The Role of EXD2 in the Maintenance of Mitochondrial Homeostasis

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I cannot believe it is time to leave. Before starting my PhD I never believed it would become such a fulfilling journey, and that the moments of frustration and despair could so easily been forgotten by the excitement of new ideas and the joy of discovering something about this universe that nobody else in the world knew. Even if it was a tiny detail about a tiny protein almost nobody knew existed! I´m grateful to have had a mentor on whose support, ideas and critical thinking I´ve always been able to count on. Thank you, Travis, for these years, I wish you Nature-papers, received grants, great students and everything else necessary to keep enjoying and making great science, nobody deserves it more than you do. I´ve also been blessed with fantastic colleagues in the lab and in the institute, and I´ve loved to come to work every day thanks to you all. Especial thanks to Katrin, Natalia and Hanna for sharing their PhD-journeys with me, in and outside the lab, my thesis is your thesis. These years would have been nothing without you, girls.

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Abstract

Mitochondrial dysfunction arising from aberrant mitochondrial nucleic acid homeostasis has been associated with various pathologies. In this thesis, we aim to characterize a putative mitochondrial exonuclease, EXD2, and the consequences of its loss in cell metabolism. For that purpose we use three approaches: 1) Biochemical assays with bacterially purified EXD2 to study its enzymatic activity, 2) *In vitro* experiments with cell lines to study the phenotype of cells with altered EXD2-levels, and, 3) *In vivo* experiments with a mouse xenograft model and *D.melanogaster* to study the consequences of EXD2-loss in tumors and at the organismal level. Our work shows, that EXD2 is a uniquely versatile mammalian exonuclease able to bind and degrade various DNA and RNA substrates. We demonstrate that loss of EXD2 in cancer cells leads to alterations in mtDNA, various metabolic changes and aberrant hypoxia signaling. We also describe how, *in vivo,* this leads to inhibition of breast tumor growth and increased lifespan in fruitflies. Taken together, our observations provide a link between mitochondrial nucleic acid maintenance and large-scale metabolic alterations influencing tumor growth and aging.

Resumen

La disfunción mitocondrial que surge de la homeostasis de ácido nucleico mitocondrial aberrante se ha asociado con varias patologías. En esta tesis, se pretende caracterizar una exonucleasa putativo mitocondrial, EXD2, y las consecuencias de su pérdida en el metabolismo celular. Para ello utilizamos tres enfoques: 1) ensayos bioquímicos con EXD2 purificado de bacterias para estudiar su actividad enzimática, 2) los experimentos *in vitro* con líneas celulares para estudiar el fenotipo de las células con niveles de EXD2 alterados, y, 3) experimentos *in vivo* con modelo de xenotrasplante de ratón y *D.melanogaster* para estudiar las consecuencias de perdida de EXD2 en los tumores y en el nivel del organismo. Nuestro trabajo muestra que EXD2 es una exonucleasa mamíferos excepcionalmente versátil capaz de unirse y degradar diversos sustratos de ADN y ARN. Se demuestra que la pérdida de EXD2 en las células del cáncer conduce a alteraciones en el ADN mitocondrial, diversas alteraciones metabólicas y de señalización hipoxia aberrante. También describimos cómo, *in vivo*, esto conduce a la inhibición del crecimiento del tumor de mama y aumento de la vida útil en moscas de la fruta. En conjunto, nuestras observaciones proporcionan un enlace entre el mantenimiento de ácidos nucleicos mitocondriales y de gran escala alteraciones metabólicas que influyen en el crecimiento del tumor y el envejecimiento.

Preface

At the center of cellular energy production and oxygen balance are the mitochondrion. The mitochondrial (mt) genome encodes 13 subunits of the oxidative phosphorylation complexes, and alterations in the mtDNA have been shown to cause large-scale metabolic alterations that lead to various pathologies, including neurodegeneration and cancer. Increased mtDNA lesions have also been associated with aging, thus making the quality of mitochondrial DNA of utmost importance to the organism. However, the pathways and mechanisms involved in the maintenance of the mitochondrial genome are still largely unknown. In this work we have explored the contribution of a previously uncharacterized protein, EXD2, to the mitochondrial homeostasis.

At the beginning of this project, little information was available as only four published papers mentioned EXD2: One of them identified EXD2 mRNA localization in the *Xenopus* germ plasm, another one suggested it was part of the Fanconi Anemia complex that functions to repair DNA crosslinks, a third reported EXD2 as a host caftor essential for Dengue virus propagation and a fourth suggested EXD2 was the *Drosophila* orthologue of the WRN nuclease-helicase important for recombination. We began this project by addressing the subcellular localization and potential role of EXD2 in crosslink damage repair. We found that the protein localized solely to the mitochondrion and its depletion did not affect sensitivity to crosslinks, making its participation in the FA complex highly unlikely.

To date, there is evidence of various DNA repair pathways functioning in the mitochondria and a number of DNA repair proteins that are being shuttled between the nucleus and the mitochondria. However, the relative importance of different repair pathways in the mitochondria and the factors that localize to the mitochondria remain to be determined. Considering the structural similarity of EXD2 to the nuclear repair protein WRN, our initial hypothesis was that EXD2 was

involved in DNA repair or replication n the mitochondrion. Consistent with this hypothesis we did confirm the localization of EXD2 to the organelle, as well as its binding to the mtDNA. We also observed that cells lacking EXD2 have increased incidence of mtDNA lesions, suggesting it may have a role in maintaining mtDNA integrity. However, as EXD2 lacks the RECG helicase domain that is essential for the known functions of WRN, it is clearly not a direct orthologue.

Studies during the past thirty years have produced three main theories of how the mammalian mitochondrial DNA is replicated. The two most recent of them describe a participation of RNA-templates in this process, but the enzymes involved in the synthesis, editing and degradation of these intermediates remain to be found. Equally unknown are the nucleases involved in tRNA- editing and the enzymatic machinery responsible for the expression and degradation of non-coding RNAs. Our biochemical analysis has shown that EXD2 is potentially one of the most versatile mammalian exonuclease/exoribonucleases identified to date. We further hypothesized that its substrate(s) might also be some species of RNA of RNA/DNA hybrids that exist in large parts of the mtDNA. In 2D analysis of mt replication intermediates we did not observe any clear alterations but are looking further using more sensitive techniques.

A role for EXD2 in RNA maintenance is supported by our cellular and *in vivo* analysis. We discovered that loss of EXD2 led to severe metabolic reprogramming *in vitro,* as well as to reduced tumor growth *in vivo.* In addition, the deletion of EXD2-orthologue in *Drosophila* led to increased lifespan and altered stress-sensitivity in the flies. In the light of current literature such severe metabolic changes cannot be explained by the mild defect in the mtDNA-quality we have observed.

Considering all our data, we propose that EXD2 is affecting mtDNA integrity and stability by processing some of the many species of mitochondrial RNA. The loss of EXD2 leads to accumulation of these RNAs and activation of a mitochondrial stress response, resulting in the metabolic phenotypes described in detail in this

thesis, including reductive carboxylation in cancer cells and lifespan extension in *Drosophila*. This work contributes to the understanding of the role of mitochondria in the context of cellular metabolism, cancer growth and organismal development and aging and how the crosstalk between them

Abbreviations

2-7DCHF	2′,7′-Dichlorofluorescin diacetate
2-HG	2-Hydroxyglutarate
2D-age	2-dimensional agarose gel electrophoresis
4-OH	4-hydroxytamoxifen
α-KG	Alpha-Ketoglutarate
ACTC1	Alpha cadrdiac muscle actin 1
Ago	Argonaute
AP site	Apurinic/apyrimidinic site
APE1	AP endonuclease 1
APTX	Aprataxin
ARS	Aminoacyl-transfer RNA synthetase
ATP	Adenosine diphosphate
BER	Base excision repair
ChIP	Chromatin immunoprecipitation
CI-CV	Complex I- Complex V
CO_2	Carbon dioxide
$CoCl_2$	Cobalt Chloride
CoQ	Coenzyme Q
COX	Cyclo-oxygenase
CR	Calorie restriction
CREB	cAMP-response element binding protein
CSC	Cancer stem cell
CTRL	Control

CytC	Cytochrome C
DDR	DNA damage response
dILP	Drosophila insulin-like peptide
Dm6744	Drosophila melanogaster 6744 (dmExd2)
DMEM	Dulbecco´s modified Eagle Medium
dNTP	Deoxyribonucleotide
Ds	Double-strand
DSB	Double strand break
DUOX	Dual-oxidase
ECM	Extracellular matric
EcR	Ecdysone receptor
EM	Electron micrograph/microscopy
ENDOG	Mitochondrial endonuclease G
ER	Estrogen receptor
ERE	Estrogen responsive element
EtBr	Ethidium bromide
ETC	Electron transport chain
EtOH	Ethanol
EXD2	Exonuclease 3´-5´domain containing 2
EXD3	Exonuclease 3´-5´domain containing 3
EXOG	Mitochondrial exonuclease G
FA	Fanconi anemia
FADH2	Flavin adenine dinucleotide
FBS	Fetal bovine serum
Fe-S	Iron-sulphur
FEN1	Flap endonuclease 1
GEO	Gene Expression Omnibus
GLUT	Glucose transporter

GPX	Glutathione peroxidase
H^+	Hydrogen-ion
H_2O_2	Hydrogen peroxide
HDR	Homology-directed repair
HIF	Hypoxia inducible factor
HRE	Hypoxia responsive element
HSP	Heavy strand promoter
HSP70	Heat shock protein 70
hTERT	Human telomerase
htmPAP	Human mitochondrial poly(a)polymerase
ICDH	Isocitrate dehydrogenase
IDH	Isocitrate dehydrogenase
IF	Immunofluorescence
IGF	Insulin growth factor
IHC	Immunohistochemistry
IIS	insulin/insulin like growth factor 1 (IGF-1) signaling
IM	Inner mitochondrial membrane
IMS	Intermembrane space
InR	Insulin receptor
LIG3	DNA ligase 3
LLR	Long loop repair
LP-BER	Long patch BER
LSP	Light strand promoter
MCM	Minichromosome maintenance
MLS	Mitochondria localizing signal
MMC	Mitomycin C
MMS	Methylmethane sulphonate
MMR	Mismatch repair

MPT	Mitochondrial transition pore
Mt	Mitochondria
MtDNA	Mitochondrial DNA
MTERF	Mitochondrial transcription termination factor
mtHSP	Mitochondrial heat shock protein
MtSSB	Mitochondrial single strand binding protein
mTOR	Mammalian target of rapamycin
MUTYH	*E.coli* MutY homologue
NAC	N-acetylcysteine
NADH	Nicotinamide adenine dinucleotide
Nc	Non-coding
nDNA	Nuclear DNA
NEIL1	Nei-endonuclease VIII-like (Homolog of *E.coli* Fpg)
NHEJ	Non-homologous end-joining
NMR	Nuclear magnetic resonance
NOX	NADPH oxidase
NRE	NRF-response elements
NRF	Nuclear respiratory factor
Nt	Nucleotide
NTH1	Nth-endonuclease III-like 1. (Homolog of *E.coli* endonuclease III)
O/N	Over night
O_2	Oxygen, molecular
O_2^-	Superoxide anion
OEX	Overexpression
OGG1	8-oxoguanine DNA-glycosylase 1
OH	Origin of replication, Heavy strand
OL	Origin of replication, Light strand
OM	Outer mitochondrial membrane

OXPHOS	Oxidative phosphorylation
PARP1	Poly-ADP-ribose polymerase 1
PCR	Polymerase chain reaction
PDH2	Prolyl hydroxylase domain protein 2
PDH	Pyruvate dehydrogenase
PEI	Polyethyleneimide
PGC-1α	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha
PKA	Phosphokinase A
PNKP	Polynucleotide kinase/phosphatase
PNPase	Polynucleotide phosphorylase
POLG	Polymerase gamma, subunit 1
POLG2	POlymerase gamma, subunit 2
POLRMT	Mitochondrial RNA polymerase
PPP	Pentose phosphate pathway
PRPP	Phosphoribosyl pyrophosphate
PTGS	Post-transcriptional gene silencing
rDNA	Ribosomal DNA
RITOLS	RNA incorporated throughout lagging strand
RNAi	RNA interference
RNAPOL	RNA polymerase
ROS	Reactive oxygen species
RPA	Replication protein A
RRM2B	Ribonucleotide reductase M2 polypeptide pseudogene 3
rRNA	Ribosomal RNA
SDH	Succinate dehydrogenase
SDM	Strand displacement mechanism
SF-TAP	Strep-flag tandem affinity purification
Sh	Short hairpin

Sir/SIRT	Sirtuin
SLIT2	Slit homolog 2 (Drosophila)
SOD	Superoxide dismutase
SP-BER	Short patch BER
sRNA	Small RNA
Ss	Single-strand
Ssb	Single-strand break
StAR	Steroidogenic acute regulatory protein
T3	3-Iodothyronine
TCA	Tricarboxylic acid cycle
TDP1	Tyrosyl phosphodiesterase 1
TFAM	Mitochondrial transcription factor A
TFB1M	Mitochondrial transcription factor 1
TFB2M	Mitochondrial transcription factor 2
TIM	Translocase of the inner membrane
TOM	Translocase of the outer membrane
TopoI	Topoisomerase 1
TOR	Target of rapamycin
UNG1	Uracil-DNA glycosylase 1
UPR	Unfolded protein response
UTR	Untranslated region
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
VHL	Von Hippel Lindau tumor suppressor
WB	Western blot
WNT7B	wingless-type MMTV integration site family, member 7B
WRN	Werner

WT	Wild type
YB1	Y-box binding protein 1
YW	Yellow white
ΔΨ	Mitochondrial membrane potential

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1. General Introduction: The mitochondria

Mitochondria are membrane-bound organelles found in eukaryotic cells that are involved in various cellular functions such as energy production, apoptosis and the regulation of cellular metabolism. Mitochondria are thought to have evolved millions of years ago when a nucleated cell engulfed an aerobic prokaryote([1],[2]). This endosymbiosis led to a mutually beneficial environment where the prokaryote was protected by the surrounding cell and provided the eukaryotic progenitor a new source of energy in aerobic conditions, facilitating the adaptation to the increasing oxygen content of the early atmosphere[3]. This bacterial ancestry of mitochondrial DNA (mtDNA) maintenance systems makes the mitochondria susceptible to antimicrobial drugs and viral nucleoside analogs. Instead of existing as a single organelle, the mitochondria can be described better as a dynamic network, undergoing constant fusion and fission events as the mitochondria move along cytoskeletal structures of the cell. Thus the number of mitochondria per cell is hard to determine, but depending on the cell type, it can vary from tens to tens of thousands (ex. oocytes). Exceptions to this are the red blood cells that extrude the mitochondria, along with other organelles during erythrocyte maturation[4], and some rare amitochondrial eukaryotic organisms[5]

Recent advances in proteomic and genomic approaches have provided a comprehensive inventory of mitochondrial proteins in various eukaryotes. The mammalian mitochondria contain over 1500 proteins, of which only 13 are encoded by the mtDNA. This makes mitochondria highly dependent on the proteins and lipids from other cellular compartments, making the active transport of these components crucial for mitochondrial function. The cell expends considerable resources to maintain the mitochondrial compartment in order to ensure the integrity of the various roles that it plays. This is demonstrated further by the fact that mitochondrial dysfunction is associated with an increasingly large proportion of

human hereditary diseases, including neurodegenerative disorders, cardiomyopathies, metabolic syndromes, cancer and obesity.

2. Structure of Mitochondria

Mitochondrial membranes

The mitochondria are surrounded by a phospholipid bilayer very similar to that of bacteria. The outer membrane (OM) separates the intermembrane space (IMS) of the mitochondria from the cytoplasm and serves as a diffuse barrier for small molecules such as sugars, amino acids and adenine nucleotides. The OM also plays a significant role during apoptosis, when it releases cytochrome c and the apoptosis-inducing factor to the cytoplasm, a point of no return in programmed cell death[6]. Molecules larger than 5000 Daltons are transported through the OM by active transport via transport proteins and specialized translocases of the outer membrane (TOM) complexes. The outer membrane also contains enzymes involved in elongation and degradation of fatty acids, oxidation of adrenaline and degradation of tryptophan (Orientations of Proteins in Membranes database, http://opm.phar.umich.edu/).

The inner mitochondrial membrane (IM) provides the major permeability barrier in the mitochondria. It is only freely permeable to oxygen, carbon dioxide and water molecules and the translocase of the inner membrane (TIM) is required for protein transport across the membrane or for insertion into the membrane itself. The structure of the IM is very complex and contains more than 150 proteins and is rich in cardiolipin, a phospholipid characteristic of bacterial membranes. The inner membrane is folded into compartmentalized structures called the cristae, to maximize the surface area for electron transport chain proteins required for energy production.

The inner membrane encloses the mitochondrial matrix containing 2/3 of the proteins in a mitochondrion. The high concentration of proteins makes the matrix viscous and creates osmotic pressure believed to be maintained by aquaporin that forms channels that regulate water transport [7]. Another factor contributing to the creation of osmotic pressure is the higher pH of the matrix (7.8 vs cytosolic 7.0).

This is an important regulator of mitochondrial function and affects directly the strength of proton gradient required for electron transport chain (ETC) as well as proper folding of various pH-sensitive proteins in the mitochondria[8]. The mitochondrial membrane potential (ΔΨ), sustained by the matrix pH and the ETC together, is a crucial regulator of the import and export of various signaling molecules and proteins through the mitochondrial membranes.

Mitochondrial genome

MtDNA organization and nucleoid structure

The first proof of the existence of the mitochondrial genome was found in the 1960s[9]. During the same year, electron micrograph (EM) pictures of chicken liver mitochondria with structures stained for DNA were published and DNA was isolated from yeast mitochondria[910]. Despite 60 years of research following its discovery, the elucidation of the processes governing mtDNA maintenance is still in its infancy.

The mtDNA is a negatively supercoiled circular DNA molecule of approximately 16,5 kilobases (kb). It is packed in structures called nucleoids and encodes for 37 genes, of which 13 are components of the respiratory complexes, 22 are transfer RNAs (tRNAs) and 2 are mitochondrial ribosomal RNAs (rRNAs) necessary for translation (Figure 1). Similar to its bacterial ancestors, the mitochondrial genome is guanine and cytosine -rich consisting of two strands of DNA, the heavy (guanine-rich) and the light strand (cytosine-rich), it lacks introns and almost all noncoding DNA is concentrated in one region. This region is called the noncoding region (NCR), and much of it is occupied by the D-loop that contains one of the two origins or replication and the two origins of transcription. The importance of the NCR to mitochondrial genome maintenance is underlined by the fact that all the characterized, partially deleted mtDNA in humans retain this structure[11].

The processing of the mtDNA is challenged by the fact that it is tightly surrounded by the mitochondrial transcription factor and DNA packaging protein mt Transcription Factor A (TFAM) that inhibits various DNA processing enzymes from binding to it[12]. How the structure of the densely packaged mitochondrial nucleoids is regulated to allow mitochondrial replication, transcription and DNA repair to occur has yet to be solved.

