in cancer cell survival and cisplatin resistance. Work in our lab showed that cisplatin-resistant cancer cell lines overexpress ASNA1 (137,138) while downregulation of ASNA1 in tumor cells leads to apoptosis and increased cisplatin and arsenite sensitivity (138,139). This stands in agreement with first observations where cisplatin-resistant cells are cross-resistant to arsenite and antimonite (140,141). These studies classified Asna1 as a very interesting potential target for platinum-based treatment to prevent failure due to resistance.

Another interesting function of ASNA1 is its involvement in insulin secretion. We showed that ASNA1 is expressed in β-cell cells of the pancreas, which are the main site for insulin secretion. Overexpression of ASNA1 in insulinoma cell lines resulted in increased levels of insulin secretion and in the opposite manner, decreased levels of ASNA1 expression led to a decrease in insulin secretion (142). This led to the conclusion that ASNA1 is rather involved in insulin secretion than production. Further, it has been shown that *Asna1* controls β-cell function and ER homeostasis via retrograde transport regulation. *Asna1* pancreatic β-cell knockdown in mice led to hypoinsulinemia, impaired insulin secretion, and glucose intolerance that rapidly developed into diabetes (143). Moreover, *Asna1* is needed for pancreatic progenitor cell survival and knockdown of *Asna1* in pancreatic progenitor cells resulted in its developmental failure (144).

Furthermore, *Asna1* plays an important role in disease prediction and modeling. It has been characterized as an important biomarker in treatment response in schizophrenia (145), differentiation between subjects with active and latent tuberculosis (146), predicting disease severity of dengue virus infection (147), a biomarker for abnormalities associated with ultra-high-risk for psychosis (148), Downs syndrome (149), amyotrophic lateral sclerosis (150), and pediatric cardiomyopathy (151).

It is still unclear if all of those functions of ASNA1 are linked to the TAP insertion function of the protein or indeed are independent and can be separated based on the oxidative state of the protein.

C. elegans ASNA-1: a multifunctional protein

Up to date, the mechanistic role of *Asna1* has been very hard to assess due to the embryonic lethality of mice lacking *Asna1* (129), pointing out the importance and necessity of ASNA1 in survival. Therefore, an *in vivo* model for studying ASNA1 functions is in great demand. Our group has used the nematode *C. elegans* for that purpose. This gave us the possibility to assess ASNA-1 functions in the absence of *asna-1*.

asna-1(ok938) mutation is kept in trans to a balancer *hT2(qIs48)* and segregate homozygous worms (*asna-1(ok938)*) from heterozygous mothers, which reach adulthood at the same time as wild-type animals. However, they are characterized by pale, small, scrawny sterile phenotype (142). The ability of worms to escape the L1 arrest, seen with *asna-1* RNAi treatment, was caused by maternal rescue. Animals with depleted both maternal and zygotic *asna-1* (m-z-) were arrested irreversibly in L1 stage (142). *asna-1(ok938)* animals were characterized by normal pharyngeal pumping rate and no defect in food uptake (142).

As mentioned, treatment of adult *C. elegans* with *asna-1* dsRNA caused F1 progeny to arrest in the first larvae stage with Z1 and Z4 gonad progenitor cells undergoing one or two rounds of division. The arrest was reversible and disappeared over time. Interestingly human ASNA1 cDNA was able to reverse the L1 arrest (142).

The expression pattern of ASNA-1, revealed by a transgene carrying GFP fused to the ASNA-1 cDNA under *asna-1* promotor, was limited to sensory neurons (ASI and ASK), intestine and hypodermis. This construct was able to rescue the scrawny phenotype of *asna-1(ok938)* animals. Rescue capacity was also observed for *asna-1* under neuronal and intestinal specific promotors (142).

Other characteristics of *asna-1(ok938)* animals are insulin signaling and secretion defect (142), as well as cisplatin and arsenite sensitivity phenotype (152). Cisplatin sensitivity was not caused by a decrease in the oral intake of cisplatin and neither by avoidance defect. Interestingly, human ASNA1 and *C. elegans* ASNA-1 were able to rescue the cisplatin sensitivity phenotype of *asna-1(ok938)* animals showing a connection between human and worm conserved mechanism in cisplatin response (152).

To summarize, *C. elegans* ASNA-1 plays a role in cisplatin sensitivity, insulin secretion, and worms' growth and development. Earlier research indicated that likely insulin secretion is not involved in cisplatin sensitivity of *asna-1(ok938)* animals (152). Considering the importance of post-mitotic cells in cisplatin treatment, as well as the ability to use *C. elegans* adult animals as an *in vivo* post-mitotic model, we were equipped with a powerful model to study the mechanism of function separation.

Aims

The main objective of this thesis was to have a better understanding of cellular pathways involved in response to cisplatin in order to address a major drawback of drug resistance. Specifically, the aims of the papers included in this thesis were:

Paper I

Separation of *C. elegans* ASNA-1 clinically relevant roles in cisplatin detoxification and insulin signaling

Paper II

Evaluation of tissue and genetic requirements of ASNA-1 in cisplatin response function and separation from its roles in growth and development of *C. elegans*

Paper III

Establishing the role of the p38 MAPK pathway in response to cisplatin by using *C. elegans* as a post-mitotic model

Methods

A brief description of the key methods used throughout the papers. More details can be found in the paper and manuscripts.

***C. elegans* maintenance and synchronization**

Worms were cultured under standard conditions at 20ºC on nematode growth media (NGM) plates unless stated otherwise and the *E. coli* strain OP50 was used as a food source. All worms used in the study were synchronized to the young adult stage. Synchronous larval populations were obtained by gravity separation. The L1 larvae were cultured for three more days at 20ºC to obtain a plate with a synchronous population of worms at the 1-day old young adult stage.

TAP targeting analysis

Live 1-day old adult animals were sedated in 2 mM Levamisole/M9 and mounted onto agarose pads. The int8 and int9 cells in the posterior intestine were imaged in all cases.

Glycosylation analysis

Protein lysate from young adult worms expressing 3xFlag::SEC-61β::opsin was split in two and one half treated with Endoglycosidase H. All samples were separated by SDS-PAGE followed by western blot and detection by immunoblotting.

Insulin assays

Worms carrying integrated DAF-16::GFP were scored based on the nuclear vs cytoplasmic GFP signal. Worms carrying integrated DAF-28::GFP were scored based on the GFP uptake by coelomocytes.

Subcellular fractionation.

Young adult animals were lysed in extraction buffer containing sucrose and supernatants were centrifuged for 1 h at 100,000xg to separate the membrane and cytosolic fractions. Proteins in both fractions were separated by SDS-PAGE followed by western blot and detection by immunoblotting.

Cisplatin plate preparation

Plates containing a desired concentration of cisplatin were prepared by adding cisplatin (from 1mg/mL stock solution) to cooled autoclaved MYOB media. Plates were allowed to solidify and were stored at room temperature in the dark. 30x concentrated OP50 from a stationary phase culture was added right before use as a 5μL spot.