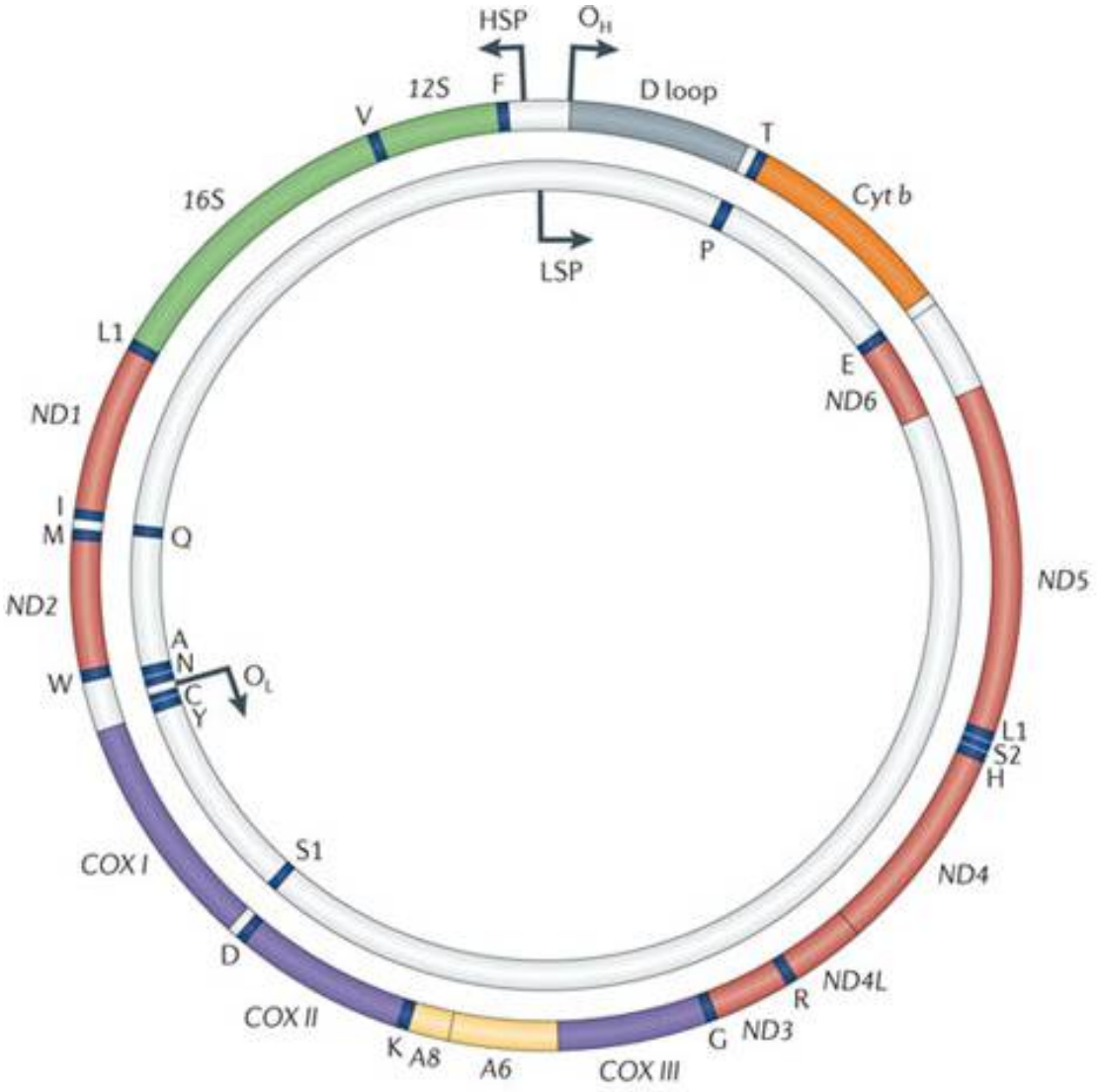

Figure 1. Mitochondrial genome encodes for subunits of oxidative OXPHOS complexes. The mitochondrial genome encodes for 13 subunits of the respiratory complexes (gene-codes), 22 tRNAs (one-letter code) and 2 rRNAs (12S and 16S) necessary for translation. MtDNA replication

starts from two origins of replication, the heavy and light strand promoter (OH and OL, respectively). The transcription start sites are the heavy strand promoter (HSP) and light strand promoter (LSP). Picture modified from Schon *et al* [13].

MtDNA replication

Chromosomal DNA replication in both prokaryotes and eukaryotes occurs via coupled leading and lagging-strand DNA synthesis, although many plasmids and viruses use alternative replication mechanisms[14]. The mechanism of mtDNA replication was first addressed in the 1970's by cesium chloride separation of different replication intermediates[15 16]. Several groups confirmed the presence of a long stretch of single stranded DNA following the initiation DNA synthesis. The resulting model, whereby leading strand synthesis started at a specific site and advanced approximately two-thirds of the way around the molecule before second-strand synthesis is initiated, was named the strand displacement mechanism (SDM) (Figure 2A)[17 18]. It differed from both nuclear and bacterial DNA replication due to the presence of such a long single-stranded intermediate[17 19]. The model of SDM was supported ten years later by Clayton and colleagues who were able to map two free 5' end sites approximately 11kb apart and on opposing strands, suggestive of strand-specific replication initiation sites[19].

Coinciding with the proposal of the SDM-theory, another group reported the existence of fully duplexed mtDNA intermediates, suggesting a resemblance to Okazaki fragments, similar to those involved in chromosomal replication[20]. However, this step-wise model of replication was largely ignored and for the next few decades the SDM theory was mostly unchallenged. This changed when a new method called neutral two-dimensional agarose gel electrophoresis (2D-AGE) was introduced to the field. 2D-AGE separates the DNA based on the shape and mass, capturing the complete series of intermediates of the replication cycle in arc- and spot- patterns on autoradiographs [21 22]. To aid the interpretation, various site-specific restriction enzymes can be used to distinguish DNA duplexes, DNA-RNA hybrids and single strand nucleic acids. Using these methods, Holt and colleagues

were able to show that the single-strand (ss) DNA previously identified was in fact resistant to single-strand specific enzymes, arguing against both the SDM and step-wise models[23]. In addition, its migration pattern on the gel resembled to that of a double-stranded (ds) DNA.

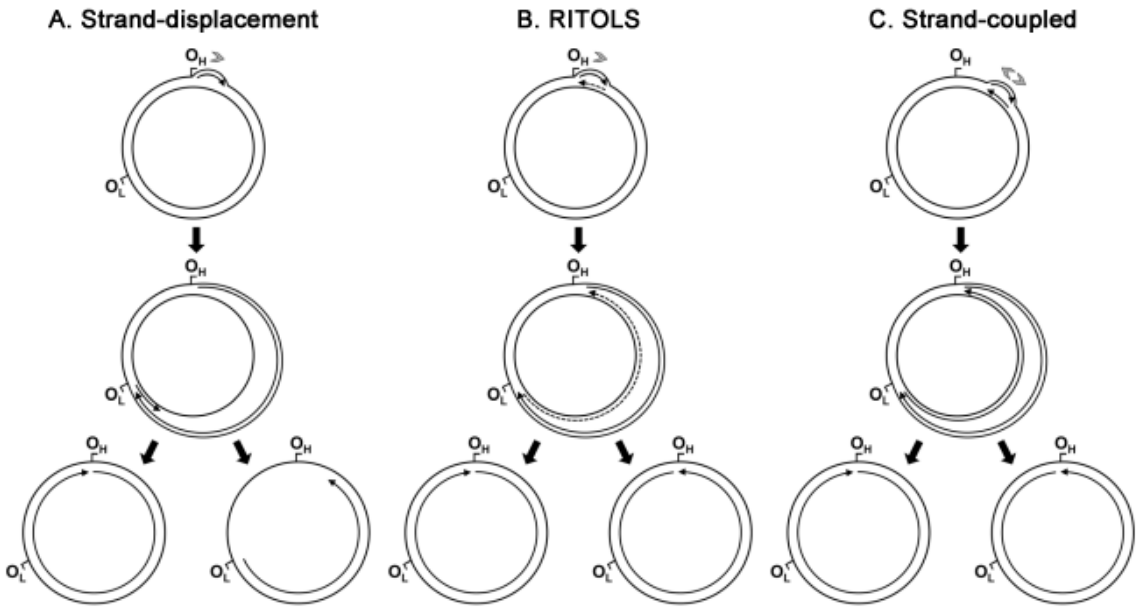

Figure 2. Proposed models for mtDNA replication. A. Replication starts in OH and proceeds continuously. Once the replication machinery passes OL-site, the lagging strand replication is initiated and proceeds in reverse direction until the whole strand has been duplicated. B. Replication starts in the D-loop area and segments of RNA are incorporated as RNA/DNA hybrids along the lagging strand. C. Replication starts at initiation zone outside of the d-loop and proceeds bi-directionally along both strands. Dashed line represents RNA/DNA hybrids, solid lines the DNA and arrowheads the direction of replication. Picture modified from McKinney and Oliveira 2013[24]

The identity of the second strand of the duplex was finally revealed when it was exposed to digestion with RNase H[25]. RNase H only degrades RNA bound to DNA, and indeed, the presence of RNA complementary to the lagging strand was subsequently confirmed, leading to a new model for mitochondrial replication

termed RITOLS (RNA incorporated throughout lagging strand) (Figure 2B)[26] . According to this new model, an RNA strand is synthetized and incorporated in the 3'-5' direction with the lagging strand, sheltering the DNA from deletions and mutations more efficiently than the mitochondrial single strand binding protein (mtSSB), also found in the replication structures. The evolutionary rationale for this might be that the RNA contains the same genetic information as the DNA, thus serving as back-up information for the repair of damage occurring during lagging strand synthesis.

Although the DNA-RNA duplex seems to be the most prevalent mitochondrial replication intermediate, a small part of the DNA appears to exist as dsDNA. The presence of two dsDNA molecules in mammalian mt has been shown to be present during recovery from ethidium bromide depletion of mtDNA. This kind of coupled leading and lagging strand synthesis is known to occur in the bacteria, and might very well be operating simultaneously with other proposed mtDNA replication mechanisms (Figure 2C)[23]. The RITOLS-theory has provoked many new questions about the identity of enzymes and factors involved in it. How is the lagging-strand RNA synthesized, incorporated and degraded? Is it used for priming or solely for protection? Which, still unidentified, enzymes are involved in these processes? And if the conventional strand-coupled DNA replication occurs in mitochondria, there must be apparatus for both priming and Okazaki fragment maturation. As part of the DNA-maintenance proteins are shared between the nucleus and mitochondria, including flap endonuclease 1 (FEN1), AP-endonuclease (APE1), DNA2 and uracil-DNA glycosylase 1 (UNG1), it is likely that additional nuclear factors will be found to be involved in the replication of the mtDNA [27 28 29].

Proteins involved in MtDNA replication

MtDNA replication is thought to be coupled to mt transcription as it is believed that transcription starting from LPS generates the RNA-primer needed to initiate mtDNA

replication, but neither the mechanism enabling the transcription machinery to switch from genome-length transcription to primer formation at the OH nor the enzyme(s) involved is known. Very few enzymes have been identified to be critical for mtDNA replication. Mt replication is dependent on the minimal replisome, consisting of five proteins; the 2 subunits of Polymerase gamma (POLy), the DNA helicase TWINKLE, the mtSSB and the mitochondrial RNA polymerase POLRMT.

POLy consists of two catalytic subunits, one with exonuclease activity (PolyA) and an accessory subunit (PolyB)[30 31]. To date, Poly is the only known polymerase devoted specifically to mtDNA synthesis but there is no clear reason why the mitochondria would need to restrict themselves to only one DNA polymerase given the importance of mitochondrial genome integrity[32]. Recently the Primase-Polymerase enzyme PrimPol was identified as a nuclear and mitochondrial factor[33]. Depletion of PrimPol in human cells led to decreased nucleoid levels and impaired recovery of Ethidium Bromide (EtBr) depleted mtDNA genomes, consistent with a role in mtDNA synthesis. However, other human cell types than those used in that study or mouse cells or tissues lacking PrimPol expression have higher levels of mtDNA[33]. The precise role of PrimPol in the mitochondrion remains to be clearly elucidated.

Although the exact role of the mtSSB has ben debated, it appears to be necessary for the replication process as flies with disrupted mtSSB have severe mtDNA depletion and defective mitochondrial respiration[34 35]. MtSSB is considered to be analogous to replication protein A (RPA) that protects single stranded DNA generated during replication and plays multiple roles in signaling and the physical recruitment of factors to replication forks. In this regard, it is interesting to note that PrimPol interacts with RPA in the nucleus and with mtSSB in the mitochondria, suggesting conserved roles in replication, bypass of oxidative lesions or recovery of stalled forks[36].

TWINKLE is a mammalian 3' to 5' helicase that unwinds the DNA during replication and its depletion directly affects mtDNA copy number[37]. TWINKLE is considered

analogous to the minichromosome maintenance (MCM) complex in the nucleus and it functions in conjunction with Poly at replication forks [38].

The most recently discovered protein of the minimal replisome is POLRMT[39]. It synthesizes RNA primers with lengths of 25-75 nucleotides (nt) that can be used by the POLy to initiate DNA synthesis in a stem-loop structure of lagging strand origin of replication. In bacteria, termination of replication and resolution of replication intermediates is a well-orchestrated process, which requires a site-specific recombination apparatus[40]. However, in mammals we merely have the suggestion that three proteins of the Mitochondrial Transcription Termination factor (mTERF) family, mTERF, mTERFD1, and mTERFD3, in some way contribute to the termination of DNA replication[41 42].

MtDNA repair

Considering the importance of mitochondria to cellular functions, maintaining the integrity of its genome is critical. The mitochondrial genome lacks canonical histones and is more highly exposed to reactive oxygen species (ROS), which is believed to be a contributing factor in the high mutagenesis rate of mtDNA. The mechanisms of how the mitochondrion repairs its DNA and the enzymes involved are still to be identified, but it is becoming increasingly apparent that the mtDNA repair relies heavily of the machinery of nuclear DNA-damage repair processes.

Similar to the nuclear DNA, the mitochondrial genome may be damaged by exogenous agents, including Ultraviolet (UV) and ionizing radiation (IR) and DNA damaging chemicals, as well as by endogenous agents that arise from cellular metabolism, such as ROS. There are four types of DNA-damage known to occur in the mitochondria: base lesions, single-strand breaks (ssb), double strand breaks (dsb) and base-base mismatches, and the pathways to repair these damages

share many mechanistic similarities with the nDNA (nuclear DNA) DNA damage response (DDR).

Base Excision repair

One of the main types of mtDNA damage is the ROS-induced base lesion. These lesions include non-bulky chemical alterations such as alkylation, deamination, oxidation or spontaneous loss of the DNA base. Once the lesion is detected it is largely repaired by the base excision repair (BER) pathway, the best characterized and probably the most important repair pathway operating in the mitochondria[43 44].

Both in the nucleus and the mitochondria the BER pathway consists of four steps; excision and strand cleavage, DNA end-processing, gap filling and ligation. However, before the first step, the site of damage has to be recognized by sensor proteins that scan the DNA. This is accomplished by DNA glycosylases that diffuse slowly along the DNA, probing the DNA for damage until it is specifically recognized[45]. Depending on the availability of the proteins, either monofunctional or bifunctional glycosylases are used. The monofunctional glycosylases include UNG1 and the *Escherichia coli* MutY homologue (MUTYH) that cleave dsDNA N-glycosylic bond, leaving an apurinic/apyrimidinic site (AP site)[46 47]. After this the APE1 cuts the phosphodiester backbone 5' to the AP-site, creating a 3' –hydroxyl (OH) end that can be used for DNA synthesis but leaving the 5' phosphate group that needs to be removed by POLy1 and to allow the consecutive ligation of the newly synthesized nucleotide via the short-patch (SP) BER. If POLy cannot process the 5' end and create ligatable ends, DNA repair will switch to long-patch (LP) BER. During LP-BER POLy creates either short 5' ssDNA flaps of uncoated nucleotides or longer flaps covered by RPA-protein that can be further removed by synergistic activity of FEN1 and human DNA2[48 49]. After this the DNA ligase 3 (LIG3) can finally link the ends and re-establish a continuous strand of DNA[50 51 52].

There are four mitochondrial bifunctional glycosylases known to date. The 8-oxoguanine-DNA-glycosylase 1 (OGG1) and a homologue of *E.coli* endonuclease

III (NTH1) are specific for dsDNA whereas homologues of *E-coli* Fpg and Nei (NEIL1, NEIL2, respectively) detect ssDNA structures with base lesions[53 54]. After detecting the lesions, the bifunctional glycosylases can incise the damaged nucleotide, and with the help of APE1 and polynucleotide kinase/phosphatase (PNKP) leaving a 3'-OH end that can be processed via the SP-BER where the POLy adds a single nucleotide to replace the damaged one and then LIG3 can proceed to seal the gap[54 55].

Indeed, the mechanisms and many of the proteins involved in BER are shared between the nucleus and the mitochondria. One notable difference between BER in the two cellular compartments is their subcellular localization. Most nuclear BER proteins are in soluble form in the nucleoplasm and reorganize in subnuclear foci after the induction of the DDR[56]. In contrast, the proteins involved in mtBER interact with the IM and the damaged DNA is only repaired when associated with the IM[57 58]. This has prompted the idea that mtDNA might scroll through a membrane-associated complex that replicates, proofreads and repairs it, making the mtDNA the mobile part of the process[59]. There is some evidence for this type of model of mtDNA processing in *E.coli*, and in yeast it has been shown that the mt replisome is tightly anchored to the mt membranes by elements in the cytoskeleton[60].

DNA break repair

Like many of the proteins involved in BER, it seems also that many DNA break repair proteins are shared between the nucleus and the mitochondria. One of the main proteins involved in SSB recognition in the nucleus is the poly (ADP ribose) polymerase 1 (PARP1) that has recently been shown to localize to the mitochondria[61]. Another dual-localizing SSB repair protein is tyrosyl-phosphodiesterase 1 (TDP1) that can process breaks or abortive intermediates formed during transcription and replication by topoisomerase-1 (TopoI). TDP1 is

also implicated in the repair of nuclear and mitochondrial DNA breaks induced by radiation, potentially through the modification of damaged 3' nucleotides[62]. Another example is Aprataxin (APTX) that repairs 5' adenylate adducts caused by abortive DNAse ligase activity in the nucleus and was recently found to possess an N-terminal mitochondrial localizing signal (MLS) and localize to the mitochondria where it has been shown to influence mtDNA homeostasis[63]. Interestingly, mtAPTX seems to be expressed most hjghly in the human brain, specifically in the cerebellum, suggesting a tissue-specific but yet unstudied role for it in mtDNA maintenance[63] [64]. Mutations in APTX underlie the human neurodegenerative disorder ataxia-ocuolar apraxia, but whether the nuclear or mt roles are more important in disease has not been established[65]. In addition to the dual-localizing ssDNA-repair proteins, mammalians possess a nuclease implicated in ssDNA repair solely in the mitochondrion; Exonuclease G (EXOG)[66]. EXOG has both endonuclease and 5'-3' exonuclease activity, and lack of EXOG leads to changes in the mitochondrial metabolism, including increase in ROS and decline in OXPHO, as a result of accumulation of SSBs[67]. A paralog of EXOG, Endonuclease G (ENDOG), also participates in the mitochondrial ssDNA repair[68]. It has been shown that upon Herpex Simplex virus 1-infection both EXOG and ENDOG participate in the depletion of mtDNA from the infected cells[69]. EndoG has an additional role during the apoptosis, when it translocates to the nucleus and triggers DNA-degradation[66].

In the nucleus, DNA double strand break (DSB) repair can occur either by non-homologous end joining (NHEJ) or by homology-directed repair (HDR) pathways[70]. Many of the factors involved in nuclear NHEJ have been reported to locate to the mitochondria and some of them seem to interact with mtDNA and proteins bound to it. However, the confirmation and identification of many of them, as well as the relevance of DSB repair to mt homeostasis, still remains to be determined[71]. Despite the presence of numerous copies of mtDNA in every cell, recombination between two mtDNA genotypes rarely occurs. However, as most of the proteins

needed for HDR pathways are found in the mitochondria, this suggests that the mitochondrial recombination apparatus may function to resolve daughter DNA molecules at the end of the replication cycle, as occurs in bacteria[40]. It has also been suggested that mitochondria may possess a viral-like mechanism, and use complementary RNA as template for homologue-directed repair processes[72].

Mismatch repair

Errors of replication that result in a mismatch or a small loop are detected and corrected by the mismatch repair (MMR) system. Mitochondrion-specific MMR has been observed in various cell lines *in vitro* and in rat mitochondrial lysates, but none of the nuclear MMR proteins have been found in the mitochondria[73 74]. This would suggest the existence of a whole set of mitochondrial enzymes operating in the MMR pathway yet to be discovered. So far the only known protein involved in the mtMMR is the multi-functional Y-box binding protein 1 (YB1) which is thought to recognize the damage and recruit the mtMMR complex to initiate repair[74]. In the nucleus after the recognition of the mismatched nucleotide, it is nicked and the following repair is guided by the daughter strand in a strand-specific way. However, similarly to bacterial MMR, mtMMR is thought to be neither nick-directed nor strand-biased, and much less efficient than nMMR [73]. One explanation for the lack of evidence for mtMMR is that the mt have an alternative repair pathway to repair mismatched nucleotides. Long loop repair (LLR) has been described in yeast and higher eukaryotes, and even in mammals, to recapitulate some of the functions that could be used for repairing mitochondrial base mismatches[75 76 77]. In the mammalian mt this mechanism seems to be totally independent of MMR proteins or other known DDR proteins, and supposedly uses the various direct repeats found in the mtDNA to process the loops[75 78].

MtDNA degradation

Without the presence of specific nucleases, DNA is a very stable molecule and its half-life has been estimated to be over 500 years[79]. However, in a living organism, when a cell becomes dysfunctional due to damage to either its DNA or other macromolecules and enters apoptosis, nucleic acids are rapidly degraded by specific, endogenous endonucleases into nucleosomal fragments[80 81]. Since each cell harbors many copies of mtDNA, the irreparable damage to one genome does not lead to cell-wide consequences. It has been reported that while consistent, long-term damage to the mtDNA leads to the activation of mtDNA repair and degradation machinery, the mutagenic consequences are minimal [82 83]. This could be explained by efficient degradation of the damaged DNA form the organelle, leading to mitochondrial dysfunction and leakage of fragments of mtDNA to the cytosol. In turn, mtDNA found in the cytosol can be mistakenly considered of viral or bacterial origin and has been shown to trigger a potent autophagic innate immune response[84]. Additional evidence for the role of autophagy in mt and mtDNA elimination comes from a study done in *C.elegans*, showing that paternal mitochondria are removed from the oocytes after fertilization by autophagy[85 86]. Whether specific mitochondrial nucleases are involved in the degradation process remains unknown. However, there is evidence that the mitochondria use salvage pathways for deoxyribonucleotides (dNTPs) more efficiently than does the nucleus, specifically during quiescence, whereas the mt of proliferating cells rely on dNTP transporters[87]. The importance of the balance in mtDNA precursor pool has been further established in context of various diseases resulting from alterations in mitochondrial dNTPs but despite great efforts, the majority of the enzymes involved in the mitochondrial nucleotide salvage pathways remain to be identified[88 89 90 91],.

Mitochondrial RNAs

Mitochondrial trancription

The transcription of the mitochondrial DNA, similarly to bacteria, creates a polycistronic mRNA-strand. Transcription occurs and in conjunction with the mitochondrial replication and translation, and the machineries participating in each of these processes have been found to reside in close proximity to each other, suggesting that they are regulated in a highly complex way at nucleoid-structures[57] [92]. The mt genome possesses three transcription initiation sites that reside in the D-loop area and its vicinity in the noncoding region. The heavy strand is transcribed from two H-strand promoters, HSP1 and HSP2, whereas there is only one LSP. Although their exact roles are still unclear, there are three main participants in the transcription initiation machinery; TFAM, POLRMT and either of the mitochondrial transcription factors 1 or 2 (TFB1M, TFB2M)[93] [94]. The mTERFs 1-4 have been implicated in both initiation and termination of transcription, but the results remain controversial. They all bind the D-loop structure, but it seems that MTERF1-2 are positive regulators of mt transcription, whereas MTERF3 inhibits it, and MTERF4 might be involved in RNA methyltransferase activity[95] [96].

The transcription of the mtDNA starting from the HSP2 and LSP creates polycistronic mRNAs with the size of the full-length mt genome. Each of the 13 protein coding and 2 rRNA coding genes are flanked by tRNAs, that serve as sites for endonucleolytic cleavage, yielding most of the mature mRNAs, rRNAs and tRNAs[97]. After this, the 5' and 3' ends are processed by RNase P and RNase Z respectively, and a CCA-triplet is added in the 3' end of the tRNAs prior to the attachment of the corresponding amino acid. The post-transcriptional modification of the mRNA and rRNA consists of polyadenylation carried out by the mt poly(A) polymerase that adds adenosines at the 3' end of the transcript. In some cases this

provides part of the stop codon and it may also act as a stabilizing factor. In addition to the HSP2 and LSP, a third transcript, the HSP1, is synthesized and consists only of the rRNA-genes.

Recently, with the development of chromatin immunoprecipitation (ChIP) and DNA footprinting methods, various nuclear transcription factors have been found to bind to mtDNA and regulate mitochondrial gene expression independently of their nuclear roles. Tumor suppressor p53 has been co-immunoprecipitated (CoIP) with the mtDNA binding TFAM and POLy and directly affects the replication and repair of mtDNA by possibly inducing conformational changes in TFAM and its ability to bind damaged mtDNA [98] [99]. Cyclic-AMP response binding element (CREB) has been shown to bind the D-loop area after phosphorylation by mitochondrial Phosphokinase A (PKA)[100].

In addition, both of the estrogen receptors (ER), a and b, have also been found to bind directly to the estrogen responsive elements (ERE) of mtDNA[101]. However, the evidence that estrogen treatment alters mitochondrial gene expression is still lacking. Some research supports the role of ERs in regulating mitochondrial function by modulating protein-protein interactions in the organelle[102], and indeed the involvement of ER in the induction of mt biogenesis through its broad effects on nuclear transcription are unquestionable. The effect of estrogen on cellular functions will be discussed in more detail further in this thesis.

Another multifunctional mitochondrial transcription factor shown to bind to the mtDNA in various locations is p43, the mitochondrial isoform of receptor for thyroid hormone tri-iodothyronine (T3). Similar to estrogen, T3 regulates a vast array of nuclear genes involved in mt biogenesis, while p43 can also bind to various elements in the mtDNA, stimulating its transcription in a thyroid hormone-dependent manner[103] [104]. Thyroid hormone has also been implicated in the translocation of adenine nucleotides across the mitochondrial membrane to support mtDNA replication[105].

Degradation of Mt mRNAs

Due to the polycistronic transcription, the mitochondria must possess a robust and highly regulated RNA degradation and maintenance system to ensure that the optimal number of each transcript is maintained and that by-products or damaged transcripts are eliminated[106]. To date, no such nuclease activity has been found in the mammalian mitochondria, although SUV3 and polynucleotide phosphorylase (PNPase) are possible candidates[107]. In eukaryotes polyadenylation of RNA transcripts acts to prevent degradation. In bacteria and yeast mitochondria, it has the opposite role, marking the RNAs for rapid degradation. To study the role of poly(A) in the mammalian mitochondrial transcripts, several groups silenced the mitochondrial poly(A) polymerase (hmtPAP), the protein responsible for the poly(A) synthesis[108 109]. The first group observed no changes in the level of transcripts and the latter the depletion of the mitochondrial poly(A) transcript pool, leading to a controversy that has not been resolved. To date, the mechanism and proteins involved in mitochondrial mRNA degradation continue to be largely unknown.

Mitochondrial non-coding RNAs

Bacteria are known to harbor various small RNAs (sRNAs) involved in transcription and protein synthesis, and many of their sRNAs are essential in stress response regulation during cold, iron repletion and sugar stress[110 111]. Therefore it was not a surprise when it was recently reported that murine and human mitochondrial genomes encode thousands of small noncoding (nc) RNAs, mostly derived from the sense transcripts of the mitochondrial genes. They also showed that, contrary to the nuclear encoded sRNAs, mitochondrial sRNAs are not spliced by Ribonuclease III DICER, and no known components of the RNA interference (RNAi) machinery are present in the mitochondrial[112]. Therefore the biogenesis of Mt sRNAs must be mediated by one or more currently unidentified mitochondrial ribonucleases. An alternative possibility is that mitochondrial sRNAs act as

epigenetic regulators and alter mitochondrial gene expression by modulating mtDNA methylation, which would also explain the lack of RNAi machinery in the mitochondria[113]

In addition to sRNAs, mitochondria seem to harbor longer regulatory RNAs that are complementary to its genome. Studies from laboratory of Burzio et al. have confirmed the existence of three long ncRNAs synthetized in the mitochondria from mouse and human cells whose expression correlates with cell proliferation[114 114] . Interestingly, they later observed that the anti-sense RNAs were down-regulated in various cancer cell lines and tumor samples, suggesting this to be a universal characteristic of tumor cells[115]. If this holds true, the next challenge will be to decipher the mechanisms regulating transcription and degradation of these RNAs and the mitochondrial enzymes involved.

There is evidence that nuclear-encoded pre-micro and mature micro-RNAs can also enter the mitochondria and target some of its genes. The process of RNA import into the organelle has been observed in various organisms, but the import mechanism of these RNAs in mammals is still completely unknown[116]. In mammalian cells the main active protein of the RNA induced silencing complex, Argonaute (Ago2) has been described to be involved in transporting 70 nt RNA (tRNAmet) out from the mitochondria, and might be involved in similar processes involving micro-RNAs[117].

The Mitochondrial Proteome

The total mitochondrial proteome consist of the 13 proteins encoded by the mtDNA and the approximately two thousand nuclear encoded proteins that are transported to the mitochondrion to support its functions.

Mitochondrial protein translation

As mentioned earlier, mitochondrial protein translation is both physically and temporally coupled to mitochondrial replication and transcription. As the mRNAs of the 13 mitochondrially encoded proteins are transcribed, cleaved and processed, the mature mRNAs are channeled from RNA polymerase (RNAPOL) to the mito-ribosomes. Mito-ribosomes are bound to the IM, apparently facilitating the co-translational insertion of the OXPHOS subunits into the inner membrane[118]. As each transcript is produced at the same rate, the regulation of protein translation must be well-coordinated with the needs of each mitochondrion. Consistent with this, many mitochondrial translational activators specific to protein-coding mRNAs have been identified in yeast and have confirmed orthologues in humans[119 120 121 122]. In order to promote translation initiation, they bind to the 3' or 5'-untranslater regions (UTR) of mRNA and interact with mito-ribosomes[123 124]. In addition to the translational activators, mitochondrial membrane potential and lipid composition of IM are also known to affect mitochondrial protein synthesis via complex mechanisms[125 126]. Equally to unrecognized/dysfunctional mtDNA, also misfolded and damaged mitochondrial proteins have to be properly handled in order to maintain the cellular homeostasis. The first line of defence is controlled by the mitochondrial proteolytic quality control system consisting of molecular chaperones and proteases that can selectively remove the damaged and excess proteins from the organelle[127]. If this is not sufficient to restore the normal mitochondrial function in the cell the most severely damaged mitochondria can be removed by a selective autophagy, termed mitophagy. Mitophagy prevents the release of pro-apoptotic signals from the damaged mitochondrion, and rapidly after the degradation of the dysfunction organelle the distribution of healthy mitochondria is restored by mitochondrial fusion/fission[128].

Protein transport into the mitochondria

The mitochondria have the machinery necessary to translate the 13 proteins it encodes. However, the whole proteome of the mitochondria consists of close to

2000 nuclear encoded proteins that are needed for its functions. These proteins are translated as preproteins in the endoplasmic reticulum or cytosol, after which they are transported to the mitochondria. The vast majority of the proteins destined to go to mitochondria have an MLS that addresses them to the organelle. In soluble proteins, the MLS is 10-80 nt long and, with few exceptions, resides in the amino-terminus of the protein[129]. Upon entering the mitochondria it is in most cases proteolytically removed, allowing the protein to fold in its proper conformation in the organelle[130 131]. The MLS of the hydrophobic proteins targeted to the mitochondria is often embedded in their mature sequence. It is suggested that the MLS may help the nascent insoluble protein to travel through the cytosol in a more soluble conformation, together with various molecular chaperones that facilitate the protein traffic to the cellular organelles. Approaching the mitochondrial OM, both types of signal peptides are recognized by receptors of the TOM complex that consists of TOM20, 22 and 70. In concert with the cytosolic chaperones, they partially unfold preproteins to fit through the import channels formed by TOMs. In yeast it has been shown that in some cases the preproteins enter the TOM in a co-translational manner, while a part of them is still being translated by the cytosolic ribosomes located close to the OM[132]. After crossing through the OM, the soluble preproteins with the N-terminal signal peptide are guided through the TIM23 with the help of the mitochondrial heat shock protein 70 (mtHSP70) that drives the import of preproteins into the matrix in an adenoside triphosphate (ATP)-dependent manner. mtHSP70 also helps them to fold into their active form in the matrix after the removal of MLS by the mitochondrial processing peptidase. The hydrophobic mitochondrial proteins that bear internal MLS are processed by the TIM22-complex that inserts them into the IM. The whole process of mitochondrial import of proteins is highly dependent on mt $\Delta\Psi$ between the negative matrix and positive intermembrane space. Thus, prevention of proton leakage during protein import is crucial to prevent the collapse of the mitochondrion.

3. Mitochondrial function

In all cell types, mitochondria are the major cellular source of NADH and participate in pyrimidine and lipid biosynthetic pathways, including the fatty acid b-oxidation pathway, which converts long chain fatty acids to Acyl-CoA. Mitochondria also regulate cellular levels of metabolites, amino acids, and cofactors for various regulatory enzymes, including chromatin-modifying histone deacetylases. Moreover, mitochondria play a central role in metal metabolism, synthesizing heme and iron-sulphur (Fe-S) clusters,which are essential components of the major oxygen carrier, hemoglobin, as well as ETC and DNA replication and repair machinery[133]. Mitochondria also have many tissue-specific functions, including heat production in adipose tissue and ammonia detoxification in the liver[134].

Energy production as ATP (Figure3)

Glycolysis

Glucose, $C_6H_{12}O_6$, is the main energy source of the cell. The energy production of the cell relies on oxidation of glucose and converting the chemical energy released from breakage of carbohydrate bonds into ATP. This ATP generation, also called cellular respiration, involves three different processes: Glycolysis, the tricarboxylic acid cycle (TCA) and ETC. Although glycolysis occurs in the cytosol and the TCA cycle and OXPHOS by ETC take place in the mitochondria, these processes are tightly regulated and interconnected by feedback activation and inhibition loops responding to changes in cellular energy status and the availability of building blocks for energy.

Glucose enters the cell via members of a family of glucose transporters (GLUT1-10). After entering the cell, it is processed during glycolysis by a series of dehydrogenases, releasing electrons and hydrogen ions (H+). The ten-step

glycolysis produces two NADH, two ATPs and two three-carbon pyruvates per one glucose molecule. Glycolysis is also involved in degrading other monosaccharides, and each enzymatic step provides an entry point into glycolysis for molecules originating from precursors such as galactose or fructose. These sugars are degraded by glycolysis in parallel to glucose, and in the absence of glucose can be used to support glycolysis. After glycolysis, the processing of the two molecules of pyruvate can proceed via two pathways. In the case of anaerobic respiration, where oxygen supply is insufficient to fuel mitochondrial respiration, glycolysis can alone produce ATP needed for basal cellular maintenance. During anaerobic glycolysis pyruvate is broken down in lactic acid fermentation that restores the NAD+ that can be reused during the next round of glycolysis. Fermented lactic acid is secreted out of the cell, leading to local acidification of the cellular environment until normal oxygen levels and aerobic respiration is restored. However, if there is sufficient oxygen supply to sustain mitochondrial respiration, pyruvate is transported into the mitochondria via specific pyruvate transporters that enter it into the TCA cycle.

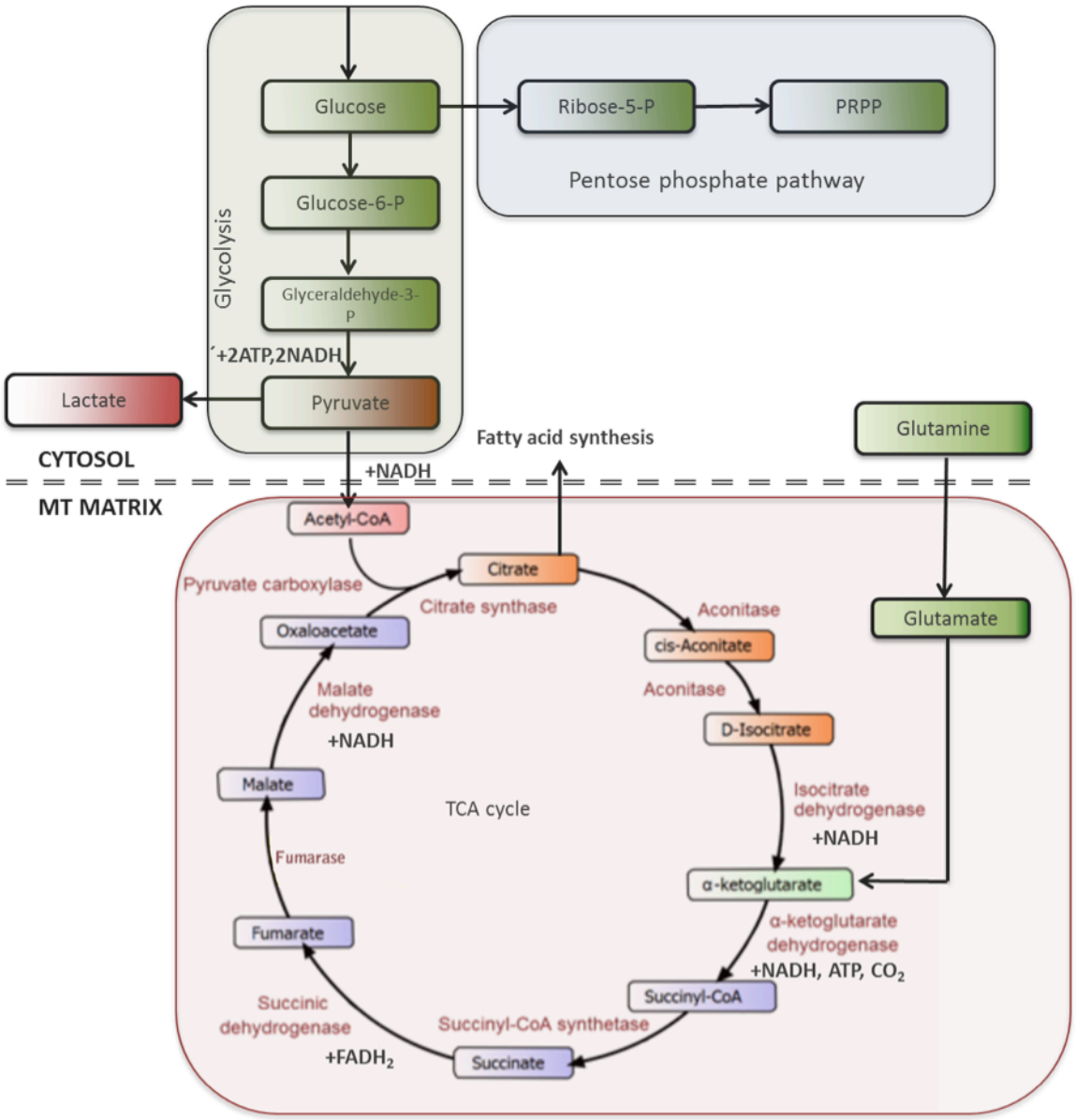

Figure 3. Cellular energy pathways. During glycolysis, glucose is catabolized into pyruvate, ATP, NADH and precursors for nucleotide synthesis in the pentose phosphate pathway (PPP) pathway. Pyruvate is then either fermented to lactate (anaerobic glycolysis) or transported into mitochondrion (aerobic glycolysis). In the mitochondria, during aerobic respiration, pyruvate is converted into Acetyl-CoA that enters the TCA-cycle. The TCA-cycle consists of a sequence of enzymatic reactions aimed to catabolize pyruvate to produce ATP, NADH, FADH2 and metabolic intermediates that participate in various cellular functions beyond energy production. PRPP, phosphoribosyl pyrophosphate.

TCA-cycle

In the mitochondrial matrix, pyruvate is converted by pyruvate dehydrogenase (PDH) to Acetyl-CoA, the first intermediate of the TCA cycle. PDH, like many other enzymes involved in metabolic regulation, is allosterically activated by its substrate, pyruvate, and inhibited by its products NADH and Acetyl-CoA, ensuring the fine-tuning of the TCA-cycle to meet the energy needs of the cell. The TCA consists of eight enzyme-catalyzed reactions used by all aerobic organisms to generate ATP, amino acid precursors and NADH. In the TCA-cycle the Acetyl-CoA is condensed with oxaloacetate by citrate synthase, forming a six-carbon citrate molecule. Citrate is then converted to isocitrate by aconitase, and further processed by isocitrate dehydrogenase to yield **α**-ketoglutarate (**α**-KG) and carbon dioxide (CO_2). This process is reversible and inhibited by ATP and enhanced by ADP and NAD, markers of low energy-state of the cell. **α**-KG is then processed by **α**-KG dehydrogenase to release the second of the two CO_2-molecules created during aerobic respiration, resulting in succinyl-CoA. This is the only non-reversible step in the TCA-cycle, preventing it from operating in the reverse direction. In the following four enzymatic steps, succinate, fumarate, malate, oxaloacetate and citrate are formed by succinate dehydrogenase (SDH), fumarase, malate dehydrogenase and citrate synthase respectively. Finally, the citrate is transported into the cytosol, where is it broken down to yield oxaloacetate and Acetyl-CoA that can be processed further to synthesize other biomolecules or return to the TCA-cycle as intermediates. The net gain of the TCA cycle is 2 ATPs, 6 NADHs and 2 FADH2s. However, many of the cycle intermediates serve as precursors for important biomolecules. For example, succinate-CoA is a precursor for the synthesis of porphyrins, a group of heme-producing proteins and oxaloacetate forms aspartate that can be converted further to other amino-acids and pyrimidine nucleotides[135].