Cisplatin sensitivity assay

L4 larvae were isolated and grown for 24h before exposure to cisplatin-containing MYOB plates. After 24h cisplatin exposure, death was scored by the absence of touch-provoked movement when stimulated by harsh touch using a platinum wire.

Auxin treatment

Animals were transferred to NGM plates containing the indicated concentration of the water-soluble auxin analog (potassium naphthalene acetic acid-KNAA). An 800μM stock of K-NAA in water was freshly prepared before use. KNAA was added to cooled NGM agar for a desired final concentration and plates were stored in the dark.

ROS estimation assays

Reactive oxygen species (ROS) levels were quantified using 2,7- dichlorodihydrofluorescein diacetate (H_2DCFDA). Fluorescence was read at the time of adding the dye to the live worm pellet and one hour after dye addition using a fluorimeter (485 excitations, 520 emissions). Initial readings were subtracted from the final readings and fluorescence was calculated.

Carbonylated protein detection

Protein carbonylation was determined by OxyBlot Protein Oxidation Detection Kit. Total extracted proteins were derivatized according to the manufacturer's instructions. All samples were separated by SDS-PAGE followed by western blot and detection by immunoblotting.

Quantitative PCR (qPCR).

qPCR was performed using KAPA SYBR FAST qPCR Kit with the comparative Ct method and normalization to the housekeeping gene.

Results and Discussion

Paper I: Alternative redox forms of ASNA-1 separate insulin signaling from tail-anchored protein targeting and cisplatin resistance in *C. elegans*

RESULTS

Work conducted in the group has established ASNA-1/TRC40 as an important cisplatin sensitivity factor. The inactivation of the protein in mammalian and *C. elegans* systems caused a severe cisplatin sensitivity phenotype (138,139,152). This pointed to the importance of the protein in studies focusing on cisplatin resistance. However, the major drawback of ASNA-1/TRC40 inactivation is impaired insulin secretion and signaling (142,143). Therefore, in this paper, we have explored the possibility of separating the important functions of ASNA-1 in insulin secretion and cisplatin resistance and hypothesized that this will allowed us to use ASNA-1 as a target to re-sensitize resistant cells to cisplatin treatment while leaving the insulin function intact.

ASNA-1 is a redox-sensitive protein

Work on the *S. cerevisiae* homolog of ASNA-1, GET3, showed that this multifunctional protein exists in two redox-sensitive states: reduced and oxidized (134). Therefore, we have speculated that separation of insulin secretion from cisplatin resistance might be possible based on the oxidative state of the protein. Our analysis showed that indeed *C. elegans* ASNA-1 was present in both reduced (ASNA-1^{RED}) and oxidized (ASNA-1^{OX}) states and formation of the oxidized form was dependent on the presence of two conserved cysteines in positions 285 and 288. Most importantly, the shift in the balance toward a more oxidized form, at the expense of the reduced form, was seen upon oxidant (H_2O_2) treatment, and the balance was restored after the removal of worms from the oxidizing environment. We concluded that *C. elegans* ASNA-1, like its homolog in yeast Get3, exists in two alternative redox states and the oxidation is not only reversible but can be altered in both directions.

ASNA-1 has a role in tail-anchored protein insertion

The most widely described function of the yeast (GET3) and mammalian (ASNA1/TRC40) homologs of ASNA-1 is the insertion of tail-anchored proteins (TAP) into the ER (143,153–156). We investigated whether worm ASNA-1 also has a role in promoting a TAP insertion. For that reason, we established the *in vivo* system where we could visualize and quantify the extent of TAP insertion. SEC-61β was chosen as a model TAP based on the mammalian homology to SEC-61β and established ASNA-1- dependent insertion into the ER membrane (107,156,157). Co-expression in the intestinal cells of GFP::SEC-61β and rough ER specific protein SP12 tagged with mCherry (mCherry::SP12), resulted in their near complete colocalization in adult *C. elegans*. Moreover, ASNA-1 was needed for proper GFP::SEC-61β localization since we detected a decrease in the colocalization between GFP::SEC-61β and mCherry::SP12 in *asna-1* protein null mutant: *asna-1(ok938)*. A defect in TAP insertion was further confirmed by the decreased amount of total GFP::SEC-61β protein level in *asna-1(ok938)* background with the reasoning that the TAP is degraded when its insertion into the ER membrane fails. Therefore, we concluded that *C. elegans* ASNA-1 plays an essential role in SEC-61β targeting into the ER.

TAP targeting defect is associated with cisplatin detoxification but not insulin secretion

The structural homology suggested that *C. elegans wrb-1* is a homolog of a well-described ASNA-1 ER membrane receptor WRB1, which is required for TAP insertion in mammalian cells (158). To confirm that, we performed Co-IP/MS/MS analysis and found that *C. elegans* WRB-1 is an interacting partner of ASNA-1::GFP. Furthermore, GFP::SEC-61β localization to the ER was disturbed in the *wrb-1(tm5938)* deletion mutant background. Moreover, *wrb-1* deletion mutants also shared the cisplatin sensitivity phenotype of *asna-1(ok938)*. We have concluded that *C. elegans wrb-1* is an ER receptor for ASNA-1 TAP insertion which plays a role in cisplatin detoxification. Moreover, those results indicated a correlation between the TAP targeting defect and cisplatin sensitivity.

As shown previously, maternally rescued *asna-1(ok938)* mutants have a sterile, small, and pale phenotype with a low level of insulin/IGF signaling (IIS) (142). Although *asna-1* and *wrb-1* mutants share GFP::SEC-61β localization defect, *wrb-1* mutants are bigger with a much better-developed germline. This indicated that indeed the TAP-targeting defect may be separated from the growth phenotype, which in *asna-1* is associated with insulin signaling defect. We used two transgenic worm strains to assess the insulin signaling and secretion status in *wrb-1* mutant animals: DAF-16/FOXO::GFP (159) and DAF-28/insulin::GFP

(142). In the *asna-1(ok938)* mutant background, DAF-16/FOXO::GFP was present in nuclei foci and no DAF-28/insulin::GFP was visible in coelomocytes. Both phenotypes indicated an IIS activity defect. On the contrary, *wrb-1* knockout did not cause any insulin signaling defect. Taken together, those findings strongly support the notion that cisplatin sensitivity and TAP insertion defect are likely linked whereas both characteristics are not connected to insulin secretion defect.