The major force driving the TCA is sugar-derived pyruvate. However, there is another source of carbon that can be introduced in to the cycle. The six-carbon non-essential amino acid glutamine can be converted to glutamate and further to **α**-KG (and vice versa) to feed the TCA cycle. Glutamine is the most abundant amino acid in the human body, and most mammalian cells use this pathway to support growth and proliferation. However, cancer cells have been shown to increase glutamine uptake in order to provide building blocks for amino acid and nucleotide synthesis and regulate the cellular antioxidant response via glutathione, as well as neutralize lactic acid in the tumor environment[136 137 138].

Oxidative Phosphorylation

The final step of cellular respiration is OXPHOS. During OXPHOS the electrons stored in the form of NADH and FADH2 during glycolysis and the TCA cycle are used in redox reactions performed by the OXPHOS complexes, leading to conversion of ADP to ATP.

The OXPHOS apparatus consists of four protein complexes (I–IV) embedded in the mitochondrial IM, which undergo conformational changes through sequential redox-reactions to release electrons (from NADH/FADH2) and to pump protons from the matrix into the IMS (Figure 4). All except complex II have mitochondrially encoded subunits, rendering them susceptible to aberrancies in mitochondrial gene regulation. The first and largest of the complexes, NADH dehydrogenase (Complex I), consists of 45 core subunits with 9 Fe-S clusters, whose biogenesis requires an army of assembly factors[139 140]. It pumps 4 hydrogen ions across the membrane per one glycolysis-derived NADH electron it accepts, and serves as a link between glycolysis, TCA cycle, fatty acid oxidation and OXPHOS. Succinate dehydrogenase (Complex II) is the smallest complex and resides on the matrix side of the IM. It shuttles electrons via three FeS clusters and heme iron of cytochrome b560 and connects the TCA cycle to the OXPHOS chain by oxidizing

succinate to fumarate. Both complexes I and II transfer electrons directly to the cytochrome bc1 complex (Complex III). Each half of dimeric Complex III consists of 11 subunits and has a binding site for the lipid-mobile carrier ubiquinol/uniquinone. It also has various heme-centers and a subunit with FeS-domain called the Rieske protein. The Rieske-protein has been shown to be important in the generation of mtROS resulting in leakage of electrons at complex III. Complex III catalyzes the reduction of cytochrome c by oxidation of coenzyme Q, and concomitantly pumps 4 electrons from the mitochondrial matrix to the IMS. In the process, two electrons are passed to a cytochrome c that transports them to the last enzyme of the OXPHOS, Cytochrome c oxidase (Complex IV). Complex IV consists of 14 subunits, of which three are synthesized in the mitochondria. It has two hemes, two cytochromes and two copper sites that together form the site of oxygen reduction. In the process of converting molecular oxygen to water, it translocates four protons across the mt membrane. Finally, the protons pumped from the matrix to the IMS during OXPHOS are transported back to the matrix by ATP synthase by using electrochemical gradient sustained by the mitochondrial membrane potential. This movement of protons is the driving force of ATP synthase converting ADP to ATP molecules[141 142].

Beyond ATP production, the IM electrochemical potential generated by OXPHOS is a vital feature of the organelle[143]. Membrane potential is necessary for other essential mitochondrial functions, such as protein import and is used to trigger molecular changes that alter mitochondrial behaviors in response to mitochondrial dysfunction. The net gain of OXPHOS is approximately 34 ATP molecules, making it the main respiratory process producing cellular energy.

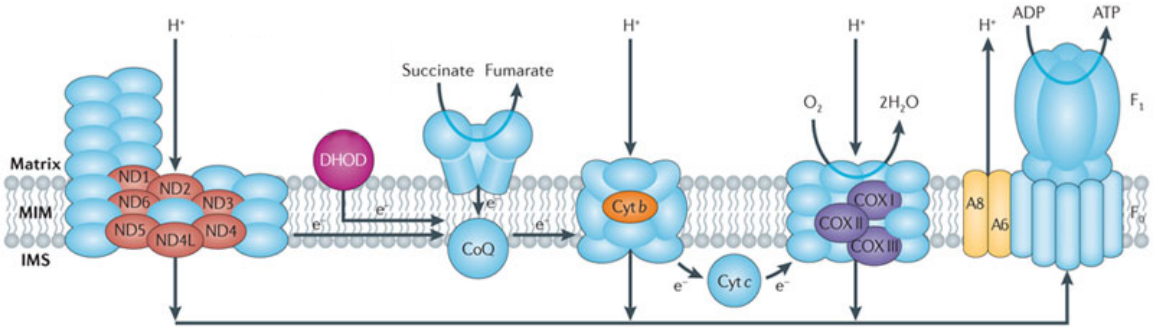

Figure 4. OXPHOS chain. The OXPHOS apparatus consists of complex I (NADH dehydrogenase-CoQ reductase), complex II (succinate dehydrogenase), complex III (ubiquinone-Cyt c oxidoreductase), complex IV (cyclo-oxygenase, COX) and complex V (ATP synthase), and two electron carriers, coenzyme Q (CoQ) and Cyt c. Complexes I–IV pump NADH and FADH2 derived protons from the matrix across the mtIM to the IMS to generate a proton gradient, while at the same time transferring electrons to molecular oxygen to produce water. The OXPHOS chain consist of 13 mtDNA-encoded proteins (see the mt genome in Figure 1 for location of corresponding sequence by colors) and 80 nDNA encoded ones (blue). The picture modified from Schon *et al.*[13]

Steroid synthesis

In addition to being the center of cellular respiration, the mitochondria are also the site of steroid hormone biosynthesis from cholesterol, a process indispensable for mammalian life. The six groups of steroid hormones are synthesized from cholesterol in a tissue-specific manner. They include; glucocorticoids and mineralocorticoids produced in the adrenal cortex, estrogens, progestins and androgens, produced in the gonads, and calciferols, produced in the kidney.

As the mitochondria, like bacteria, are cholesterol-poor, it needs to be transported into the organelle prior to hormone synthesis. This steroidogenic cholesterol may be produced de novo in the endoplasmic reticulum, or derived from circulating

lipoproteins via endocytosis of lipid droplets. Once inside the cell, cholesterol is believed to be transported to the mitochondria with the help of steroidogenic acute regulatory (StAR) proteins, that all harbor high-affinity cholesterol-binding domains and appear to play important roles in cellular cholesterol homeostasis[144 145 146]. In the mitochondria, cholesterol is embedded in the OM, and following cAMP-induction, specific steroidogenic pools of cholesterol are mobilized[147]. This mobilization is preceded by a pH-induced activation of StAR-proteins that move the cholesterol to the IM where cholesterol side-cleavage enzyme (P450scc) system processes it to yield pregnenolone. Pregnenolone then exits the organelle and is processed in the cytosol to yield the mature steroid hormones. Mutations in StAR-proteins lead to the accumulation of mitochondrial cholesterol in various tissues, and for example, in the ovary, it can cause loss of fertility due to impairment in estrogen synthesis soon after puberty[148 149].

Additional functions

Apoptosis, heme synthesis, Ca2+ storage

Programmed cell death (apoptosis) is crucial for tissue homeostasis, and when deregulated can contribute to immunodeficiency and cancer. The cell has two distinct pathways for apoptosis. The intrinsic apoptotic pathway involves pro-apoptotic receptors on the cell surface that detect extracellular ligands that trigger a cascade of intracellular events leading to cell death. The extrinsic (mitochondrial) apoptotic pathway is activated in response to intracellular events, such as DNA damage and cellular stress. Whether this pathway leads to apoptosis depends on the balance of pro- and antiapoptotic Bcl-2 family proteins released from the mitochondria[150 151]. The release of more proapoptotic proteins favors apoptosis and changes the structure of the mitochondrial membrane, making it permeable to cytochrome C (CytC)[152 153]. The leak of CytC to the cytosol to form a multi-protein

complex, the apoptosome, is considered the point of no-return in the mitochondrial apoptotic pathway. The apoptosome initiates a cascade of apoptotic caspases that lead to activation of DNA degrading proteins and subsequent cell death[154]. Currently, the intrinsic apoptotic pathway is being widely studied for its potential role in blocking tumor growth[151].

Hemoglobin is responsible for delivering oxygen into our cells. Heme synthesis begins in the mitochondria, when the tetrapyrrole ring of heme is formed by the condensation of glycine d with Succinyl-CoA from the TCA cycle. The product, g-aminolevulinic acid, is then exported to the cytosol, where its synthesis towards heme continues. However, in the last step, the heme precursor returns to the mitochondria where an iron-sulfur protein, ferrochelatase, introduces iron to produce mature heme. Heme plays an important role in transcriptional control and the activity of various proteins and pathways. Defects in mitochondrial heme synthesis are known to cause metabolic and oxidative stress, accumulation of iron and cell death[155 156 157].

Calcium is important for various cellular processes and signal transduction, and disruptions of intracellular Ca2+signaling are known to lead to osteoporosis and underlie Alzheimer´s disease. Most of the cellular Ca2+ is stored in the ER and the mitochondria that coordinate the release of calcium ions into the cytosol. Ca2+ uptake into the mitochondria occurs via the IM uniporter driven by $\Delta\Psi$. Once in the matrix, it participates in NADH-production by activating the first enzymes of the TCA cycle; PDK, isocitrate dehydrogenase (ICDH) and α-KG dehydrogenase[158]. Increasing calcium-levels has also been shown to activate electron transport and ATP-synthase even more efficiently than low ATP/ADP[159]. Another way Ca2+ affects ATP-production is by activating the mitochondrial permeability transition (MPT) pore in the IM[160]. While high intra-mitochondrial calcium concentration is sufficient for the MPT to open, various factors, such as drugs and injuries, can activate the MPT. However, even in these cases, calcium acts as a cofactor facilitating the opening of the pore. When the MPT pore opens, the mitochondrial

membrane polarization decreases, leading to a decrease in the ETC. And open MPT pore also allows free flux of molecules smaller than 15kDa, leading to osmolar pressure and various mitochondrial and cellular stress responses, often followed by apoptosis or necrosis[161].

4. Regulation of Mt Function

Nuclear factors regulating Mt biogenesis

Due to its central role in various cellular pathways, balanced mitochondrial function is of the utmost importance to organismal integrity. To ensure this, the cell has developed a complex network of connections from nucleus to mitochondria, and vice versa (retrograde signaling), that are governed by a few main regulators; Nuclear respiratory factor 1, 2 (NRF-1, NRF-2), PPAR (peroxisome proliferator-activated receptor-gamma coactivator-1a) (PGC-1α) and TFAM, These nuclear proteins are able to control mitochondrial biogenesis in response to changes in cellular stress and energy demands [162].

Transcription factors NRF-1, 2, TFAM and PGC-1a

NRF-1 was first discovered as an activator of the cytochrome c gene[163]. Now we know it is a ubiquitously expressed nuclear transcription factor that binds to NRF-1 response elements (NRE) in the promoters of various nuclear-encoded mitochondrial genes in order to regulates mitochondrial biogenesis[164]. It plays a critical role in combining nucleo-mitochondrial interactions by initiating transcription of nuclear-encoded mtDNA-specific transcription factors including TFAM, TFB1M and TFB2M[165]. It responds to redox signaling pathways through post-translational modifications and through its specific interaction with transcriptional co-activators. Its target genes include most subunits of the ETC complexes, proteins of the mtDNA transcription and replication machinery, enzymes of heme biosynthesis, mitochondrial protein import, hundreds of genes involved in cell cycle regulation, metabolism, DNA replication and transcriptional regulation[166].

Interestingly, cell cycle regulator Cyclin D has been shown to inhibit NRF-1[167]. Although for decades mitochondrial dynamics was considered to be uncoupled

with cell cycle progression, recently it has been shown that the dynamic changes in the mitochondrial network are indeed affected by the cell cycle phase, although the significance of this still remains to be studied. Notably, how the expression of NRF-1 itself is regulated is not well known. Thyroid hormone, as well as estrogen, have been reported to increase the mRNA-expression of NRF-1 via genomic interactions, although the response elements for these proteins are not present in the promoter of NRF-1[168 169]. However, inhibitors of mt oxidant production prevent the estrogen-induced expression of NRF-1 target genes involved in breast cancer growth, induce NRF-1 expression and delay cellular proliferation, suggesting that estrogen induces NRF-1 expression through ROS[170].

NRF-2 is a transcription factor coordinating expression of approximately 10% of known cytoprotective genes. Under basal conditions it is ubiquitinated and proteosomally degraded, but under cellular stress, it translocates to the nucleus, activating antioxidant, drug metabolizing and anti-inflammatory genes, as well as influencing the metabolism of the cell. An upregulation of NRF-2 target genes redirects glucose and glutamine into anabolic pathways that produce reducing equivalents (NADPH) for maintaining glutathione in its reduced (active) state and providing building blocks for fatty acid, nucleotide and amino acid biosynthesis. These together lower oxidative stress and inflammation[171 172 173].

TFAM is essential for the maintenance of mtDNA, as it is shown to be tightly packed along the length of the whole mitochondrial genome[174]. Indeed, mtDNA copynumber has been shown to be directly affected by the expression of TFAM. Both NRF-1 and PGC1-α (below) stimulate transcription of TFAM, which in turn activates mtDNA transcription[175].

PGC-1α. One of the first insights into the nuclear regulation of mitochondrial function came from studies showing that PGC-1α induces mitochondrial biogenesis in brown fat and muscle cells in response to cold temperature and high-carbohydrate diet[176 177 178]. PGC-1α is a member of the PGC-family of proteins whose members all have a central role in control of cellular metabolism. Today

PGC-1α is considered the master regulator of mitochondrial biogenesis and it plays an important role in coupling physical stimuli from exercise and hormonal regulation to a metabolic response[179] [180]. It is a co-transcriptional factor that induces mitochondrial biogenesis by activating transcription factors such as NRF-1 and 2, thyroid hormone, glucocorticoids and oestrogen-related receptors 'that control the expression of mt genes involved in mitochondrial fatty acid metabolism and b-oxidation among others[181]. PGC-1α is also shown to induce the expression of TFAM, providing direct means of coordinating nuclear and mitochondrial transcription[175] [164].

Hormonal regulation of Mt function

Not only are mitochondria the starting site of steroid hormone synthesis, but their function is also under hormonal regulation. During the past ten years, a growing body of evidence suggests that estrogen is a major regulator of mitochondrial function. It has been even been proposed that the estrogen-related extension of life-span of women might actually be due to the effects of the hormone on mitochondrial respiration[182].

Current evidence suggests estrogen to affect mitochondrial function at both the genomic and non-genomic level. At the genomic level, after the induction of estrogen ER-α and ER-β, are translocated to the nucleus where they directly activate co-regulators and bind EREs as described earlier. Many of the genes involved in mitochondrial biogenesis are known to harbor EREs in their promoter regions, making them responsive to changes in estrogen levels. The reported non-genomic effects of estrogen involve post-translational changes mediated by cell-surface ERs but are much less studied[183] [184] (Levin 2001). There is recent evidence involving various primary and transformed cell lines showing 10-20% of total cellular ERs reside in the mitochondria, where they directly bind to sequences resembling EREs in the mtDNA. These "mtEREs" are found within specific genes

and in the D-loop area and indeed, mitochondrial transcription has been shown to be increased in response to estrogen-treatment, although it has not been shown to occur directly via mtEREs. Pinpointing the exact effect of estrogen on mitochondrial function is challenging, because estrogen exposure causes various changes in the cell. For example, in endothelial cells, estrogen rapidly increased cAMP-levels and stimulated release of Ca2+ and nitric oxide (NO), all shown to affect mitochondrial function[185 186 187].

Contrary to its effect in mitochondrial transcription, estrogen has been shown to inhibit the function of mitochondrial respiratory complexes. Whether this inhibition happens at the post-translational level is not clear. Mitochondria are known to have kinases able to phosphorylate mitochondrial proteins, but whether this happens in response to estrogens is still unknown. One explanation to explain the lower ETC activity could be that the diffusion of 17B-estradiol through the mitochondrial membranes changes their fluidity and affects the function of ETC complexes embedded in the IM, similarly to the thyroid hormone[188].

Retrograde signaling

Retrograde regulation (also referred to as mitochondrial stress signaling) is the general term for mitochondrial signaling. Until 1996 the notion that the mitochondria was an isolated organelle responsible for cellular energy production, was challenged by the discovery that cytochrome C release mediates apoptosis[189]. First proof that the signals coming from the mitochondria were able to regulate nuclear gene expression came from studies done with rho0-cells. Rho0-cells are created by culturing cells in the presence of EtBr that intercalates in the mtDNA and prevents it from being replicated, resulting eventually in a cell line void of mtDNA. Studies showed that the depletion of mtDNA led to elevated $\Delta\Psi$ and cytosolic Ca^{2+}, alterations in cell morphology and an increase in the mRNA-levels of nuclear encoded mitochondrial genes. These together suggested the existence

of a stress-inducible regulatory connection between the two genomes[190] [191] [192]. A similar retrograde signaling was observed in various studies, where changes in nuclear transcription were inhibited by using Ca2+ chelator in cells experiencing mitochondrial stress, confirming the role of Ca2+ in retrograde signaling[193].The current model for retrograde signaling is, that mitochondrial stress induced by drugs, oxygen depletion, mutagens, mtDNA depletion or any other factor, disrupts the $\Delta\Psi$ which in turn leads to lower incorporation of Ca2+ into the mitochondria, reducing ETC capacity and ATP-production. Due to insufficient ATP, the Ca2+ efflux out from the cell is prevented, and cytosolic Ca2+ concentration increases, activating calcium-responsive phosphatases and kinases and subsequent gene activation in the nucleus.

ROS as signaling molecules

It was originally thought that only phagocytic cells were responsible for ROS production through their role in host cell defense mechanisms. However, now we know there are numerous potential endogenous sources of ROS depending on the cell and tissue type. Historically, ROS have been considered as harmful byproducts of cellular metabolism and even the culprits for aging, but during the last decade, major insights have been made into the role of ROS as a crucial signal transducer in the cell.

ROS are produced as a by-product of mitochondrial respiration and function as critical second messengers in various cellular pathways, including retrograde signaling. Mitochondrial ROS are biologically important in a variety of physiological systems, including adaptation to hypoxia, regulation of autophagy, regulation of immunity, regulation of differentiation, and longevity.

It has been found that the major determinant of mtROS is the redox state of the ETC. Inhibition of the ETC causes electron carriers to be in a reduced state, increasing the propensity to create superoxide. Another significant factor involved

in mtROS production is the mt membrane potential and pH-gradient across the IM that create the proton motive force necessary for electron transport, and an increase in either of them results in increased mtROS production[194]. Mitochondrial ROS is mainly generated in the form of superoxide anion (O^{2-}) when the molecular oxygen required by ETC complexes I and III loses an electron. This “leakage” occurs relatively frequently in healthy mitochondria, but is strongly induced in the presence of mitochondrial dysfunction. As ROS are considered cytotoxic, their levels must be tightly regulated by multiple defense mechanisms involving anti-oxidants and ROS-scavenging enzymes such as superoxide dismutases (SOD 1-3s), catalase and glutathione peroxidase (GPX)[195].

Increasing concentrations of Oxygen (O_2) in the mitochondrial matrix induces an activating conformational change in SOD enzymes, resulting in rapid dismutation of the oxygen radical into molecular oxygen and hydrogen peroxide (H_2O_2). H_2O_2 can pass easily through mitochondrial and cellular membranes and acts as the main ROS-signal from the mitochondria, regulating the redox-state of the cell[196 197 198]. To ensure its proper neutralization in each cellular compartment, the SODs are distributed to many parts of the cell; SOD1 is located in the IMS and cytosol, SOD2 in the mitochondrial matrix, and SOD3 in the extracellular matrix. The further neutralization of hydrogen peroxide into water and oxygen results in the action of enzymes with catalase activity or they can be processed by redox-sensitive peroxidases. Peroxidase activity requires oxidizing of other substrates like NADH and glutathione, and so the ratio between reduced/oxidized glutathione can be used to detect alterations in the anti-oxidant and redox state and signaling of the cell.

The pathways by which mitochondrial ROS signal to the nucleus have been under tedious investigation for the past two decades and are still not fully understood. In 1998 it was shown that H2O2 released by the mitochondria was able to activate transcription factor Hypoxia-inducible Factor 1α (HIF-1 α) under low oxygen conditions, and later that the mitochondrial release of H_2O_2 was activating the

mitogen-activated protein kinase pathway, tumor suppressor p53 and the NF-Kβ transcription factor[199] [200]. Increasing evidence suggests that the molecular mechanisms behind the posttranslational changes regulated by H_2O_2 are in its ability to oxidize thiol groups on cysteine residues of proteins. Further reactions with glutathione reductase form inhibitory disulfide bonds that are commonly found in the structures of many redox-regulated proteins, for example PTEN and MAPK phosphatases[201] [202] [203].