Cisplatin sensitivity and TAP targeting defect are caused by changes in ASNA-1 redox balance

Analysis of *wrb-1* mutants provided the first line of evidence for function separation between IIS and cisplatin response. However, to obtain more direct evidence we have focused on single point mutant in *asna-1*: *asna-1(ΔHis164)*. We found that worms with this single point mutation showed a cisplatin sensitivity phenotype comparable with null *asna-1* mutants, while still maintaining wild-type levels of ASNA-1 protein. Moreover, *asna-1(ΔHis164)* mutants also demonstrated the TAP targeting defect and decreased steady-state protein levels of GFP::SEC-61β which further confirmed TAP insertion defect. Having in mind that ASNA-1 exists in two alternative redox states, we have tested the redox balance in worms expressing ASNA-1$^{\Delta His164}$::GFP from a transgene. Higher levels of the oxidized form of ASNA-1 were found in the ASNA-1$^{\Delta His164}$::GFP expressing worms in comparison to the worms expressing ASNA-1::GFP. Taken together, we conclude that deletion of Histidine 164 results in a shift in the balance towards higher levels of oxidized form of the protein which in consequence leads to TAP targeting defect and cisplatin sensitivity phenotype, while leaving insulin secretion intact. This line of evidence suggests that ASNA-1 functions are separable based on the redox status of the protein. We concluded that cisplatin function and TAP targeting function of ASNA-1 did not depend on the oxidized form of the protein but are maintained by reduced form of ASNA-1.

ASNA-1 oxidation is promoted by cisplatin-induced reactive oxidative species

ROS generation by cisplatin in mammalian cells has been a well-established phenomenon (160,161). We found that in worms as well, cisplatin treatment also promoted ROS generation and induced oxidative stress response genes *gcs-1* and *gst-4*. Knowing that ASNA-1 is a redox-sensitive protein we sought to examine the influence of cisplatin on the redox state of ASNA-1. Cisplatin exposure, at the concentration that significantly decreased survival of *asna-1* null mutants without affecting the wild-type worms, resulted in the shift of the balance to-

wards a more oxidized form of ASNA-1. Thus, cisplatin promoted ROS generation in *C. elegans* which resulted in changes in the redox balance.

Cisplatin selectively delocalizes an ASNA-1 dependent TAP from the ER

Having in mind the role of the reduced form of ASNA-1 on TAP targeting and cisplatin resistance we sought to determine whether those two functions were directly linked. Cisplatin exposure of wild-type worms resulted in SEC-61β targeting defect. Surprisingly, the same cisplatin treatment regime did not affect the delocalization of two ASNA-1 independent proteins: CytB5.1 (*cytb-5.1*) and SERP-1.1 (*serp-1.1*). Next, we have tested the impact of cisplatin on insulin function. After cisplatin exposure, we have detected no insulin secretion defect, which led to the conclusion that the effect of cisplatin on GFP::SEC-61β delocalization was likely not a result of general toxicity or effects on membranes. Knowing the consequences of cisplatin treatment on ASNA-1 dependent TAP localization and ASNA-1 oxidation, we asked whether the targeting defect of GFP::SEC-61β in cisplatin-treated worms required ASNA-1. Comparison of GFP::SEC-61β delocalization defect in *asna-1(ok938)* animals with and without cisplatin treatment showed that there was no difference between those two conditions. Hence, reduced TAP targeting caused by cisplatin was dependent on ASNA-1 since no additional effect after cisplatin treatment was seen. Given that, we propose the model where cisplatin exposure leads to ROS generation, in turn causing a shift in the balance between redox forms of ASNA-1 (towards a more oxidized form of the protein), and in consequence, diminished GFP::SEC-61β targeting while leaving insulin secretion unaffected (Figure 6).

DISCUSSION

ASNA-1 is a highly conserved multifunctional protein with functions in tail-anchored protein insertion, cisplatin detoxification, and insulin secretion. In this paper, we aimed to establish if essential ASNA-1 functions in cisplatin detoxification and insulin secretion can be separated. We have discovered that *C. elegans* ASNA-1, like its yeast homolog GET3, exists in two redox-sensitive states: ASNA-1^{OX} and ASNA-1^{RED}. Moreover, we were able to separate ASNA-1 functions based on the oxidative state of the protein. In the course of our analysis, using molecular and genetic tools, we provided evidence to strongly support the idea that the reduced form of the protein is needed for TAP insertion and cisplatin detoxification while the oxidized form of ASNA-1 is necessary to maintain proper insulin secretion.

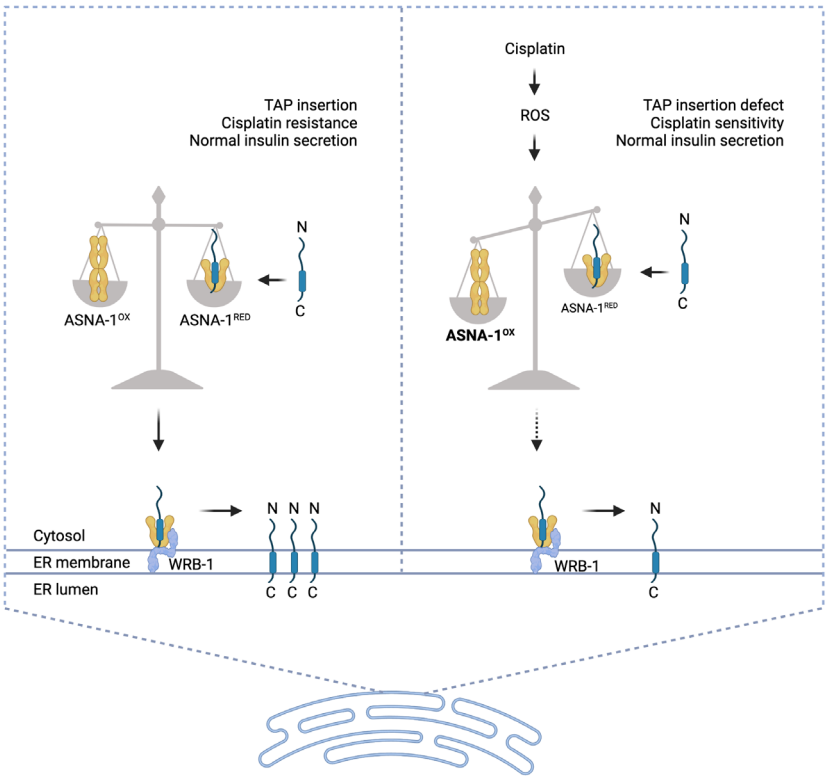

Figure 6. Balance between the redox forms of ASNA-1 assures proper tail-anchored protein insertion and cisplatin resistance. *Proposed model suggesting that under normal physiological conditions, ASNA-1 is present in both redox forms: reduced (ASNA-1^{RED}) and oxidized (ASNA-1^{OX}). The reduced form of the protein participates in the insertion of TAP into the ER, while the oxidized form of ASNA-1 assures proper insulin secretion. Cisplatin exposure leads to ROS generation and in consequence disturbance in the redox balance towards more ASNA-1^{OX} at the expense of ASNA-1^{RED}. This shift in the redox balance impairs TAP targeting into the ER without affecting insulin secretion and leads to cisplatin sensitivity. Created with BioRender.com*