The redox-regulation of disulfide bond formation in certain proteins has been shown to be dependent on the mitochondrial ROS via its effects on the acidification of cytosol, having direct functional consequences for the cell[8].

It was recently discovered that two groups of enzymes, the NADPH oxidases (NOXs) and dual oxidases (DUOX), mediate critical physiological and pathological processes through the generation of ROS as their sole function, underlining the importance of ROS as a crucial cell signaling molecule[204]. ROS created by the NOX/DUOX- family of proteins are formed in the extracellular space of phagosomes and transported into the cytosol where they participate in signaling cascades and/or is neutralized.

In addition to their beneficial function in cellular homeostasis, out of control ROS production has the potential to cause extreme damage to the cell. This is seen during increased environmental stress (heat, radiation etc.) when levels of ROS can increase dramatically[205]. When free oxygen scavengers are not able to neutralize ROS, they can directly attack cytosolic lipids, membranes and nucleic acids, causing irreversible damage contributing to multiple pathologies, including aging (discussed later).

Cellular response to hypoxia

Although able to survive short periods with low oxygen supply, aerobic organisms rely on oxygen for their energy production. When mammalian cells are under low

oxygen concentration (0.3%-3%) they immediately initiate transcriptional and non-transcriptional responses to increase oxygen intake and reduce its usage. These changes are from large part induced by mtROS, in a manner not yet known, but the effector transcription factors are the family of HIFs. Heterodimers HIF-1 and HIF-2 both consist of HIF-1α and HIF-1β (ARNT) proteins. Under normal oxygen conditions, HIF-1α is hydroxylated at its two proline residues by PDH2 (prolyl hydroxylase domain protein 2), which targets it for proteosomal degradation by the Von Hippel-Lindau (VHL) tumor suppressor[206] (Figure 5). When oxygen drops below 5%, PDH2, that requires oxygen as a co-substrate, is inhibited and HIF-1α stabilized and transported to the nucleus where it dimerizes with ARNT and binds hypoxia responsive elements (HREs) in the genome. Together they regulate an array of oxygen-sensitive genes, many of them involved in reprogramming of cellular metabolism and restoration of oxygen supply[207]. However, the whole scale of HIF-1α/ARNT targets remains unresolved, as gene expression profiling fails to clearly identify direct vs indirect targets[208 209]. More recent studies using ChIP and genomic tiling microarrays (ChIP-chip) identified over 500 genes with HIF-1α and ARNT binding regions, but the identity of these proteins remains to be validated[210].

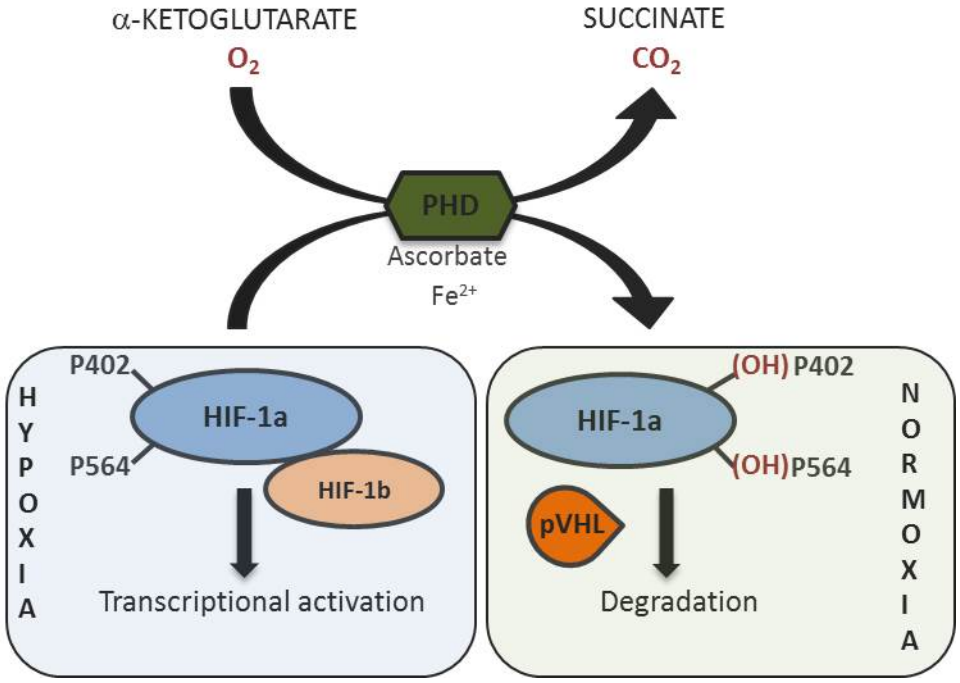

Figure 5. HIF-1α stability is dynamically regulated by oxygen availability. Under normoxia, two prolyl sites (P402, P564) of HIF-1α are hydroxylated by prolyl hydroxylases (PHD). After this, the VHL protein ubiquitinates HIF-1a to target it for proteosomal degradation. During hypoxia, PHD remains inactive, HIF-1α accumulates and translocates to the nucleus where it binds HIF-1β and regulate transcription of its target genes.

As inhibiting HIF1-signaling in tumors can be used as a therapeutic approach to treat cancer, various inhibitors have been developed against it. Many of them are aimed to inhibit PHD. The PHDs belong to a family of α-KG –dependent dioxygenases that use O_2 as a cosubstrate to add a hydroxyl group to HIF1 proline residues. In addition to an absolute requirement for O_2, ferrous iron (Fe(II)) is required for the activity of PHD, and many hypoxia-mimetics such as $CoCl_2$ (Cobalt Chloride) function by chelating Fe(II) and leading to inhibition of PHD function and subsequent HIF1-stabilization[211]. Another possible target site for therapeutics aimed at HIF-1 is ascorbate. During the hydroxylation of PHD, α-KG is converted into succinate simultaneously to the oxidation of Fe(II) to Fe(III). In order for the PHD to regain functionality and the state of normoxia returned, iron needs to be converted back to its reduced state by ascorbate. Thus, intracellular ascorbate and Fe(II)-levels can directly affect the state of HIF-1 in the cells[212].

Mitochondria as oxygen sensors

Mitochondria have long been suspected to play an oxygen-sensing role since they bind O_2 at Complex IV and they are the primary site of O_2 consumption in the cell (Bunn, Poyton 1996). The very first evidence suggesting mitochondria to be involved in O_2 sensing during hypoxia came from Rho0-cells[213]. These cells, devoid of mtDNA, were unable to stabilize HIF-1α under hypoxia due to the lack of functional ETC and mtROS production. Similar phenotypes were seen in wild type cells when treated with ETC-inhibitors, confirming the role of mtROS in the induction of the hypoxic response[214]. The same study found evidence that HIF-1

activation requires the presence of mtROS in the cytosol, and that prior to triggering HIF-1 activation, mtROS enters the cytosol via anion channels. Later, by using Complex III subunit cytochrome b mutant cells, it was shown that specifically the loss of Complex III-derived ROS –production was responsible for the hypoxic response in cells[215 216]. This was confirmed by using small molecule inhibitors of ETC and genetic silencing of regulators of mtROS, that inhibited HIF activation under hypoxia. Controversially, Complex III inhibitor Antimycin A was not able to block stabilization of HIF-1α under hypoxia despite inhibiting electron flux through complex III, suggesting that changes in redox state of complex III are not the primary mechanism regulating HIF-1α activation[217]. The effect of mtROS on the stabilization of HIF-1α was also shown in response to non-hypoxic activation of HIF-1α, suggesting a direct regulatory mechanism between them[218].

However, how mtROS specifically inhibits HIF-1α and HIF-2α hydroxylation and resulting HIF activation is still an open question. Also, how mitochondria sense oxygen depletion remains under debate, but based on their studies Chandel et al hypothesized that “Complex III must possess inherent sensitivity to O2, allowing it to adjust its generation of ROS inversely with the O2 tension”[214]. Indeed, hypoxia induces a strong increase in the translation of mt complex III subunit, supporting this hypothesis[219 220].

One possible explanation could involve structural changes occurring in ETC complexes during hypoxia. It has been shown in yeast, that subunit composition is regulated by COX5a and 5b gene transcription in response to high and low oxygen, respectively[221 222]. Interestingly, mammalian cells seem to limit ROS-production by switching from using COX4-2 to COX4-1 during chronic hypoxia[223]. In addition to changes in the usage of single ETC subunits, there is structural evidence that entire ETC complexes interacts with each other to form a functional “respirasome”[224]. Assembly of the tightly packed respirasome is considered to minimize the leakage of oxygen radicals from the ETC and decrease levels of mtROS[225]. Since mtROS is shown to regulate hypoxic response, these structural

changes in ETC could very well be part of the mechanism. Thus while there is clear evidence that mtROS plays a vital role in activation of hypoxia signaling in the cell, the mechanisms involved in cellular oxygen-sensing remain an enigma.

5. Mitochondria and Disease

Mitochondrial dysregulation in diseases

Human mitochondrial disorders are a genetically heterogeneous group of diseases, caused by mutations in mitochondrial and/or nuclear DNA, which encompass almost all fields of medicine[226]. To date we have seen mitochondrial diseases affecting almost any organ system, and manifesting at any age. Currently, there is no cure for mt disorders, and available treatments are directed at relieving symptoms[227]. Mitochondrial diseases display both clinical heterogeneity and have tissue-specific manifestations, as indicated by the fact that mutations in the same mitochondrial protein complex can lead to contrasting disease phenotypes. For example, defects in respiratory complex I can lead to optic nerve atropy in adults and in infants to subacute necrotizing encephalopathy[228 229]. The most common nuclear mutations associated with mitochondrial diseases are associated with DNA POLG and manifest as early-onset hepatocerebral disorder, juvenile catastrophic epilepsy, or adult-onset ataxia-neuropathy syndrome[230 231 232 233]. Another example of clinical variability was shown in the recently characterized disease group linked to defects in mitochondrial aminoacyl-tRNA synthetases (ARS2s). ARS2 defects promote a wide array of phenotypes, including cardiomyopathies, cerebral white matter disease, hearing loss and ovarian dysfunction[234 235 236]. The nature of the molecular defect can often explain variations in the severity and onset age of these diseases, but the variability in tissue-specific manifestations is harder to explain. Possible explanation could involve the patient's complement of protective and risk alleles playing part in the development of the disease.

Recently, a mutation in a gene encoding for a p53–inducible ribonucleotide reductase M2 polypeptide pseudogene 3 (RRM2B) was found to be mutated in a group of patients suffering from mtDNA depletion syndrome. Although not detected in the mitochondrion, RRM2B is affects directly mitochondrial replication and

copynumber by controling the pool of dNTPs available for nucleic acid synthesis[237]. Interestingly, the EEM2B -deficient mice suffer from loss of mtDNA depending on the tissue, highlighting the tissue-specific effects of changes in mitochondrial copynumber and DNA mutations[238].

Phenotypic variability associated with mtDNA-linked diseases is also due, in part, to the high copy number of mtDNA in mammalian cells, which can consequently contain both mutant and wild-type mtDNA populations (heteroplasmy). The pathogenic threshold of mutated mtDNA has been estimated to be approximately 80%, but the variability in mitochondrial heteroplasmy in diseases is high[239] and varies between species[238]. While mtDNA mutations in tRNA genes present high clinical variability independent from heteroplasmy, in the case of protein-coding gene mutations, the degree of heteroplasmy correlates with the severity of phenotypes[240 241].

Mitochondria and cellular metabolism in cancer

Recent work has linked tumor suppressors and oncogenes directly to metabolic sensing and regulation, and has consequently indicated that altered cellular metabolism is a contributory factor in the onset and progression of cancer. Cancer cells reprogram the use of two key catabolic molecules, glucose and glutamine via signaling pathways containing known oncogenes including myc and tumor suppressors, such as p53[242]. These signaling pathways contribute to the so called Warburg effect in cancer cells by shunting glucose into glycolysis and glutamine toward glutaminolysis for the purpose of producing building blocks for rapid proliferation[243]. In cancer cells, mitochondria play a central role via the TCA cycle in the catabolism of glutamine. The altered metabolism of cancer cells raises the possibility that treatments that shift metabolism toward OXPHOS could be therapeutically effective against cancer.

Warburg effect; cancer cells switch to aerobic glycolysis

Increases in glycolysis and lactic acid fermentation are common phenomena during anaerobic respiration. However, over 80 years ago Otto Warburg described how tumor cells consume high amounts of glucose and produce lactic acid even in the presence of oxygen[243]. This cancer-specific phenomenon was termed the Warburg effect, and ever since its discovery, a great effort has been made to understand the mechanisms of how cancer cells induce glycolysis and how we could exploit this in therapy. For decades it was thought that increased glycolysis in cancer cells was compensatory for reduced mitochondrial energy production due to mtDNA mutations. Later it was shown that the mitochondria of most cancer cells are still able to produce plentiful amounts of ATP.

Oncogenic mutations affect metabolism

In order to explain the mechanisms underlying the switch from anaerobic glycolysis to aerobic glycolysis in cancer cells, various oncogenes have been identified as metabolic regulators. The AKT/PKB kinase is an oncogene that is able to enhance glucose uptake and activate aerobic glycolysis, whereas the transcription factor MYC is able to activate virtually all glycolytic genes by binding directly to their promoters[244]. MYC is also involved in enhancing mitochondrial respiration and ROS-production that might contribute to mitochondrial genomic instability and result in increased dependence of glycolysis for ATP-production[245 246]. Expression of the proto-oncogene H-RAS is also able to induce lactate production, suggesting an increase in glycolytic flux[247]. In addition, accumulation of the tumor suppressor p53 during hypoxia enhances apoptosis, and mutations in p53 have been suggested to confer a survival advantage to tumor cells under low oxygen

conditions as well as contributing directly to the switch towards aerobic glycolysis[248 249 250].

Oncogenic mutations in mitochondrial proteins

Mitochondrial metabolic enzymes have been identified as tumor suppressors and inactivating mutations in SDH and fumarase cause the accumulation of succinate and fumarate, respectively, which can inhibit α-KG-dependent enzymes including PHD, leading to inappropriate HIF-1α activation[251]. Also TCA-enzymes isocitrate dehydrogenase 1 and 2 (IDH1, 2) that catalyze the conversion of citrate to isocitrate and α-ketoglutarate in the TCA-cycle are frequently mutated in brain tumors and leukemias[252 253]. The mutated enzymes produce 2-hydroxyglutarate (2-HG) a potential oncometabolite that has been shown to prevent histone demethylation in progenitor cells, resulting in a blockage to differentiation. This might potentially lead to an accumulation of more "stem-cell-like" tumor-cells[254]. The potential of IDH-mutations to alter gene expression was confirmed when Turcan et al found that IDH1 mutant astrocytes undergo DNA hypermethylation leading to gliomas and solid tumors[255]. Back to back with these results, Koivunen *et al* found that a 2-HG (R)-enantiomer was able to decrease HIF levels, leading to enhanced tumor proliferation[256].

Hypoxia contributes to the tumor environment

In addition to oncogenic activation of aerobic glycolysis, the activation of HIF-1 family members also significantly contributes to the conversion of glucose to lactate. It was first reported 60 years ago that tumors grow in a pattern around blood vessels, and if the distance from the closest vessel grew larger than oxygen diffusion in the tissue was possible, the cells necrosed[257]. These findings already suggested that tumors must have developed ways to adjust to this constant

hypoxic state and instead of dying of necrosis grow even further. Further insight into this phenomenon came later, when it was noticed that the hypoxic tumor areas were resistant to radiotherapy that required oxygen-derived ROS to induce cell death[258]. Very soon after this Vaupel and colleagues found direct evidence that low oxygen tension in tumors was associated with increase in metastasis and poor survival in patients[259 260].

During the following decade, great efforts were made to reveal the complex network of HIF-regulated cellular pathways that contribute to the malignancy of hypoxic tumors. These studies elucidated how hypoxia induces the selection of subpopulation of cells within the tumor, that have increased expression of drug-resistance genes[261 262], selection for apoptosis-resistant clones[249], increase in expression of genes involved in tumor invasion and metastasis[263], and increased genomic instability, including reduced DNA-damage repair[264 265 266]. Tedious investigations into mechanisms and unifying theory of where these biological changes could originate from culminated into discovery and characterization of cancer stem cells (CSCs)[267 268 269 270]. CSCs are characterized by the expression of stem cell markers, self-renewal and clonogenic growth and potential to differentiate into diverse cell types giving rise to heterogeneous tumor tissue. Interestingly, it was shown that the CSCs have higher DNA-repair capacity than the more differentiated cells, making them more resistant to radiotherapy and suggesting there might be a connection between hypoxia and the stemness state of cancer cells[271]. Indeed, the connection was confirmed in the context of neuroblastoma, breast cancer and leukemia, which upon hypoxia acquired a more stem-cell–like state[272 273 274 275].

Similarly to normal tissue, hypoxia signaling in cancer cells is primarily regulated by HIF-1 complex. It was shown that ectopic expression of HIF-1 α in highly aggressive breast cancer line MDA-MB-231 (referred as MDA231 from now on) increased bone metastasis with increased blood vessel formation and reduced osteoblast differentiation, whereas inhibiting HIF-1α reduces metastasis[276]. Today

the general consensus is that high HIF-1α promotes tumor growth, whereas reduced HIF-1 leads to inhibition of tumor growth[277 278]. Similar tumor-enhancing effects and poor patient prognosis has been observed with HIF-2 overexpression[279].

In addition to intracellular changes, hypoxia contributes to the tumor environment through modifying the expression of many components of the extracellular matrix (ECM). ECM consists of tightly regulated complex network of macromolecules that commonly becomes disorganized in diseases such as cancer. ECM anomalies are known to promote cellular transformation and metastasis by deregulating behavior of stromal cells, facilitating tumor-associated angiogenesis and inflammation, creating a tumorigenic microenvironment. It has been demonstrated that HIF-1 regulates ECM remodeling by activating collagen prolyl and lysyl hydroxylases required for collagen synthesis, folding and stability[280 281].

6. Mitochondria and aging

As human life expectancy is steadily rising, increasing resources are being used to identify and fight the underlying causes of age-related phenomena, such as physical and mental decline, increased risk of metabolic syndrome, cancer and neurodegenerative disorders. Today, many genes known to coordinate cell growth and differentiation are known to be involved in these processes, and various theories have been made in order to explain the age-related deterioration of the organism.

Cellular pathways implicated in aging

To date, a plethora of mutant genes involved in conserved pathways regulating growth, energy metabolism, nutrient sensing and reproduction, have been shown to increase longevity in model organisms (Figure 6)[282]. The majority of these genes encode components of 1) the insulin/insulin like growth factor 1 (IGF-1) signaling pathway (IIS), 2) the target of rapamycin (TOR) pathway, 3) the Sirtuin (Sir) family. Interestingly, all of these pathways have been shown to be influenced by calorie restriction (CR) to some extent, and dietary restriction has been acknowledged as the only mechanism increasing longevity in all tested organisms from yeast to mammals[283]. There is also strong evidence that the deterioration of cellular maintenance accompanying aging is caused by the accumulation of misfolded and damaged proteins during ones lifetime. One of the mechanisms by which CR increases longevity is its ability to activate autophagy in the cell to recycle damaged proteins and cellular organelles. Consistent with this, various studies support the notion that autophagy can sustain longer life by degrading dysfunctional macromolecules, thus acting as a cellular “cleaning lady”[284 285 286].