The first line of evidence for the separation of clinically relevant ASNA-1 functions came from our analysis of *wrb-1* mutant. This finding was consistent with several other publications which elegantly established not all ASNA-1/ASNA1/Get3 functions require WRB-1/WRB/Get1 or its interaction partner Get2 (in yeast) – the counterpart of mammalian CAML. In *S. cerevisiae,* Get1/2 mutants displayed not only a sporulation defect, which was not seen in Get3 mutants, but exhibited high sensitivity to DNA-damaging agent - hydroxyurea (HU), while Get3 mutants did not share this severe sensitivity phenotype (162). Moreover, it has been shown that Get3 is co-regulated with the pro-

teasome/ubiquitin/NPL4 pathway and displays genetic interactions with it, while Get1 and Get2 did not (162). This provided further evidence of Get3 having a Get1/Get2 independent role. Several reports have shown an additional role for GET3 as a holdase chaperone that binds to aggregated proteins under oxidative stress conditions and ATP depletion (133,134). Structural rearrangements upon oxidation of GET3 led to masking of the hydrophobic binding domain required for TA protein binding as well as substantially reduced its ATPase activity: both the binding and ATPase properties of GET3, which are essential for its interaction with WRB/GET1, were completely dispensable for the function of oxidized Get3. Voth *et al.* (2014) showed that oxidized GET3 also acts as a general chaperone that can refold denatured citrate synthase in vitro in the absence of GET1. Using a Get3 (I193D) mutant they demonstrate that the chaperone activity is independent of tail-anchored protein targeting (134). These lines of evidence demonstrated that under conditions of oxidative stress, Get3 has functions that did not require Get1. Given this literature, we were able to model how ASNA-1 in worms would act in the absence of WRB-1. Moreover, our analysis of *asna-1(ΔHis164)* mutants provides very strong independent lines of evidence that again point towards the same conclusion of genetically separable functions.

ASNA-1 belongs to the group of proteins, like PRDX-2, IRE-1, or GLB-12, whose activity changes with oxidation (163–165). In those proteins change in the activity is achieved due to redox-sensitive cysteines or disulfide bonds. In *C. elegans* ASNA-1 two conserved cysteines are responsible not only for maintaining the redox balance but are necessary for protein activity. Mutation in those two conserved cysteines resulted in a lack of ASNA-1^{OX} form of the protein. It has been shown that the GET3$^{C285S;C288S}$ form of the protein fails to dimerize and leads to the presence of an inactive form of the protein in a monomeric state (134,154). This limits the use of the protein with mutated cysteines residues. Consequently, mutation of conserved cysteines residues in ASNA-1 affects all protein functions and makes it difficult to study the reduced form of the protein (ASNA-1^{RED}) in the absence of ASNA-1^{OX}. We proposed that ASNA-1^{RED} drives the cisplatin detoxification function of the protein. However, we cannot completely rule out the possibility of the ASNA-1^{OX} having a role in cisplatin detoxification.

Paper II: Analysis of tissue and genetic requirements of ASNA-1 for growth, reproduction and cisplatin response in *C. elegans*

RESULTS

In Paper I we have shown that *C. elegans* ASNA-1 is a redox-sensitive protein that promotes TAP-targeting and cisplatin resistance separately from insulin secretion. This conclusion was based on the analysis of ASNA-1 ER receptor null mutant *wrb-1*, though more direct evidence came from analysis of sterile *asna-1(ΔHis164)* point mutant. In this paper, we sought to establish the genetic and tissue requirements of ASNA-1 in the separation of cisplatin detoxification function from functions in growth and development. The analysis of single point mutant *asna-1(A63V)* allowed us to separate the ASNA-1 clinically relevant functions even further without compromising the growth and reproduction phenotypes.

ASNA-1 is broadly expressed protein in somatic and germline tissues

Analysis with ASNA-1 extrachromosomal transgene has shown that ASNA-1 is a broadly expressed protein with a strong GFP signal seen in the intestine, hypodermis, and sensory neurons (142). However, to obtain a more detailed description of the ASNA-1 expression pattern we have created a strain expressing mNeonGreen in the chromosomal ASNA-1 locus. We observed strong ASNA-1::mNeonGreen signal in pharynx, neurons, muscles, intestine, developing vulva, proximal and distal gonad cells, oocytes, spermatheca, sperm, and the most proximal gonadal sheath cells. We have not only confirmed the previously characterized somatic tissue expression pattern (142) but also established germline and somatic gonad as likely important places for ASNA-1 function.

Somatic ASNA-1 expression is necessary for *C. elegans* larval development

Taking into consideration the broad expression pattern of ASNA-1 in somatic and germline tissues, we asked if somatic ASNA-1 knockdown alone will lead to larval arrest seen formerly in *asna-1(RNAi)* animals (142) or whether the L1 arrest requires depletion of ASNA-1 in the germline as well. Taking advantage of the auxin-inducible degron system (166,167) we created the strain carrying ASNA-1::mNeonGreen::AID (*syb2249*) and TIR1 pan-somatic driver *ieSi57*. This allowed us to study the consequences of ASNA-1 knockdown in all somatic tissues.

Firstly, somatic ASNA-1 depletion in adult animals had little effect on adult animals but led to arrest in first larval stage (L1) of their progeny. Interestingly, we observed fluorescent signal in Z2 and Z3 germline progenitor blast cells in the arrested larvae, whereas they were lacking the ASNA-1::mNeonGreen::AID signal in all previously characterized somatic tissues. Secondly, somatic ASNA-1 depletion in L1 larvae animals did not allow for their further development and animals remained arrested as L1 larvae. As expected, the auxin-treated animals did not show any ASNA-1::mNeonGreen::AID signal in all previously characterized somatic tissues. Curiously, auxin treatment of L1 animals allowed for further germline development, and the ASNA-1::mNeonGreen::AID signal was seen in many more germline cells. This stands in contrast with only two ASNA-1::mNeonGreen::AID positive Z2 and Z3 germline progenitor blast cells when worms hatched in the presence of auxin. We concluded that somatic ASNA-1 expression is necessary for *C. elegans* larval development, entry into the L2 stage, as well as germline development.

Next, we tested if adult worms with somatic depletion of ASNA-1 will share cisplatin sensitivity phenotype of deletion mutants *asna-1(ok938)*, which reach adulthood besides its scrawny phenotype. Surprisingly, we did not observe cisplatin sensitivity phenotype. The same experiment was set up for strains with depletion of ASNA-1 in germline and gut. Here as well no enhanced sensitivity phenotype was observed.

Multi-tissue requirement of ASNA-1

Previously, our work has established an important role of ASNA-1 expression in neurons and intestine. Those two tissue-specific promotors driving *asna-1* expression were able to rescue the scrawny *asna-1* body size phenotype and force dauer exit (142). Moreover, we had established the gut as an important place for ASNA-1 function in correct tail-anchored protein (TAP) insertion into the ER (168). Thus, we asked if gut-specific ASNA-1 depletion will affect the development of *C. elegans*. First, gut-depletion of ASNA-1 adult animas resulted in developmental delay of their progeny. Second, gut-depletion of ASNA-1 in L1 larvae did not cause any developmental delay. We concluded that ASNA-1 in the intestine is needed for proper development and growth. However, the lack of such a severe phenotype as seen by the somatic depletion indicated rather a multi-tissue requirement of ASNA-1 for the development of *C. elegans*.

Our analysis of the ASNA-1 expression pattern has indicated that germline tissue will likely be an important place for ASNA-1 function. Additionally, *asna-1(ok938)* animals have been previously characterized as sterile animals point at a severe germline defect (142). Therefore, we asked if germline-specific depletion of ASNA-1 will also result in a larval arrest as seen after somatic de-

pletion of the protein. First, germline-depletion of ASNA-1 adult animas resulted in fewer progeny hatched with no apparent development or growth defect. Second, germline-depletion of ASNA-1 was carried out in L1 larvae, and here as well no evident defect was visible. We concluded that the germline expression of ASNA-1 largely did not affect the growth and development of *C. elegans* and the defect was not as severe as one caused by somatic-depletion of ASNA-1.

Taken together, somatic expression of ASNA-1 plays a major role in the growth and development of *C. elegans*, more than ASNA-1 expressed in germline or gut alone. Moreover, separation of ASNA-1 function might be possible not only based on the oxidative state of the protein (168) but also based on the tissue-specific expression.

***asna-1(ΔHis164)* mutants are likely sperm or oocyte defective**

As described in in paper I, *asna-1(ΔHis164)* mutants carry a deletion of single histidine at codon 164 in ASNA-1. Deletion of histidine led to a shift in the balance between redox forms of ASNA-1 toward the more oxidized version of the protein. This change in the balance resulted in TAP insertion defect and cisplatin sensitivity phenotype. Additionally, a transgene expressing *asna-1*$^{\Delta His164}$::GFP was able to rescue the *asna-1* deletion mutants growth phenotype (152) but did not rescue the cisplatin sensitivity phenotype, indicating the importance of the amino acid in ASNA-1 insulin/IGF-1 signaling (IIS) function. We measured directly IIS activity by analysis of two markers: DAF-16::GFP and DAF-28/insulin::GFP. Analysis of both reporters in the *asna-1(ΔHis164)* mutants background showed that there was no defect in IIS activity, since DAF-16::GFP was present in the cytoplasm and DAF-28/insulin::GFP visible in coelomocytes.

Another characteristic of *asna-1(ΔHis164)* mutants is the sterility phenotype. Although animals are visibly bigger than *asna-1(ok938)* mutants, both do not produce progeny. We sought to establish the extent of the germline defect in both, *asna-1(ok938)* and *asna-1(ΔHis164)* animals. Deletion mutants *asna-1(ok938)* are small, pale, scrawny, and had a severe gonad migration defect. No oocytes or eggs were produced, no sperm was observed in the animals. By contrast, the *asna-1(ΔHis164)* point mutant produced sperm and unfertilized oocytes, as well as non-fertilized embryo-type structures in the uterus. Knowing that presence of sperm is necessary for ovulation, transit through the spermatheca, and entry of oocytes into the uterus (54), we concluded that *asna-1(ΔHis164)* mutants are likely oocyte or sperm defective with possibly immature spermatids or primary spermatocytes present in *asna-1(ΔHis164)* mutants.

***asna-1(A63V)* mutant separate insulin signaling and cisplatin function of ASNA-1 without compromising growth phenotype**

Data from our previous study has established that *C. elegans* ASNA-1 exists in two redox-sensitive states: reduced (ASNA-1^{RED}) and oxidized (ASNA-1^{OX}) (168). The balance between those two states is important for proper TAP insertion and cisplatin detoxification whereas shift towards a more oxidized form of the protein leads to defect in TAP targeting to ER as well as cisplatin sensitivity (Figure 6). This imbalance however left insulin function driven by ASNA-1 largely unaffected. We established that clinically relevant ASNA-1 functions in cisplatin response and insulin secretion are separable and separability is possible due to the existence of two alternative redox forms of the protein. Those conclusions were mainly drawn based on the analysis of sterile *asna-1(ΔHis164)* mutants. Here we wondered if we will be able to separate ASNA-1 functions without compromising the growth and reproduction phenotypes of *C. elegans*. Therefore, we obtain a set of seven strains generated by the million mutation project (169), each carrying a single amino acid change in ASNA-1 alongside the other mutations in other genes. After cisplatin survival analysis we focused on the analysis of one strain. *asna-1(gk592672)* strain carries alanine to valine change in conserved position 63, which corresponds to alanine 82 in human ASNA1/TRC40. Because of the heavy mutational load in the *asna-1(gk592672)* containing strain, extensive outcrossing was performed in order to evaluate if the mutation in ASNA-1 is indeed responsible for the cisplatin sensitivity phenotype. The outcrossed strain, hereafter called *asna-1(A63V)*, was as sensitive to cisplatin as a complete *asna-1* deletion mutant and this phenotype was rescued by transgene-expressed wild-type ASNA-1::GFP. We concluded that alanine in position 63 in ASNA-1 protein is an important site for cisplatin sensitivity function of ASNA-1 and heavy mutational load in *C. elegans* is not a cause of cisplatin sensitivity. Although as cisplatin sensitive as the *asna*-1 deletion mutant, *asna-1(A63V)* animals did not share the insulin signaling/secretion defect of *asna*-1*(-)* mutants, nor the enhanced ER stress phenotypes. The animals exhibited a wild-type lifespan, were fertile with normal brood sizes, and had properly developed germline. This opened a possibility for ASNA-1 function separation without jeopardizing growth and development function, as seen in *asna-1(ΔHis164)* mutants. Furthermore, *asna-1(A63V)* animals displayed significantly defective TAP targeting and membrane fractionation showed decreased membrane association of the ASNA-1^{A63V}::GFP protein compared to that seen in for ASNA-1::GFP. Insertion of SEC-61β into the membrane (using the glycosylation assay) was decreased in *asna-1(A63V)* mutant background while steady-state level of the SEC-61 β protein remained unchanged. Non-reducing SDS-

PAGE analysis revealed higher levels of the ASNA-1(A63V) protein in the oxidized state.

We concluded that the A63V mutation in ASNA-1 leads to a shift in the balance towards more oxidized ASNA-1, and in consequence cisplatin sensitivity and TAP insertion defect while leaving insulin secretion unaffected. This confirmed the results obtained before with *asna-1(ΔHis164)* mutants and allowed us for ASNA-1 function separation without compromising the germline of *C. elegans*.

DISCUSSION

The purpose of the current study was to determine if essential functions of ASNA-1 in cisplatin detoxification and insulin signaling might be separated without negatively affecting the growth and development of *C. elegans*. We were able to dissect these two functions by targeting single amino acid in the ASNA-1 and showed that *C. elegans* strain carrying A63V mutation was sensitive to cisplatin, had mild TAP targeting deficiency but what is important, did not show any signs of IIS nor growth defect. This allowed us to study ASNA-1 functions without jeopardizing the growth or development of the worms. In the course of the analysis, we were able to establish the requirements for tissue expression of ASNA-1 in the development of the worms and the importance of somatic ASNA-1 expression on germline tissue.