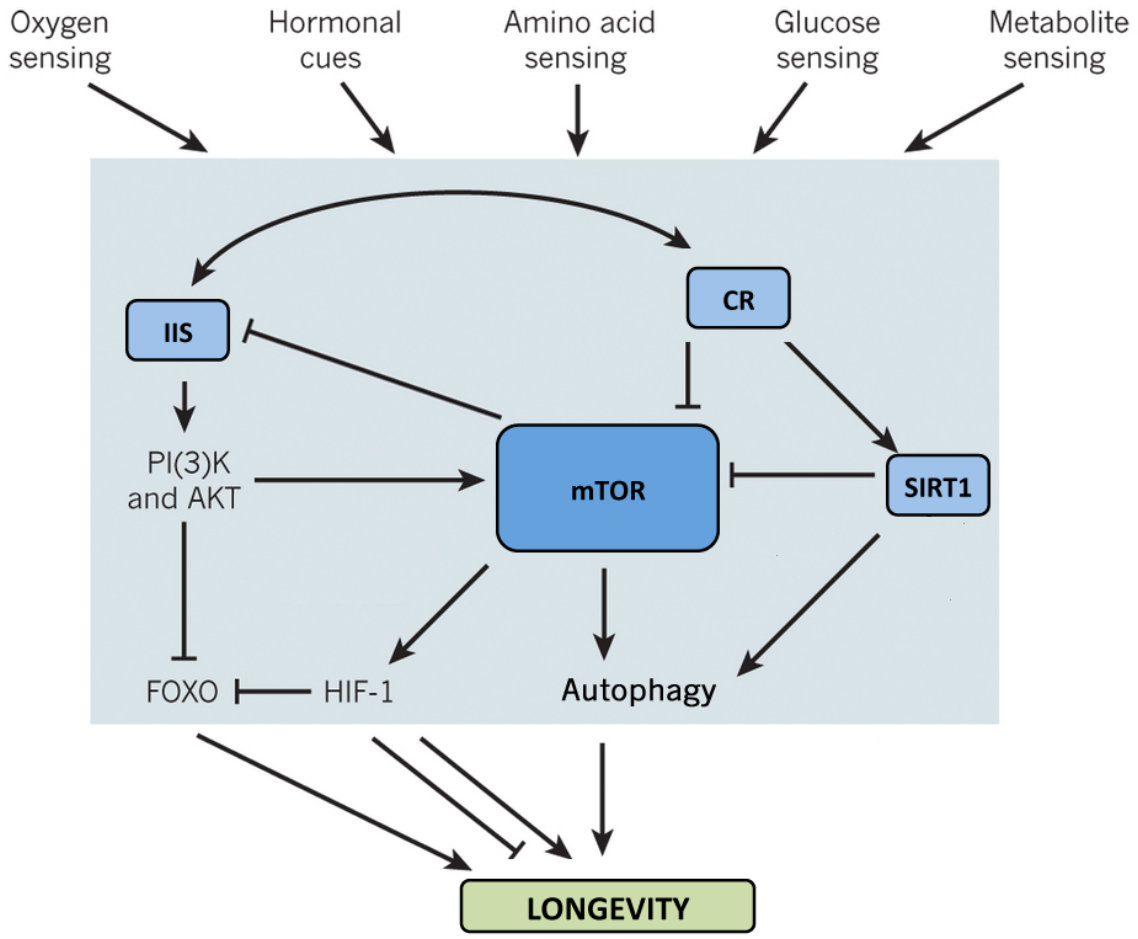

Figure 6. Longevity pathways. Insulin signaling, calorie restriction, mTOR and Sitruin pathways are involved in the longevity of various organisms. They are highly interconnected and respond to various environmental cues such as oxygen levels, nutrients and hormones and are believed to lead to increased lifespan mainly via increased stress response leading to autophagy. Figure modified from Johnson *et al.*[287]

Insulin signaling

The first pathway implicated in longevity was the insulin/IGF1-pathway[282]. The reduced activity of *C.elegans* ortholog of the insulin receptor, Daf-2, nearly doubles the lifespan of worms, and mutations affecting the downstream PI3K/AKT kinase cascade equally extends their lifespan. Similar effects were seen in the fruit fly

model where a decrease in insulin receptor levels or inactivating mutations in various downstream proteins, resulted in increased longevity. Inhibition of insulin signaling changes lifespan by modifying gene expression through the transcription factor FOXO (*Drosophila* and mammals) and DAF-16 (*C.elegans*). Downstream genes shown to be regulated differently in response to decreased insulin signaling include stress-response genes such as catalases, glutathione-S-transferases and metallothioneins, as well as chaperones, lipases and channels, that partially also affect the mTOR/autopgahy pathways (Figure 6).

Studies performed in *C.elegans* have identified, that the reproductive system is also able to regulate Insulin/IGF-1 signaling[288]. In these studies, neutralizing the germ line cells increased lifespan, but only in the presence of the somatic gonad. This germline ablation is thought to extend lifespan by activating the steroid hormone pathway and DAF-16/FOXO by somatic gonad-dependent mechanisms[288 289]. The ability of insulin to regulate progenitor cells draws an intriguing, yet rather unstudied connection between a more “stem-like” state of the cells and longevity. However, although CR has been shown to prevent cancer cell proliferation directly via reduction in insulin/IGF-1-pathway, to date, the evidence as to whether reduced activation of the insulin pathway is involved in stem cell pool exhaustion or increased self-renewal remains controversial[290]. In humans, decreases in insulin receptor activity, AKT kinases and FOXO1 and FOXO3a have been linked in increased lifespan in various studies[291 292 293 294].

TOR pathway

The conserved, nutrient sensing, serine/threonine protein kinase TOR and its accompanying pathways, have recently emerged as key factors in lifespan and healthspan regulation from yeast to mice[295 296 297]. The TOR pathways are involved in co-ordination of growth, development and aging in response to changes in environmental nutrient and amino acid availability and act by blocking salvage

pathways, such as autophagy, when food is plentiful. Due to the high conservation of the TOR pathway through species, *D.melanogaster* has been widely used to study the effects of TOR-pathway perturbation at the organismal level. In response to nutrients, TOR stimulates translation by activating ribosomal subunit S6 kinase and inhibiting translation inhibitor Eukaryotic translation initiation factor E4 binding protein 1 (4E-BP). Inhibiting S6 kinase, by CR or specific inhibitors, reduces translation leading to longer organismal lifespan[298 299]. Interestingly, this reduction in translation does not affect the protein levels of ETC complexes and, in fact, mTOR inhibition is accompanied by increased mitochondrial respiration that is required for increased lifespan[300 301]. In both worms and flies, inhibition of TOR increases resistance to environmental stress by enhancing autophagy and other tissue maintenance pathways. The transcriptional changes induced by TOR are FOXO/daf-16 independent,, suggesting distinct mechanisms for the CR-induced changes involved in insulin and TOR-signaling[302].

Sirtuins

Sirtuins are a group of NAD+ -dependent protein acetylases that function as anti-aging genes in yeast, worms, and fruit flies, and have been linked with several diet-induced physiological changes in mammals. Whereas the lower organisms possess only one Sir-protein, Sir2, in mammals, seven members of the Sirtuin-family have been identified. Two of these localize to the nucleus (SIRT1 and 6) and three are mitochondrial (SIRT4, 5, 7), whereas one (SIRT3) can be found in both compartments and one seems to be purely cytosolic (SIRT2). The mammalian SIRT1 homolog, yeast Sir2, has been shown to extend yeast lifespan by two mechanisms: by suppressing the formation of toxic rDNA (ribosomal DNA) circles and by regulating cellular antioxidant defense[303 304]. The presence of elevated rDNA has also recently been detected in mammalian cells with mutated WRN (Werner) syndrome, a helicase/exonuclease that has been found to be involved in

their degradation[305]. Interestingly, patients lacking WRN protein are predisposed to diabetes, cancer and show premature aging due in part to lack of WRN-dependent telomere maintenance[306 307 308]. It remains to be studied if the accumulation of extrachromosomal rDNA occurs during the aging of mammals, and if WRN or other nucleases are involved in the process.

Although yeast Sir2 was shown to function by deacetylating histones, a large number of the substrates of the human orthologue, SIRT1, are non-histone nuclear proteins with varying functions[309 310]. SIRT1 seems to be involved in regulating the DNA-damage response through the deacetylation of p53 and FOXO proteins, metabolism via targeting PGC-1α leading to increase in mitochondrial biogenesis, and in reducing inflammation via NF-kB inhibition[311 312 313 314]. In addition to SIRT1, also other members of the human Sirtuin-family have been implicated in metabolic regulation. The mitochondrial SIRT3 seems to participate HIF-1a stabilization in cancer cells, SIRT4, SIRT5 and SIRT7 in fatty acid oxidation and mitochondrial gene expression, and SIRT 6 has been shown to act as tumor suppressor through metabolic reprogramming of cancer cells[315 316 317 318 319 320].

Taken together SIRT-family of proteins regulates various cellular pathways to promote efficient metabolism of glucose that might explain its effects in the longer healthspan of organisms.

Oxygen metabolism in lifespan extension

The evidence for the roles of hypoxia and oxygen signaling in the context of aging is contradictory. In humans it was shown that hypoxic HIF-1 activation promotes telomerase (hTERT) expression in the placenta, and that it also contributes to the extension of lifespan of human primary lung fibroblasts[321 215]. However, in *C.elegans* both, overexpression and inactivating HIF-1 have been linked to

increase in lifespan of the organism by altering in a complex manner the signaling of all pathways; DAF-16/FOXO, SKN-1/NRF and DAF-2[322].

Age-related changes in the mitochondria

Mitochondria undergo various changes during aging. First of all, age-related changes in the mitochondrial morphology include decreased number of mitochondria, increases in their size and disruption of the cristae structures in various tissues of the body[323]. It has also been shown that mitochondrial fusion and fission dynamics are affected in a similar manner during senescence and neurodegenerative diseases, suggesting a possible connection between the maintenance of the mitochondrial network and aging[324]. The OXPHOS capacity is also affected by age. Various biochemical studies on mitochondria revealed that the activity of OXPHOS complexes declines with age in the mammalian brain, skeletal muscle, liver and skin[325 326 327 328 329], and similar results were obtained with other vertebrates and flies[330 331 332]. The underlying reason was suggested to be a reduction particularly in ADP-stimulated respiration and decreased $\Delta\Psi$ with increasing leakage of protons[325 333]. The age-associated decrease in levels of mtDNA, respiratory proteins and OXPHOS function could result from lower rates of mtDNA transcription and mitochondrial protein synthesis. Indeed, it has been shown that steady-state levels of mtRNA and protein synthesis decrease during aging, and that this reduction closely correlates with the reduction of mtDNA-levels and ATP-production rate of mitochondria[334 328 329].

Quality of mtDNA is also known to decrease with age as somatic mutations, including deletions, duplications and point mutations, accumulate in the heteroplasmic pool of mtDNA[335 336]. Especially prone to point mutations seems to be the D-loop area, leading to changes in replication and transcription rate by hindering binding of enzymes crucial in these processes[337 338]. MtDNA deletions seem to occur mostly in the area between D-loop and COX1-gene, and

accumulation of high levels of deletions has been observed to precede enzymatic and morphological abnormalities of mitochondria both in skeletal muscle and neurons[339 340]. These observations strongly support the hypothesis that mtDNA mutations and deletions might be the cause of age-dependent deterioration of mt function.

The underlying causes for accumulation of mtDNA defects with age have been attributed to ROS. Many lines of evidence show that specifically oxidative damage to nuclear and mtDNA increase with age[341 342]. It has been assumed that close proximity to sites of ROS production, lack of protective histones and limited (observed) capacity for repair DNA damage render mtDNA highly susceptible to oxidative damage. Indeed, the levels of oxidative modifications in mtDNA in various tissues have been found to increase with age[343 344 345]. In addition, the level of these modifications correlated with glutathione oxidation and lipid peroxidation, indicators of intracellular oxidative conditions, indicating an age-dependent increase in oxidative stress[346 347].

The question of whether the observed increase in oxidative damage in the context of aging was due to an increase in superoxide production or a decreased antioxidant capacity of the cell was addressed with the MnSOD-mouse model. These animals exhibited neonatal lethality associated with severe mt dysfunction and increased oxidative damage[348]. The MnSOD heterozygotes had partially reduced scavenging activity throughout their lives coupled with increased oxidative damage to the mitochondria[349]. However, levels of several aging biomarkers, as well as the lifespan of these animals, were equal to their WT littermates, suggesting that increased mtROS production is not sufficient for accelerated aging[350].

On the other hand, mice that overexpressed a mitochondrially targeted catalase exhibited prolonged lifespan associated with attenuated oxidative damage to mtDNA and a subsequent decrease in age-related mtDNA alterations[351].

Premature aging syndromes

As described earlier, levels of mitochondrial ROS and mtDNA mutations seem to have an important role in aging and lifespan determination of animals. However, whereas the mice lacking functional proofreading exonuclease activity of POLGA accumulate elevated levels of mtDNA mutations and deletions, and exhibit premature aging phenotype with shortened lifespan, they did not show increased mitochondrial oxidative stress[352 353]. In fact, all markers for oxidative damage and antioxidant defenses were similar between mtPOLGA mice and their WT littermates[354]. Interestingly, the reason for premature aging of these animals was revealed to be increase in age-induced apoptosis due to heavy load of mtDNA mutations[353]. Alternative mechanisms behind aging of POLGA-mutator mice have been proposed to involve decreased stability of OXPHOS complexes due to dysfunctional subunits encoded from the mtDNA, and existence of linear DNA molecules derived from the damaged mtDNA interfere with replication[355 356]. To date, the exact mechanism by which an accelerated mtDNA mutation rate results in premature aging remains unclear.

Also the deficiencies in the nuclear genome maintenance can lead to premature aging. Every time a cell undergoes division, the telomeres at the end of each chromatid lose some of the terminal nucleotides. Shortening of telomeres leads to replicative senescence and a block in cell division, as continuing replication might lead to irreparable chromosomal fusions leading to apoptosis or contribute to various malignancies including cancer and aging. Thus, telomeric length can be used as a method in defining cellular (and to certain extent organismal) age, and how to maintain telomeres has become a target for intense study in cancer and aging.

RecQ family proteins

Various genes are known to be involved in maintenance of the telomeres, and if mutated, lead to premature aging syndromes (progerias). Germ-line defects in three of the five known members of the highly conserved family of RecQ helicases give rise to genetic disorders with cancer predisposition and/or premature aging. One of these is the WRN protein, which is unique in the RecQ-family by possessing also 3'-5' exonuclease-activity. The roles of WRN include DNA repair, replication, transcription and telomere maintenance. All of the identified WRN mutations in patients lead to synthesis of truncated protein unable to enter the nucleus, resulting in complete loss of function, as WRN has not been detected in any other cellular compartment. At the cellular level, patient fibroblasts show premature replicative senescence with chromosomal translocations and deletions. WRN null mice exhibit phenotypes reminiscent of human syndrome, such as early-onset myocardial fibrosis, cancers and metabolic syndrome, but no premature aging was observed[357]. This might be due to fundamental differences in length and maintenance of mouse and human telomeres[358]. However, crosses with TERT deficient animals exacerbates the defects associated with WRN deletion, suggesting a telomere-dependent deterioration of organism by time, specifically the development of metabolic diseases such as diabetes. Interestingly, it was recently shown that telomere dysfunction activates p53-dependent repression of PGC-1a, leading to mitochondrial dysfunction and finally metabolic failure of the TERT-deficient animals[359]. P53 was also implicated in shortening of telomeres in mice with activating mutation in the C-terminus of p53[360]. These might partially explain the age-related decrease in telomere length and metabolic fitness of the organisms.

Interestingly, a recent study shows that depleting WRN in both mouse and human cells led to increased stabilization of HIF-1 through induced mtROS from Complex III and decreased Vitamin-C derivate, ascorbate, levels[361]. In another study, aging of helicase-mutans WRN-mice was reversed and HIF-1a-levels normalized

following treatment with Vitamin C[362]. Opposite to humans, mice and most other vertebrates can synthesize *de novo* Vitamin C, which may explain the observed differences in the phenotypes between human and mouse WRN-models. These results reveal a novel pathway regulated by WRN that might contribute to the pathophysiology of WRN independently from DNA and telomere maintenance.

Although there is no evidence for WRN to localize to the mitochondrion another member of the same helicase family, RecQ4, has been shown to be present in mitochondrial fractions. In accordance with this, the patients with defective RecQ4-helicase presented with increased expression of various mitochondrial genes and increased mtDNA damage[363]. However, compared to the severity of phenotype of cells with dysfunctional mitochondrial helicase TWINKLE, that is essential for mtDNA replication, RecQ4 is likely to play a more specialized role. Nevertheless, the RecQ-family of proteins seems to participate in various pathways involved in aging in addition to their roles in maintaining genomic integrity.

Materials and Methods

Cell culture

Human cells were cultured in Dulbecco´s modified Eagle Medium (DMEM, Invitrogen 41966) supplemented with 10% (v/v) fetal bovine serum (FBS, Sigma F7524-500ML) and 100U/ml penicillin/streptomycin (Reactiva 01030311B000). Puromycin and Hygromycin were used for selection of stable transformed cells.

Cell line	Medium	Puromycin selection	Hygromycin selection
HEK297	DMEM10%	10ug/ml	100ug/ml
U2OS	DMEM10%	10ug/ml	200ug/ml
RPE	DMEM10%	10ug/ml	N/A
MCF7	DMEM10%/RPMI 10%	10ug/ml	100ug/ml
MDA231	DMEM10%/RPMI 10%	10ug/ml	200ug/ml

Table 1. Media used for cell lines. RPMI was used with MCF7 and MDA231 -cells during Hypoxia-induction. Hygromycin-selection was used to create complementation –cell lines.

Short hairpin RNA-mediated knockdown

Stable short hairpin (sh) RNA expression. Viral production and infection: 293T-cells were seeded in 10-cm plates 24h prior to transfection. Transfection was done by adding mixture of 10ug shRNA, 2ug REV, 6ug RRE and 2ug VSV-G plasmids with 78ul Polyethyleneimide (PEI) and 900ul 150mM NaCl on the cells O/N. 16h lated the media was changed and 48h and 72h later the target cells were infected. For lentiviral stable cell lines the following shRNA´s were used form Sigma MISSION lentiviral library: EXD2 TRCN0000051631 (NM_018199.1-155s1c1), TRCN0000327822 (NM_018199.2-1001s21c1), TRCN0000312684 (NM_018199.2-938s21c1) and shRNA against GFP was (SCH005) and sh non-targeter (shNT, SHC016) . 3 days after infection, the cells with integrated shRNA were selected with Puromycin (Sigma P8833-25MG) for 5 days and maintained in the normal growth medium.

Subcloning and expression of EXD2

Human EXD2 was subcloned by using Gateway recombination cloning system (Invitrogen). First, cDNA of EXD2 was amplified by primers shown in table 5, and cloned into pENTR/D-TOPO vector. From this vector it was subcloned using LR clonase reaction into the backbone of gateway-compatible retroviral pLPC-C-SF-TAP –vector or pBABE-vector.

Mutagenesis

QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies 210518-5) was used according to the manufacturers instructions to introduce inactivating mutation A<T to the exonuclease domain of EXD2-gene in the pLPC-C-SF-TAP –vector. The polymerase chain reaction (PCR) mixture was prepared with 5ul of 10x reaction buffer, 100ng of SF-TAP-EXD2, 125 ng sense primer 125ng, antisense primer, 1ul of dNTP mix, 1.5 ul QuikSolution reagent, ddH2O to a final volume of 50ul and 1ul of QuikChange Lightning Enzyme. The primers used are shown in table 5. The supercoiled parental dsDNA was digested by 2ul of DpnI restriction enzyme (Stratagene) for 5 min at 37^{o}C, and X-Gold Ultracompetent Cells (45ul) were transformed with the DNA (5ul) by heat-shock. 500ul of LB-media was added followed by incubation at 37^{o}C for 1h, after which the reaction was spread on agar plates with kanamycin resistance and incubated at 37^{o}C O/N. Mutant plasmids were purified from the positive clones using The PureLink® Quick Plasmid Miniprep Kit (Invitrogen). The sequence of constructs was confirmed by sequencing by internal primer and large-scale plasmid purification was performed by PureYield ™ Plasmid Maxiprep System (Promega). Primers used for mutagenesis presented in Table 2.

Retroviral overexpression

For production of retrovirus, 293T-cells were seeded in 10-cm plates 24h prior to transfection. Transfection with the gene of interest was done by adding mixture of 10ug pLPC-plasmid, 9ug GAG-Pol and 1ug VSV-G plasmids with 78ul PEI and

900ul 150mM NaCl on the cells O/N. Control plasmid was pLPC-N-myc (Addgene 12540). 16h later the media was changed and viral supernatants were collected 48h and 72h later and placed on target cells for O/N infection. Selection was done with Puromycin (Sigma P8833-25MG) for 5 days and the cells were maintained in the normal growth medium.

Transient overexpression of GFP-fusion protein

RNA was extracted from U2OS-cells and cDNA synthesis was performed as described in the manual (Invitrogen, 11146-016), and used to amplify the desired part of EXD2-gene with RT-PCR to create GFP-fusion proteins for expression of full length EXD2 (EXD2-GFP), EXD2 without MLS (EXD2-MLS-GFP) and only the MLS fused with GFP (MLS-GFP) (Table 5) . The PCR-products were gel-purified and cloned into C-GFP-TOPO -vector as described in the manual (Invitrogen, 48-2001). The plasmids were sequenced to verify the presence of correct insert. HEK293-cells were seeded in 10-cm plates 24 h prior to transfection. The cells were transfected with 5ug (EXD2-GFP) or 10ug (EXD2-MLS-GFP and EXD2MLS-GFP) with 78ul PEI and 900ul 150mM NaCl for O/N. 48 hours after transfection the cells were treated 4h with 5uM MG132 (Sigma, C2211) and proteins were processed as described above for IF and WB.