Experiments using an auxin-induced degron system (166,167) gave us an opportunity to assess the tissue requirements of ASNA-1. However, we are aware of the limitation of this system where we cannot fully assess the extent of the knockdown in particular tissues neither fully recreate the full spectrum of germline or cisplatin defects seen in *asna-1(ok938)* (142,152). However, this system allowed us to uncover the phenotypes hidden by a maternal rescue in *ok938* animals like the ability to reach adulthood, bigger size, and development of the germline. Also, this included a likely role for ASNA-1 in both the somatic gonad and the germline and the finding that ASNA-1 was present in germ cells from the earliest divisions.

Earlier research has also established the importance of neuronal and intestinal expression of ASNA-1 in growth by showing that *asna-1* driven under one of those two promotors was able to rescue growth defect seen in *asna-1(ok938)* animals (142). It would be important to study the effect of neuronal depletion of ASNA-1 on the growth of worms as well as the effect on cisplatin detoxification ability taking into consideration that neuronal promotor driving *asna-1* was not able to rescue the cisplatin sensitivity phenotype of *asna-1(ok938)* animals. Interestingly, the *C. elegans* intestine is considered to be the main tissue important

for detoxification in worms (170). Our analysis indicated an important role of the intestine expression on the development of *C. elegans*. However, intestinal depletion of ASNA-1 was not able to reproduce the cisplatin sensitivity phenotype of *asna-1(ok938)* deletion mutants. Further analysis will be required to evaluate if the intestinal driven *asna-1* will be able to rescue the sensitivity phenotype of *asna-1(ok938)* as well as an effect of simultaneous depletion of ASNA-1 in two or more tissues.

Paper III: The innate immune system promotes cisplatin chemo-resistance in post-mitotic *C. elegans* via activation of the p38/MAPK pathway

RESULTS

Only 1-10% of intracellular cisplatin can be found in the nucleus, which in consequence will lead to DNA damage in proliferating cells. Strong scientific evidence shows that cisplatin has multiple cellular targets beyond DNA (19–21). Moreover, a big part of the tumor contains non-dividing cells (2,23). This allowed us to ask: what is the consequence of cisplatin treatment on non-dividing cells? Work performed in paper I and paper II has been predominantly performed on 1-day old adult animals giving us a glimpse of how important it is to model cisplatin-induced cytotoxicity and the effects of this chemotherapeutic on post-mitotic cells. Therefore, in this paper, we have explored further the effect of cisplatin on post-mitotic cells with a focus on the characterization of important biological targets of cisplatin.

p38 MAPK pathway is important for cisplatin resistance of post-mitotic cells

Work in the mammalian cell culture system has established ROS as an important factor modulating cisplatin sensitivity (69,160,171). Our previous work also showed cisplatin-induced ROS generation in adult *C. elegans* (168). Therefore, we wondered how important ROS detoxification is in protection from cisplatin. In this paper we have focused on p38 MAPK pathway since it has a key role in ROS protection. We have analyzed cisplatin sensitivity of 1-day old adult animals carrying a single mutation in components of the p38 MAPK pathway and successfully showed that *sek-1(km4)*, *sek-1(syb2311)* and *pmk-1(km25)* mutants had enhanced sensitivity phenotype when treated with cisplatin. Moreover, somatic depletion of SEK-1 in adult animals also led to increased cisplatin sensitivity phenotype showing that the depletion of SEK-1 in post-mitotic somatic cells was sufficient for producing cisplatin sensitivity and the proliferating germline did not contribute to this phenotype. We concluded that indeed p38 MAPK pathway is an essential component in protection from cisplatin-induced death in adult animals.

Induction of ROS detoxification genes does not assure cisplatin survival of post-mitotic worms

The SKN-1/NRF2 transcription factor is a crucial downstream target of the p38 MAPK pathway and drives the expression of phase II detoxification genes

in response to increased ROS levels (85). We hypothesized based on the sensitivity phenotype of *sek-1* and *pmk-1* deletion mutants, as well as ROS induction upon cisplatin, that induction of those antioxidant genes might be necessary to protect adult worms from cisplatin-induced death. Indeed, cisplatin exposure led to an increase in mRNA level of detoxification genes in the wild-type background. Moreover, we did not detect any induction of those genes in *skn-1* deletion mutant background upon cisplatin exposure. Surprisingly, mutants in *skn-1* and *daf-16*, another key transcription factor regulating ROS detoxification genes, did not show sensitivity to cisplatin treatment. Taken together, these results suggest that ROS levels generated upon cisplatin exposure were not sufficiently high to cause death, and elimination of ROS by detoxifying enzymes was likely not important for the survival of adult worms after cisplatin exposure.

Cisplatin treatment increased the abundance of the innate immune system proteins

Having established an important role of p38 MAPK pathway upstream components and ruled out a role of ROS as a cause of cisplatin-induced death in adult animals, we wished to discover biological processes involved in the response of post-mitotic cells to cisplatin. Therefore, quantitative proteomics analysis of 1-day old adult animals exposed to cisplatin for six hours was carried out in comparison to untreated control. Overall, proteomics analysis revealed a robust response to cisplatin. Reactome enrichment analysis revealed that differentially abundant proteins fell into multiple categories and the innate immune system was the most prominent among those.

The innate immune gene induction, driven by the p38 MAPK pathway, assures cisplatin survival

Bearing in mind the importance of the p38 MAPK pathway in immune response, we tested cisplatin sensitivity of *nsy-1* and *tir-1* mutants. Both proteins are upstream components of p38 MAPK pathway, and the *tir-1(qd4)* mutant has a specific and essential role in immune response (75). We found that *nsy-1(ag3)* and *tir-1(qd4)* animals were characterized by high cisplatin sensitivity. Moreover, the well-established immune genes controlled by p38 MAPK cascade (76,172,173) were not only highly upregulated upon cisplatin exposure but also *pmk-1* dependent. We concluded that all components of the p38 MAPK pathway involved in the innate immune response are essential for assuring the survival of adult worms on cisplatin.

Cisplatin resistance requires the ATF-7 transcription factor

To determine further the importance of the innate immune pathway activation in cisplatin response, we assessed the role of ATF-7. ATF-7 is a key downstream transcription factor of the p38 MAPK pathway in *C. elegans* and has a crucial role in the PMK-1-mediated intestinal innate immune response (76). We found that *atf-7(qd22)* mutants, which are characterized by diminished expression of PMK-1-dependent immune genes, were sensitive to cisplatin and this phenotype was even further enhanced by *pmk-1* deletion. On the contrary, *atf-7(qd22 qd130)* mutants, which allow for partial immune gene expression, were not sensitive and suppressed the *sek-1* and *pmk-1* cisplatin sensitivity phenotype. This indicated the downstream role of ATF-7 in the p38 MAPK cascade.

The epidermal p38 MAPK innate immunity pathway does not play a role in cisplatin response

p38 MAPK cascade has a pivotal role in innate immunity both in the intestine and in the epidermis (hypodermis) via modulation of different transcription factors and separate sets of immune effectors (83,174). We, therefore, sought to establish if specifically perturbing the epidermal arm of the innate immune response would also affect survival on cisplatin. With this intention, we examined mutants in three genes participating in epidermal immunity. All three mutants were as resistant to cisplatin treatment as wild-type animals. This supported the notion that the epidermal arm of the p38 MAPK signaling cascade did not play a significant role in cisplatin response.

Rapid and sequential phosphorylation of SEK-1 and ATF-7 by cisplatin

As mentioned previously, ATF-7 is a p38 MAPK pathway-dependent transcription factor and is activated by phosphorylation. To further explore the molecular mechanism of cisplatin-driven innate immunity response, we monitored the phosphorylation status of ATF-7 after cisplatin exposure. We found the increased phosphorylation levels, as well as total levels of AFT-7, after six hours of cisplatin exposure, and no phospho-ATF-7 was detected at earlier time points. Phosphorylation of ATF-7, as well as total levels of ATF-7, were *pmk-1* dependent. Furthermore, we detected increased ATF-7 in the nuclei of intestinal cells.

Given that phosphorylation of the ATF-7 depends on activation via phosphorylation of SEK-1, we hypothesized that SEK-1 phosphorylation after cisplatin treatment should be detected at earlier time points. Indeed, we have detected increased levels of phospho-SEK-1 already after ninety minutes of cisplatin treatment, as well as increased total levels of SEK-1 at the same time point. Based on this evidence we concluded that cisplatin induces rapid and sequential phosphorylation of SEK-1 and ATF-7.

SEK-1/p38 immune effectors are essential modulators of cisplatin survival
To explore further the mechanism of innate immune-driven cisplatin response and determine whether the innate immune effector proteins have a functional role in this response, a second proteomics analysis was carried out. We performed proteomic profiling of 1-day old *sek-1(km4)* adults with and without cisplatin treatment and compared the result to proteomics profiling of wild-type animals. We have discovered a significant change in the proteome of cisplatin-treated animals and found that immune response proteins were the most prominent class of proteins with changed abundance. We hypothesized that identified proteins, which participate in immune response, will be crucial for protection from cisplatin-induced death. Indeed, the qPCR analysis showed the induction of four genes: *clec-4*, *catp-3*, *dod-24*, and *lys-1*. Those were characterized by our second proteomics profiling as upregulated upon cisplatin exposure in wild-type animals, *sek-1* dependent, and not induced by cisplatin in *sek-1* mutants background. Moreover, mutants in those four genes were characterized by an increased cisplatin sensitivity phenotype. Taken together our results support the idea that in adult *C. elegans* systematic immune response in intestinal cells leads to protection of somatic tissues against cisplatin toxicity (Figure 7). Contrary to previous studies, this study did not find a significant role of ROS or detoxification genes in protection from cisplatin of post-mitotic cells.

DISCUSSION

In this paper, we have excluded cisplatin-induced ROS generation as the cause of cisplatin sensitivity of post-mitotic cells. Instead, we characterized the p38 MAPK cascade driving immune response as indispensable for cisplatin sensitivity.

The use of *C. elegans* in cisplatin sensitivity studies has gained interest in recent years. This simple and easy to propagate nematode has human homologous pathways allowing the research community to study the cisplatin effect *in vivo* and draw conclusions. As early as 2006, Collis *et al*., showed that *C. elegans fcd-2*, involved in nucleotide-excision repair and regulation of DNA replication, is important for cisplatin sensitivity (175). Further, another group has identified genes involved in double strand break repair (*rpa-2*, *mus-101*, *drh-3*, *emb-4*, *rad-51*), chromatin cohesion (*pqn-85*), and RNA processing and trafficking (*npp-15*) as important for protection from cisplatin (176). This was followed by characterization of *polq-1*, involved in interstrand cross-link, and *lem-3* involved in DNA damage response as sensitivity factors for cisplatin sensitivity (177,178). Knockdown of the above-mentioned genes resulted in the embryonic lethality phenotype of *C. elegans*. Taking into consideration that all genes mentioned are

linked to DNA-damage response, our research was able to characterize a gap in the current knowledge about cisplatin action. We established the impact of cisplatin on post-mitotic cells and found new sensitivity factors for cisplatin response that do not belong to the DNA damage pathway.

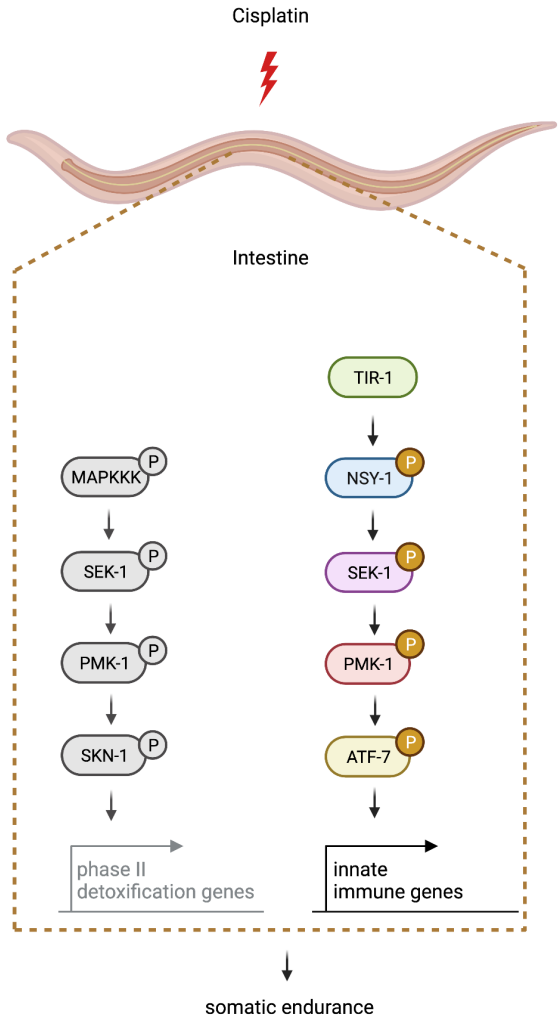

Figure 7. Model for cisplatin activation of the p38 innate immune pathway in the intestine of adult C. elegans. *Based on our data, we propose that p38 immune signaling pathway activation in the intestine regulates an immune-specific ATF-7 transcription factor. This in consequence leads to innate immune genes expression and somatic endurance (right pathway). Surprisingly, the ROS induction via cisplatin treatment, which in consequence led to activation of detoxification genes via SKN-1 transcription factor, was not important to promote survival (left pathway, marked in grey). Created with BioRender.com*

Another study had characterized *daf-16* and *polh-1* mutants as cisplatin sensitive (179). This stands in contradiction to our study, where *daf-16* mutants showed an increased resistance phenotype. The difference in the results might be explained by the stage of exposed animals. While García-Rodríguez *et al.,* (2018) score cisplatin sensitivity by body length on cisplatin-exposed larvae (179), we scored cisplatin-induced death by lack of touch-provoked movement of adult animals. Therefore, the response of developing larvae to cisplatin significantly differs in adult animals only further proving the need for more studies focused on post-mitotic cells.