MUTANT		Sequence
EXD2	sense	ATGTCTAGACAGAACTTAGTG
	antisense	CAGACAGCTGGATGGGAAGATC
DEAA	sense	GATTTTCCAGTACTTGGAATTGCCTGTGAGTGGGTAAATTTGGAAG
	antisense	CTTCCAAATTTACCCACTCACAGGCAATTCCAAGTACTGGAAAATC
CXXC	sense	TGATGGTTAAAGAGAACCTGTcTGTAGTGTcTGGCAAGAGAG
	antisense	CTCTCTTGCCAgACACTACAgACAGGTTCTCTTTAACCATCA
EXD2-GFP	sense	ATGTCTAGACAGAACTTAGTG
	antisense	CAGACAGCTGGATGGGAAGATC
EXD2-MLS-GFP	sense	ATGCGGATCCTTAAAGCAAAGGTGG
	antisense	ATGGAGTGGGATCAAATCGAGCCCTT
MLS-GFP	sense	CCACCTTTGCTTTAAGGATCCG
	antisense	GCAAGGGCTCGATTTGATCCCACTCT

Table 2. Sequences of the primers used in site-directed mutagenesis to generate exonuclease dead protein and GFP-EXD2 -fusion protein with truncations.

Cell proliferation and survival assays

MTT-assay

For MTT-assays were done according to the cell proliferation kit (Roche, 11465007001) manual. U2OS- cells were plated on 96-well plate in duplicates at density of 10 000 cells/well. The following day the media was aspirated and 100ul of media with the indicated concentrations of Mitomycin C (MMC) or Methylmethane sulphonate (MMS) were added, and the cells incubated O/N at 5% CO_2 at 37^{o}C. The next morning 10ul of MTT-reagent was added onto each well and the plates were placed back in the incubator. After four hours 100ul of the solubilizing buffer was added and the plates were returned to the incubator until next morning, when the solubilized MTT absorbance was read by using multiwell spectrophotometer.

Glutamine sensitivity was measured on MCF7-cells by plating 10 000 cells/well in triplicates. The next day the cells were rinsed with PBS and 100ul of media lacking

glutamine was added. The cells were incubated at 5% CO_2 at 37^{o}C. for 72h, and the MTT-assay was carried out as described above.

3T3-assay

Cells were continuously replated every 3 days at density of 3 x 10^5 per 6-cm dish and the total number was counted each passage.

Cell cycle analysis

200 000 U2OS-cells were plated day prior to analysis on 6-cm dish and the next day cells were harvested by trypsinization. Trypsin was inactivated by the growth medium from the cells (to include also dead and mitotic cells that detach easily) in 15ml tube and washed twice with PBS and centrifugation at 1000g. To fix the cells, they were gently resuspended in 200ul PBS, and 5ml of ice-cold 70% ethanol (EtOH) was added drop-wise while gently vortexing tubes. Cells were left to fix O/N at -20 ^{o}C. The following day cells were spun down at 1000g and EtOH was poured off. Cells were resuspended vigorously in 1ml PBS and washed twice. 300ul of Propidium Iodide (PI) (25ug/ml) was added supplemented with 0.1mg/ml RNase A in PBS and the samples were incubated at RT for 30min and analyzed by flow cytometry immediately.

Colony forming assay

For the colony forming assay to assess glutamine sensitivity on U2OS-cells, 500 cells were seeded on 6cm plates in triplicates. The next day the plates were rinsed with PBS and media lacking glutamine was added. The cells were incubated at 5% CO_2 at 37^{o}C for 12 days, after which the media was poured away and colonies stained with Crystal Violet. Colonies were counted and normalized to the number of colonies on the plate with full media.

Isolation of total proteins and nucleic acids

Genomic DNA isolation and Q-PCR for mtDNA copynumber

200 000 cells were plated on 6-well plate and collected 48 hours later on 515ul of lysis buffer (75mM NaCl, 50mM EDTA, 0.02% SDS, 0.4 mg/ml Proteinase K). The solution was transferred to 1.5ml eppendorf tube and incubated at 50C for 2h. 1 volume of isopropanol was added, tubes mixed and left in the fridge O/N. The next day the tubes were centrifuged at 4C 8500g for 30 min. Pellet was washed with 70% EtOH and left to airdry. To determine mtDNA copynumber DNA was resuspended in TE and diluted to 25-30ng/ul for Q-PCR. SyrbGreen (Applied Biosystems, 4309155) mastermix was used together with primers (Sigma) and normalized to ActB-levels ("ActB DNA") in the following reaction: 5ul SybrGreen, 2ul H_2O, 2ul Primer (10uM), 1ul DNA.

DNA isolation and protocol for long PCR

The protocol has been described in detail by Santos *et al.*[364]. In brief, 1 million cells were collected and DNA was isolated by PureLink Genomic DNA Mini Kit (Invitrogen, K1820-01). Series of dilutions were prepared from DNA-samples and quantified by using PicoGreen dye (Molecular Probes, P-7581). The dilution where PicoGreen fluorescence displayed linear correlation between DNA concentrations was selected for experiments (7.5ng). After this, the number of PCR cycles was optimized to find linear template amplification range (17 cycles). For the 9kbp long PCR program we used Hot KOD start DNA polymerase kit (Novagen, 71086) as described in the manual, with the exception of using 2.5ul of DNA polymerase. To normalize the results to the mtDNA content, a shorter piece of mtDNA was amplified. The primers used were:

LONG F: 5´-TCTAAGCCTCCTTATTCGAGCCG- 3,

LONG R: 5´-TTTCATCATGCGGAGATGTTGGATGG- 3

SHORT F: 5´-CCCAGCTACTACCATCATTCAAGT- 3´

mRNA isolation and Q-PCR

Total mRNA-levels: RNA was collected by phenol-chloroform- extraction and 1ug was used for cDNA-synthesis. Subsequenctly 1/20 was used for real-time PCR. mRNA-levels were detected by using Taqman probes from Applied Biosystems (*EXD2* Hs00217045_m1, *ACTB* 4352935E, *ESR1* (Hs01046818_m1), *GAPDH* 4333764T, *PCG1* Hs01016719_m1*, *TFAM* Hs01082775_m1*) together with Taqman master mix (AB).

Mitochondrial mtRNA-levels: RNA was treated with DNAse after extraction to remove mtDNA: 25mM MgCl2 was added to final concentration of 5mM and 10U of RNAse free DNA (Sigma, D4263-5VL) was added. The mixture was incubated at 37C for 60 min after which the DNAse was heat-inactivated at 70C 10 min. 1ug of RNA was used for cDNA synthesis according to the manufacture´s instruction (Applied Biosystems, 4387406). For determining mitochondrial mRNAs the possible DNA-contamination was controlled by cDNA-synthesis reaction without reverse transcriptase followed by Q-PCR. For housekeeping control GAPDH or ACTB spanning the introns were used.

For microarray-analysis: 5 000 000 U2OS-cells stably expressing shRNA against EXD2 and GFP were plated in duplicates on 10cm tissue culture plates. 24 hours later the RNA was collected with 1 ml TRI-reagent (Sigma, T9424-200ml), passed deveral times through a pipette and transferred to a 2 ml tube. 200ul chloroform was added, mixed and incubated 5 min at RT, followed by 15 min centrifugation at max speed at 4C. The aqueous layer was transferred to another tube and 875ul of RLT buffer (Qiagen) was added and mixed, after which 625ul pf 100% EtOH was added into the tube. The sample was loaded into an affinity column and the extraction was finished according to the RNA-kit´s instructions. The purified RNA was analyzed with Affymetrix-platforms (Human prime view and/or GeneChip Human Genome U133 Plus 2.0).

	Forward	Reverse
ND2	TAGCCCCCTTTCACTTCTGA	GCGTAGCTGGGTTTGGTTTA
ATP8	ATGGCCCACCATAATTACCC	CTTTGGTGAGGGAGGTAGGTG
ATP6	CGCCACCCTAGCAATATCAA	TTAAGGCGACAGCGATTTCT
CO3	GCCCTCTCAGCCCTCCTAATG	GTGGCCTTGGTATGTGCTTTCTCG
ND3	CCCTCCTTTTACCCCTACCA	GGCCAGACTTAGGGCTAGGA
ND4	TCCTCCTATCCCTCAACCCCG	CAATCTGATGTTTTGGTT
ND6	CCCCGAGCAATCTCAATTACA	TGATTATGGGCGTTGATTAGTAGTAGTT
CYTB	GGGGCCACAGTAATTACAAA	GGGGGTTGTTTGATCCCGTTT
12S rRNA	CATCAAGCACGCAGCAATGCAG	GTTAATCACTGCTGTTTCCCGTG
16S rRNA	CCAAGCATAATATAGCAAGGAC	CTTAGCTTTGGCTCTCCTTG
ND1	CCCTAAAACCCGCCACATCT	GAGCGATGGTGAGAGCTAAGGT
ActB DNA	TCCTCCCTGGAGAAGAGCTA	GAAGGAAGGCTGGAAGAGTG
ActB cDNA	CCTCGCCTTTGCCGATCC	AAGCCGGCCTTGCACATGC
GAPDH cDNA	CCTCGCCTTTGCCGATCC	AAGCCGGCCTTGCACATGC

Table 3. PCR-primer sequences

Total protein extraction and western blotting

Cells were rinsed with PBS and collected in denaturing lysis buffer (50mM HEPES, 16mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 1x protease (Roche 04693132001) and phosphatase (Sigma P5726) inhibitors). Samples were sonicated 15s-15s-30 min, spun down at +4C 20 min 12 000g and quantified with DC Protein assay (Biorad, 500-0114). For detection of HIF-1a 1-1-1–buffer (1mM EGTA, 1mM EDTA, 1%SDS) was used to lyse the cells and sonication was replaced by 20 min incubation on ice. 50ng of total protein was boiled 10min with loading dye (50mM Tris-Cl (pH 6.8), 0.3% SDS, 0.1% bromophenol blue, 10% glycerol, 0.4% b-mercaptoethanol) and separated by SDS-PAGE followed by transfer to nitrocellulose membrane. Membranes were blocked for 20min in PBST/5% milk and primary antibody (in PBST- 5% milk) was added for either 1h at RT or O/N at +4C. Membranes were washed 3x w/PBST and incubated in secondary antibody (in PBST- 5% milk) 1h at RT. After 3x PBS-T wash ECL-reagent (Amersham, RPN2132) and X-ray –film (Fujifilm, 47410 19236) were used to detect the signal. The antibodies used are listed in the tables 2 and 3.

In hypoxia-experiments the cells were placed in an incubator with 1% O_2, 5% CO_2 37^{o}C for O/N and proteins were collected the next morning according to the following: to detect HIF-1α the lysis buffer (1mM EDTA, 1mM EGTA, 1%SDS, inhibitors) was added directly on the cells inside the incubator to prevent HIF-1a destabilization. After this the cells were pipetted up and down until viscous, and boiled at 99^{o}C for 10 minutes. Hot samples were vigorously pipetted and spun down at high speed for 10 minutes, and supernatant was used for protein quantification.

Isolation of cytosolic, mitochondrial and nuclear fractions.

Isolation of cellular compartments were made according to the manufacturers instructions with Strandard cell fractionation kit (abcam, ab109719). For large-scale mitochondrial purification used in Co-IP and ChIP-experiments the cells were lysed in the protocol-specific lysis buffer (see details further) and nuclear fraction was isolated and discarded by centrifugation on 600g for 15 minutes.

Purification of Strep-Flag tagged EXD2 from 293T-cells

WT and DEAA-mutant EXD2 were cloned into a vector containing a C-teminal Strep/FLAG tandem affinity purification (SF-TAP) tag and expressed stably in 293T-cells. The tag can be used in one step on in tandem where the SF-TAP fusion protein is first eluted from the Strep-Tactin with desthiobiotin and then FLAG peptide is used for elution of the SF-TAP-fusion protein from the affinity matrix against FLAG M2. Prior to purification the nucleus was excluded from the solution by centrifugation at 600g for 10 minutes. The cytosolic supernatant was then incubated in overhead tumbler with 100ul pre-washed anti-FLAG-M2 magnetic beads for 1h at 4^{o}C. Beads were washed once with 500ul of wash buffer (30mM Tris pH 7.4, 150mM NaCl, 0.1% NP-40, protease inhibitor cocktail (Roche) and phosphatase inhibitors (Sigma)) and twice with TBS-buffer (30mM Tris pH 7.4, 150mM NaCl, protease and phosphatase inhibitors). SF-TAP proteins were eluted

from the FLAG-M2 beads with 500ul of Flag elution buffer (30mM Tris pH 7.4, 150mM NaCl, 200ug/ml flag peptide (Sigma)) by incubating 20 minutes at 4^{o}C in the overhead tumbler. The different aliquots retained during the experiment and the final TAP fractions were quantified using the Lowry assay (BioRad).* Only FLAG-purification was used due to loss of protein during tandem purification.

Chromatin immunoprecipitation from 293T-cells

4 confluent 15cm 293T-cell plates were used for each experiment. Proteins were crosslinked to DNA by adding formaldehyde drop-wise directly to the media to final concentration of 0.75% and rotated gently at RT for 10 min. Glycine was added to final concentration of 125mM and cells were incubated 5 min with shaking at RT. Cells were rinsed twice with 10ml cold PBS and scraped into 5ml cold PBS in 50ml tube. Nuclear fraction was excluded by centrifuging at 600g 10 minutes. The cytosolic and mitochondrial fraction was centrifuged at 1000g for 5 min and the remaining pellet was resuspended in TNG150 buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 1% Tween-20, 0.5% NP-40, protease and phosphatase inhibitors). Sonication was not used and the protocol was continued as strep-affinity purification. Briefly, the pellet was incubated with Strep-tactin resin for 1h at 4^{o}C in overhead tumbler, after which the resin was washed three times with cold wash buffer in Illustra Microspin Columns (GE Healthcare). Desthiobiotin elution buffer was added to 1x and the columns were incubated 10 min at 4^{o}C in overhead tumbler. To elute the proteins bound to the Strep-resin columns were centrifuged briefly at 100g. To minimize RNA contamination elute was treated with Rnase A and incubated at 37 ^{o}C for 1h. After this the crosslinking was reversed by adding Proteinase K and SDS and heated in 50 ^{o}C for 4h with an increase of temperature to 65 ^{o}C for O/N incubation. The next day DNA was isolated by method described under “genomic DNA isolation”. The cell cycle was analyzed based on the incorporation of PI to the DNA.

Microscopic techniques

Immunofluorescence microscopy

Primary and secondary antibodies used in this thesis for immunofluorescence and western blot (WB) analysis are detailed in tables 2 and 3. Cells were seeded on 8-well chamber slides (Labtech), 10 000/well. Next day they were washed twice with PBS, fixed with 4% formaldehyde for 10 minutes, permeabilized with 0.2% Triton-X for 10 minutes and incubated 60 minutes in primary antibody-PBST-10% FBS. After PBST washes secondary antibodies were added with DAPI for 60 minutes in PBST-10% FBS. Secondary antibodies used: Alexa Fluor 488 goat anti mouse IgG (Molecular Probes A11001), Alexa Fluor 594 goat anti mouse IgG (Molecular Probes A11005), Alexa Fluor 488 goat anti rabbit IgG (Molecular probes A11008) Alexa Fluor 594 goat anti rabbit IgG (Molecular Probes A11012), DAPI (Sigma, D9542-19MG). Slides were washed in PBS, mounted with CC Mount (Sigma C9368-30ML) and coverslipped. Visualizing was made with Confocal Microscope Leica SPE and Leica DMI6000B.

Antibody	Species	Company	Dilution
Flag	Mouse	Cosmo Bio Co (NMDN002)	WB 1:1200, IF 1:500
EXD2	Rabbit	Sigma (HPA005848-100ul)	WB 1:500, IF 1:250
TOM20	Mouse	Santa Cruz (sc-17764)	IF 1:250
Cyt C-FITC	Mouse	Becton Dickinson (560263)	IF 1:500
GFP	Rabbit	Abcam (ab6556)	WB 1:1000, IF 1:500
HIF-1α	Rabbit	Abcam (ab51608)	WB 1:1000
OXPHOS Cocktail	Mouse	Abcam (ab110413)	WB 1:1000
GAPDH	Mouse	Millipore (MAB374)	WB 1:1000
Lamin A	Rabbit	Santa Cruz (sc-20680)	WB 1:250
ERα	Rabbit	Santa Cruz (sc-543)	WB 1:250
DNA	Mouse	Novus (NB110-89473)	EM 1:100
Flag	Rabbit	Sigma (F7425)	EM 1:200

Table 4. Primary antibodies. Primary antibodies used for WB, IF and EM.

Antibody	Source	Dilution
Rabbit HRP	Thermo Scientific	WB 1:30000
Mouse HRP	Thermo Scientific	WB 1:30000
Rabbit Alexa Fluor 488 (green)	Invitrogen	IF 1:500
Rabbit Alexa Fluor 594 (red)	Invitrogen	IF 1:500
Mouse Alexa Fluor 488 (green)	Invitrogen	IF 1:500
Mouse Alexa Fluor 594 (red)	Invitrogen	IF 1:500

Table 5. Secondary antibodies used for IF and WB

Electron microscopy

293T-cells overexpressing EXD2-SF-TAP were grown to 80% confluency and submitted for labeling to the Electron Microscopy core unit of Parc Cientific Barcelona.

Mitochondrial experiments

Membrane potential

To measure *mitochondrial membrane potential*, 100,000 U2OS-cells were seeded on 24-well plate in triplicated 24 hours prior to the analysis. The following day the cells were harvested and 200,000 cells were counted and suspened in 1ml of fresh medium. Cell suspension was stained with 2.5ug/ml JC-1 (Sigma, T4069) during 20 minutes at room temperature in dark. After the incubation cells were washed 2 x 5min with PBS and resuspended in 0.3ml of PBS and immediately analyzed with flow cytometer by using channels FL1 and FL2. The ratio FL1/FL2 (aggregates/monomeric) was measured to present the membrane potential.

Mitochondrial mass

To detect *mitochondrial mass* we used Mitotracker Deep Red (Molecular Probes, M22426) –staining. 100 000 cells were seeded 48h prior to the analysis on 6-well plates in triplicates. On the day of analysis the cells were incubated for 30 minutes with 250nM Mitotracker probe in normal growth medium at 5%O2 in 37C. The cells were trypsinized and washed with warm PBS once and analyzed with Flow cytometry immediately.

Measurement of ROS

ROS-visualization: 100 000 cells were plated and incubated at 37C for 15 min in 10uM 2´7´-difluorescin diacetate (2-7DCHF) (Sigma, D6883). Following this the cells were incubated 15 minutes in warm complete media at 37C after which the cells were washed once with PCS, trypsinized and FACSed immediately (Channel FL1).

Seahorse-assay

Seahorse-assay. To measure the cellular respiration by Seahorse-assay U2OS-cells were seeded at 10 000-30 000 cells/well (Seahorse Biosciences, 100777-004) and cultured for 48h prior to the analysis. The analysis was done according to

the manufacturer´s instructions in DMEM supplemented with 25mM D-glucose, 1mM pyruvate, 4mM glutamine and during the analysis the following final concentrations of reagents were added: Oligomycin(1uM), FCCP (0.4uM), Rotenone (1uM), Antimycin A (1uM) (Seahorse Biosciences, XF Cell Mito Stress Kit, 101706-100).

Metabolomics analysis

Heavy-glucose labeling

24h prior to labeling 6 million U2OS-cells stably expressing the desired constructs were seeded on 15-cm tissue culture plates on triplicates. The following day the cells were rinced twice with PBS and 20ml media containing heavy glucose or glutamine was added to the cells. The media used was: DMEM w/o glucose, w/o glutamine, 10% HFBS, 1x L-glutamine, 1x Penstrep and 2.7g/l ^{13}C6-D-Glucose (Cambridge Isotope Laboratories, CLM-1396-1). Cells were incubated for 6 hours in the media with heavy glucose, after which 2ml of the media of each plate was snap frozen for further analysis. The cells were collected by trypsinization and 10 000 000 cells were pelleted and snap-frozen, after which the metabolites were extracted for nuclear magnetic resonance (NMR) analysis (Centre for Omic Sciences, Yanes Lab).

Fruitfly methods

Food and housing

Fruitflies were fed with food containing 200 g autolysed yeast powder, 50 g sugar, 15 g agar, 30 ml nipagin (100 g/L), and 3 ml propionic acid made up to 1 litre of distilled water. The CR food medium contained 100 g autolysed Brewer's yeast powder, 50 g sugar, 15 g agar, 30 ml nipagen (100 g/L), and 3 mL propionic acid

made up to 1 liter of distilled water. For antioxidant-diet N-acetyl cysteine (NAC) was added to the medium to final concentration of 5mg/ml and new media was prepared every week.