An inflammatory reaction to other agents than pathogens is called a sterile inflammation (180). In this process activation of damage-associated molecular patterns (DAMPs), driven by cell or tissue damage, initiates the immune response through activation of pattern recognition receptors (PRPs) like include Toll-like, NOD-like, and C-type lectin receptors (181). It has been shown that survival following traumatic brain injury in *Drosophila* was increased by induction of NF-κB innate immune response transcription factor Relish (182). Taking into consideration the ability of cisplatin to induce the innate immune response, we propose that cisplatin exposure of adult *C. elegans* will lead to stimulation of DAMPs and in consequence initiation of immunity response through activation of PRPs like CLEC-4 or CLEC-66.

Conclusions

The main objective of this thesis was to have a better understanding of the cellular pathways involved in response to cisplatin. This is particularly important in the context of drug resistance, which is the major drawback for the use of cisplatin in the clinics. We focused in this thesis on modelling the response in the post-mitotic cells since growing scientific evidence indicates their role as one of the main reasons for resistance in solid tumors.

In **paper I** we showed that *C. elegans* ASNA-1 is a redox-sensitive protein and exists in two states: reduced and oxidized. The reduced form of the protein has a role in TAP targeting, like its yeast homolog. The oxidized form of the protein is likely involved in insulin secretion however, more direct evidence is necessary. Separation of ASNA-1 functions based on the oxidative state of the protein allowed us to increase sensitivity to cisplatin treatment without compromising growth or insulin singling in *C. elegans*. We showed that *wrb-1* is an ER located receptor involved in ASNA-1-dependent TAP insertion. Further, insulin secretion was not dependent on the TAP function of the protein. Direct evidence for function separation came from the analysis of *asna-1(ΔHis164)* mutant, which preferentially exists in the oxidative state, had strong cisplatin sensitivity phenotype a well as TAP targeting defect. Mutants with a deletion of Histidine in position 164 of *C. elegans* ASNA-1 displayed an improved body size and germline development s in comparison to *asna-1(-)* worms. However, the mutants were sterile which made the analysis rather challenging. Next, we sought to establish if insulin and cisplatin function separation was possible without compromising germline development.

In **paper II** we screened for viable *asna-1* point mutants provided by the million mutation project (169). We found that introducing a point mutation in position 63 of ASNA-1 protein, which changes alanine to valine (A63V), will result in increased oxidation of ASNA-1 protein and cisplatin sensitivity phenotype. Surprisingly, this mutation did not affect the IIS activity of the worms nor the germline development. Overlapping phenotypes of *asna-1(ΔHis164)* and *asna-1(A63V)* only furthered confirmed the function separation but allowed us to study it without compromising the germline. In paper II we also examined the expression pattern of ASNA-1. We discovered that the somatic expression of ASNA-1 is necessary for larval development and found strong indications that

presence of ASNA-1 in few tissues simultaneously is needed for resistance to cisplatin and larval growth.

In **paper III**, we discovered a previously unknown mechanism of cisplatin function in post-mitotic cells which involved activation of the highly conserved p38 MAPK pathway and innate immunity-related response. In the course of the analysis, we were able to establish that not only components of *C. elegans* p38 MAPK cascade were very sensitive to cisplatin (*nsy-1*, *sek-1*, *pmk-1*) but also upstream and downstream components of the pathways (*tir-1*, *atf-7*, *clec-4*, *catp-3*, *dod-24*, and *lys-1*). In total, we discovered nine new cisplatin sensitivity factors. Unexpectedly, we excluded the ROS-induced p38 MAPK pathway branch as a significant cause of cisplatin sensitivity in post-mitotic cells and allowed us to focus on innate immune response driven by the cascade. This led us to the analysis of the ATF-7 transcription factor and we were able to show its rapid and sequential activation by cisplatin treatment. We were able to show the earliest known molecular landmark for cisplatin exposure measured by SEK-1 phosphorylation already 90 min after cisplatin treatment. Furthermore, the proteomic approach allowed us to characterize single immune effectors as cisplatin sensitivity factors for post-mitotic cells.

We believe that this thesis provides substantial evidence for understanding signaling pathways involved in the modulation of cisplatin response as well as it elucidates significant differences in the response of post-mitotic cells to cisplatin treatment.

Future perspectives

In our study we demonstrate that the signaling cascade of TIR-1/NSY-1/SEK-1/PMK-1 is required for resistance against cisplatin toxicity. However, the mechanistic insight into how the p38 MAPK pathway is activated by cisplatin exposure still remain largely unanswered. A recent global profiling study has showed a wide range of redox regulation in *C. elegans* by distinct redox forms of cysteine (183). Importantly, they showed that redox-sensitive cysteines are also essential for p38 MAPK activation in the pathogen resistance and antioxidant response. It would be interesting to understand if cysteine modification in SEK-1 and PMK-1 is also an important factor for cisplatin detoxification in non-dividing cells. This would allowed us to get an insight into the mechanism of p38 MAPK cascade activation.

In Paper III, we explored the role of the p38 MAPK pathway in resistance against cisplatin and proteomic analysis was carried out. Samples were submitted for a quantitative mass spectrometry analysis to compare the protein abundances between the groups. Surprisingly, proteomic profiling showed that the level of 43 transcription factors (TFs) was differentially regulated in *sek-1(km4)* mutants in comparison to the wild-type animals. From those TFs, 29 were found to be differentially abundant in *sek-1(km4)* treated with cisplatin allowing us to speculate that another transcription factor might be important in driving cisplatin detoxification in SEK-1-dependent manner. In the future, it would be beneficial to assess the importance of those transcription factors in cisplatin response and its functional dependence on *sek-1/MAPKK*.

This thesis aimed to establish pathways involved in cisplatin response. For that purpose, we have used *C. elegans* as post mitotic model. The simplicity of the organism and presence of conserved pathways in worms and human were the biggest advantages when choosing the model for this particular study. However, validation of the result in the mammalian cell culture is of great demand. The 2D cell culture, although provided so far valuable insight on tumor development and mechanisms of therapeutic actions (184), still lacks phenotypic and genetic heterogeneity of the original tumor (185). Alternative for translating results obtained in this thesis into humans might be growing tissues *in vitro* in 3D as organotypic structures or so-called organoids. They have been successfully established for variety of patient-derived tumor tissues like breast (186), bladder (187), ovarian (188) and more. Their important feature is the ability to phenotyp-

ically and genetically mirror intra-tumor heterogeneity making it a promising tool in many translational applications including our study of modeling cisplatin response in post-mitotic cell.

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