For lifespan experiments, flies were maintained 30 per vial at 25°C, 65% humidity, on a 12h: 12h light: dark cycle and number of dead flies was counted every three days.

Hypoxia sensitivity of flies was assayed in the hypoxia-chamber with 0.6% O_2, 5% CO_2 at 25^{o}C. Flies were put on 50ml tubes capped with cotton and set on the chamber for 30min. After this the tubes were placed vertically and the recovery time wan measured as the number of flies able to stand and climb 2cm up.

Hypothesis:

We hypothesize that EXD2 is a mitochondrial exonuclease whose enzymatic activities are important for mitochondrial homeostasis, development and cancer cell metabolism.

Objectives:

1. Determine the subcellular localization of EXD2.
2. Establish the enzymatic activity and substrate-specifity of EXD2
3. Characterize the effect of EXD2 deficiency on mitochondrial homeostasis and metabolism
4. Determine the effect of EXD2 deficiency on breast cancer growth
5. Determine the impact of EXD2 deficiency on normal development

Results

Identification of EXD2, an evolutionarily conserved exonuclease

EXD2 (Exonuclease 3'-5' domain like 2) is an evolutionarily conserved gene that appeared early in the eukaryotic lineage but is largely absent in Nematoda (Figure 7).

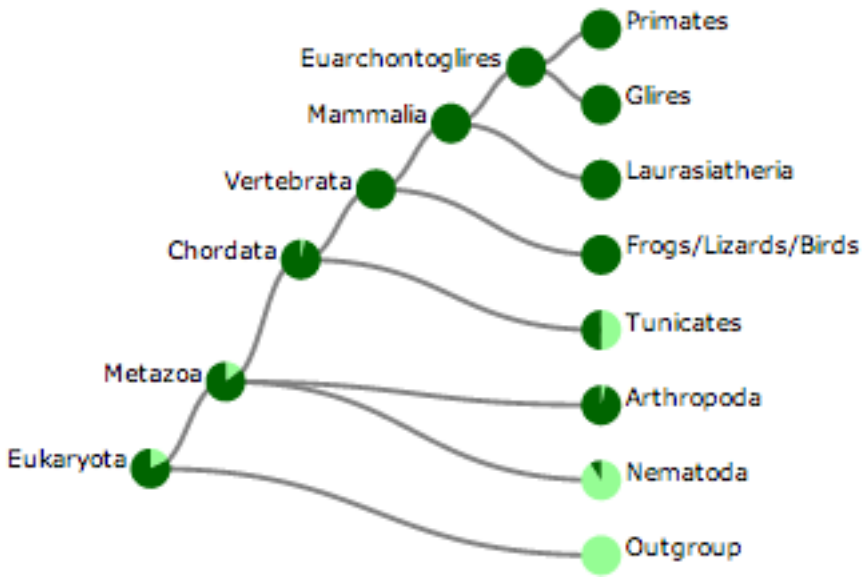

Figure 7. Evolutionary conservation of the EXD2 gene in eukaryotes. Dark green indicates percentage of gene presence and light green its absence[365].

EXD2 possesses a putative 3' to 5' exonuclease domain based on sequence analysis. The exonuclease domain shares high sequence similarity to the DNAQ-like exonucleases, especially to WRN (Werner's syndrome) protein (Figure 8).

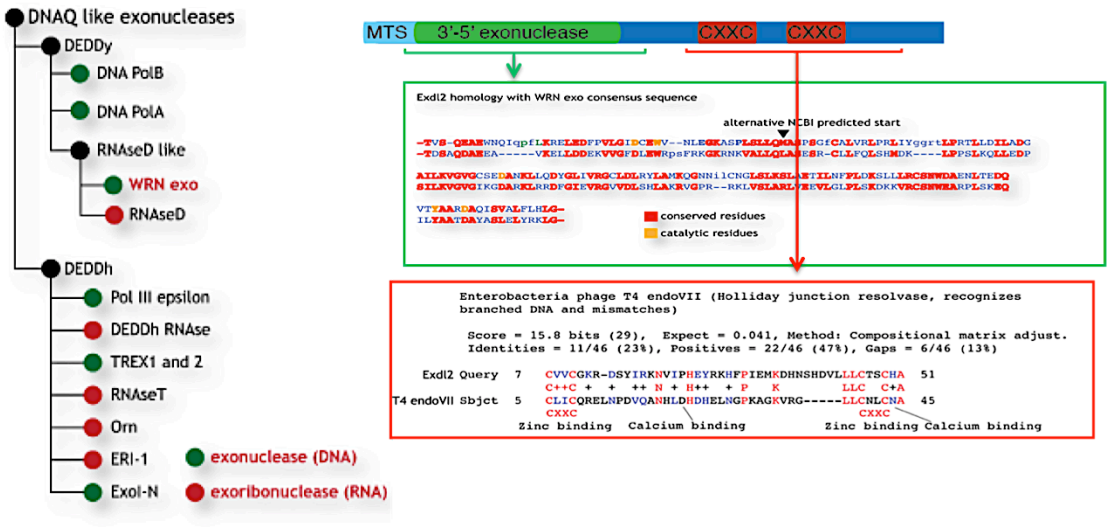

Figure 8. EXD2 is a putative 3'-5' exonuclease with sequence similarity to WRN. Left panel shows related nucleases of the DNAQ-family. Green circles indicate exonucleases and red circles indicate exoribonucleases. Upper right panel shows sequences of conserved putative domains of EXD2, with conserved residues (red) and essential catalytic residues (orange) compared to the consensus WRN sequence family. Lower right panel shows a blast alignment of the C/terminal CXXC –motifs to page HNH resolvase.

The family of DNAQ- like (or DEDD) exonucleases is a structurally conserved group of 3'-5' exonucleases that can catalyze the excision of nucleosides from the 3'-terminal end. The superfamily consists of DNA and RNA-processing enzymes such as the proofreading domains of DNA polymerases, other DNA exonucleases, RNase D, RNase T, Oligoribonuclease and exoribonucleases. The DnaQ-like exonuclease domain contains three conserved sequence motifs termed ExoI, ExoII and ExoIII, which are clustered around the active site and contain four conserved acidic residues that serve as ligands for the two metal ions required for catalysis. Most metallonucleases use Mg(II) as a cofactor, though a few enzymes feature Ca(II) or Zn(II). Depending on the variation of motif III the DnaQ-like family is classified as DEDDy or DEDDh exonucleases with structures YX(3)D or HX(4)D, respectively. The functional difference between these variants remains unclear.

DnaQ-like DEDDy exonucleases are further divided based on their additional activities into DNA polymerases or RNaseD-like exonucleases. The RNaseD

domain is named after RNaseD, one of the seven exonucleases identified in *E.coli*. In *E.coli* it is involved in the 3' processing of stable tRNA molecules by adding the 3'CCA sequence to tRNAs[366]. RNaseD activity has been linked in other organisms to post-transcriptional gene silencing (PTGS) and RNA interference, resulting in cleavage of target mRNAs and their degradation[367].

One of the members of the DnaQ-like DEDDy family proteins is the WRN. WRN has been shown to be critical for the maintenance of the nuclear genome by participating in NHEJ and HR[368 369] and cells from WRN patients have increased chromosomal aberrations[370], abnormal telomeres, increased telomere loss[371 372 373], and premature replicative senescence. These together contribute to premature aging, predisposition to cancer, cardiovascular diseases and diabetes in Werner's syndrome patients[374]. Curiously, most of the pathological manifestations of WRN are considered to result from genetic instability due to aberrant helicase activity of the protein, whereas the significance of the exonuclease activity to its function remains unclear. It is proposed that the 3'-5' exonuclease activity of WRN is involved in the maintenance of CG-rich DNA-structures, such as the telomeres, and repair of specific secondary structures, such as G-quadraplexes often found in telomeres[375]. The exonuclease has also been shown to degrade dsDNA, DNA/RNA hybrids, DNA from gaps or nicks and to remove terminal mismatched nucleotides[376 377]. There is evidence that interaction with tumor suppressor p53 enhances the helicase activity and suppresses the exonuclease activity of WRN, coupling the two enzymatic activities with cell cycle regulation[378 379].

Another known DnaQ-like DEDDy-family protein is *C.elegans* MUT7. MUT7 is a cytosolic/nuclear exonuclease that has been implicated in mRNA degradation via RNA interference during the germline development of *C.elegans*[380 381]. Based on sequence similarity, Exd3 (3'-5' exonuclease domain-like 3) is proposed to be the mammalian homolog for MUT7 but no studies have been published on the protein.

Although the function of EXD2 is not immediately clear, a large number of organisms express a homologoue, including the most widely used model

organisms with the exception of *C.elegans* (Figure 9). The high conservation of EXD2 amongst diverse species suggests that it may play important roles in basic cellular functions.

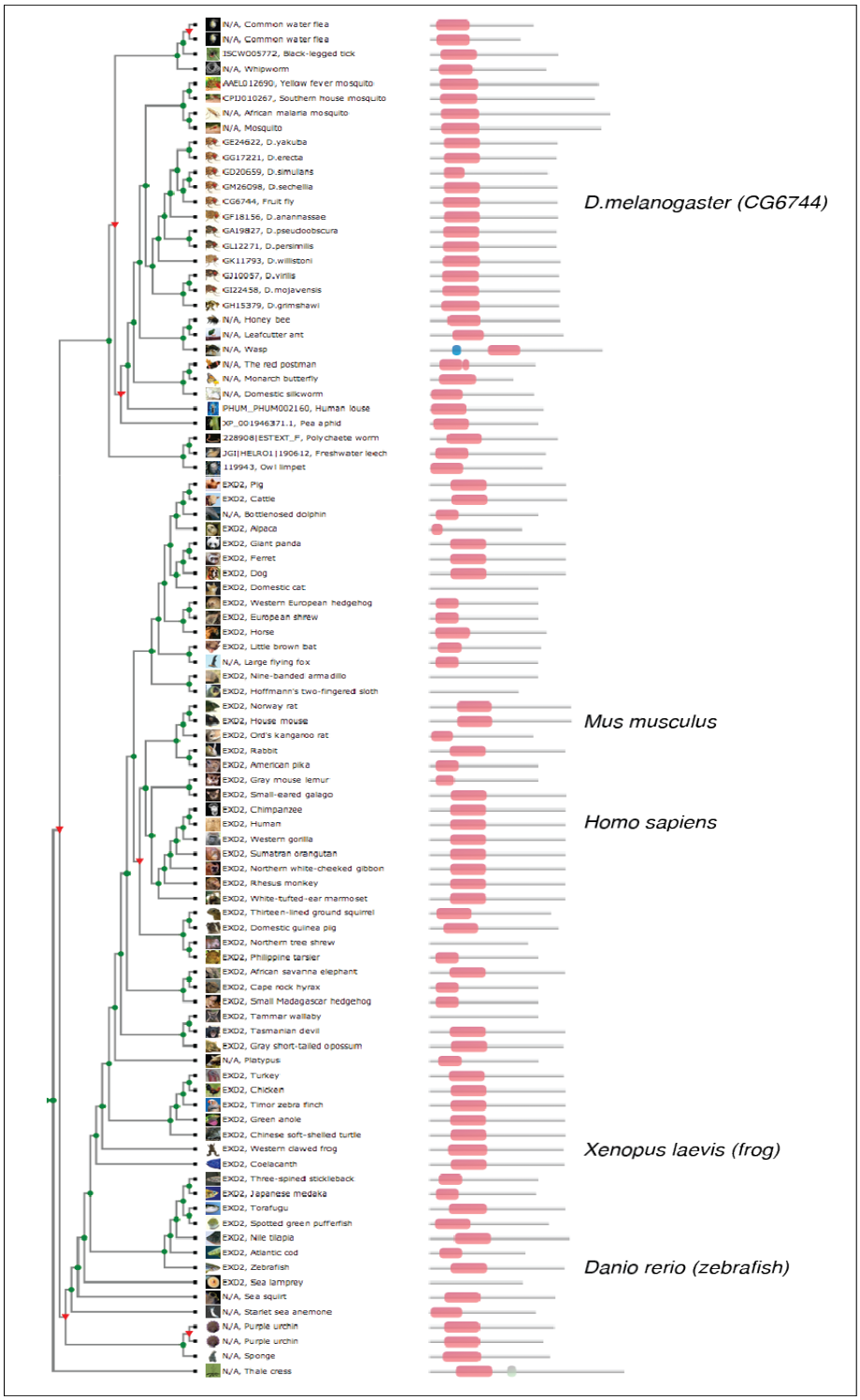

Figure 9 (Previous page). Evolutionary conservation of the EXD2 gene in eukaryotes. Gene tree of EXD2 in Eukaryota with exonuclease domain indicated by the pink box. Sequences from model organisms aligned in Figure 14 are noted[382].

In addition to its putative exonuclease domain, EXD2 also harbors a conserved putative endonuclease-domain with limited similarity to the HNH family endonucleases (Figure 8). This domain harbors two CXXC-domains suggestive of Zinc-finger motifs known to perform many functions through interactions with nucleic acids[383]. Alternative structure predictions also suggest these domains may be related to multiheme cytochromes that bind hemes through coordinated cysteine and histidine residues (Figure 10).

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CLUSTAL 2.1 multiple sequence alignment


Zebrafish       FEPSGRPDSQQD-YYLTAKENLCVVCGKADSYIRKNIVPHEYRRHFPIEMKDHNSHDILL 59
Fugu            FEPSGRPDSEKD-YYLTAKENLCVVCGKVDSYIRKNIVPHEYRRHFPSEMKDHNSHDILL 59
Coelacanth      FEPSGRPESQVD-YYLTVKENLCVVCGGRESYIRKNVVPHEYRRHFPLQMKDHNSHDVLL 59
Human           FEPAGRPESPGD-YYLMVKENLCVVCGKRDSYIRKNVIPHEYRKHFPIEMKDHNSHDVLL 59
Fly             FEPAGRAVGDVGRFYQTIKKNQCVVCGDRDAYIRKNVVPREYRKHFPLVMKSHTSDDVLL 60
                ***:**. .  . :*   *:* *****  ::******::*:***:***  **.*.*.*:** 

Zebrafish       LCTSCHA- 66
Fugu            LCTACHAA 67
Coelacanth      LCTACHAA 67
Human           LCTSCH-- 65
Fly             LCPTCH-- 66
                **.:**
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Figure 10. Clustal alignment of the predicted EXD2 C-terminal domain shows high conservation. A multiple sequence alignment generated by CLUSTAL 2.1 shows many invariant residues in organisms from fly to man, including the CXXC-motifs.

Among the most conserved sequences are the active sites of enzymes and the binding sites of protein receptors. Interestingly, when EXD2-sequence is aligned with homologues across species, various un-identified sequences share similarity, indicating possible additional enzymatic activities or functional domains in EXD2 (Figure 11).

Taken together, EXD2 is an uncharacterized protein with sequence similarity to the exonucleases of the RecQ-family, with particularlyhigh homology to the exonuclease-domain of WRN. EXD2 also harbors other highly conserved domains, including potential endonuclease or Zinc-finger domains, as well as additional motifs with yet uncharacterized functions.

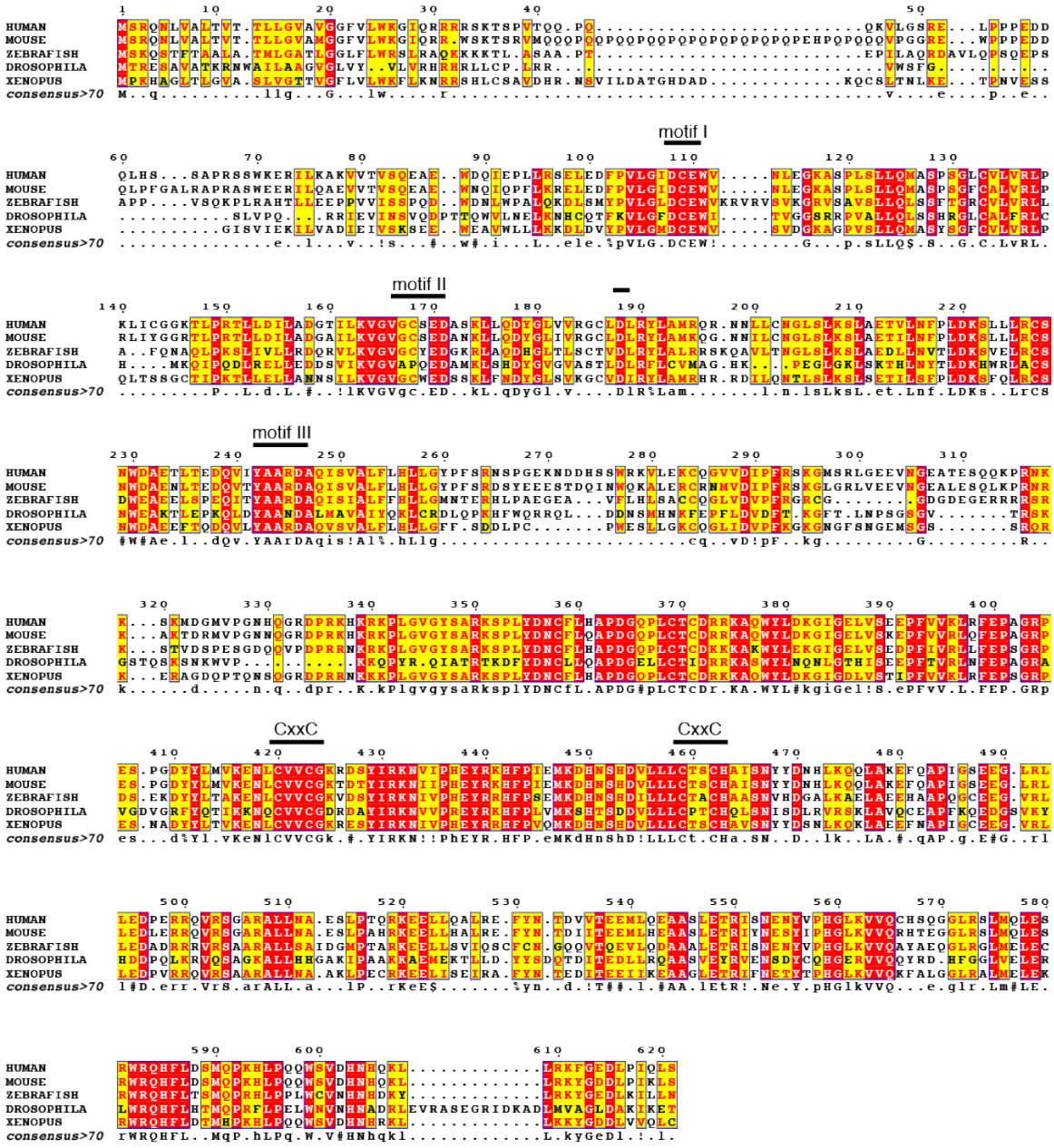

Figure 11. Conservation of EXD2 at the amino acid level. Amino acid sequences of EXD2 orthologues were obtained from Uniprot and aligned using the T-Coffee server. Clear conservation of the exonuclease domain (motifs I-III) and the C-terminal portion of the protein containing the CXXC motifs is evident. Graphics were generated in ESPript and domains notated manually in illustrator.

EXD2 localizes to the mitochondria

To determine the subcellular localization of EXD2, we performed immunofluorescence (IF) against the endogenous protein and observed a filamentous cytoplasmic signal reminiscent of mitochondrial networks. Mitochondrial staining of EXD2 was confirmed by co-staining with the mitochondrial protein Cyt-C or the mitochondria-specific dye Mitotracker (Figure 12B). This was interesting, as there are only three known mitochondrial exonucleases identified to date; EXOG and ENDOG possessing 5'-3' exonuclease activity and POLG that has both polymerase and 3'-5' proofreading exonuclease activity[66 68 384]. We queried the sequence of EXD2 in Mitoprot (http://ihg.gsf.de/ihg/ mitoprot.html), an online tool for predicting mitochondrial protein localization based on the presence of a hydrophobic signal peptide, and results suggested high probability (65%) for human EXD2, and even higher probabilities of the zebrafish (92%) or the Drosophila orthologues (95%), to be localized to mitochondria. Sequence alignment revealed the putative MLS to be conserved across species, suggesting it to be an important part of the protein (Figure 11, 61 first residues, and 12A). In addition, this sequence bore similarity to the N-terminal signal peptide of the mitochondrial polymerase POLG (Figure 12A).

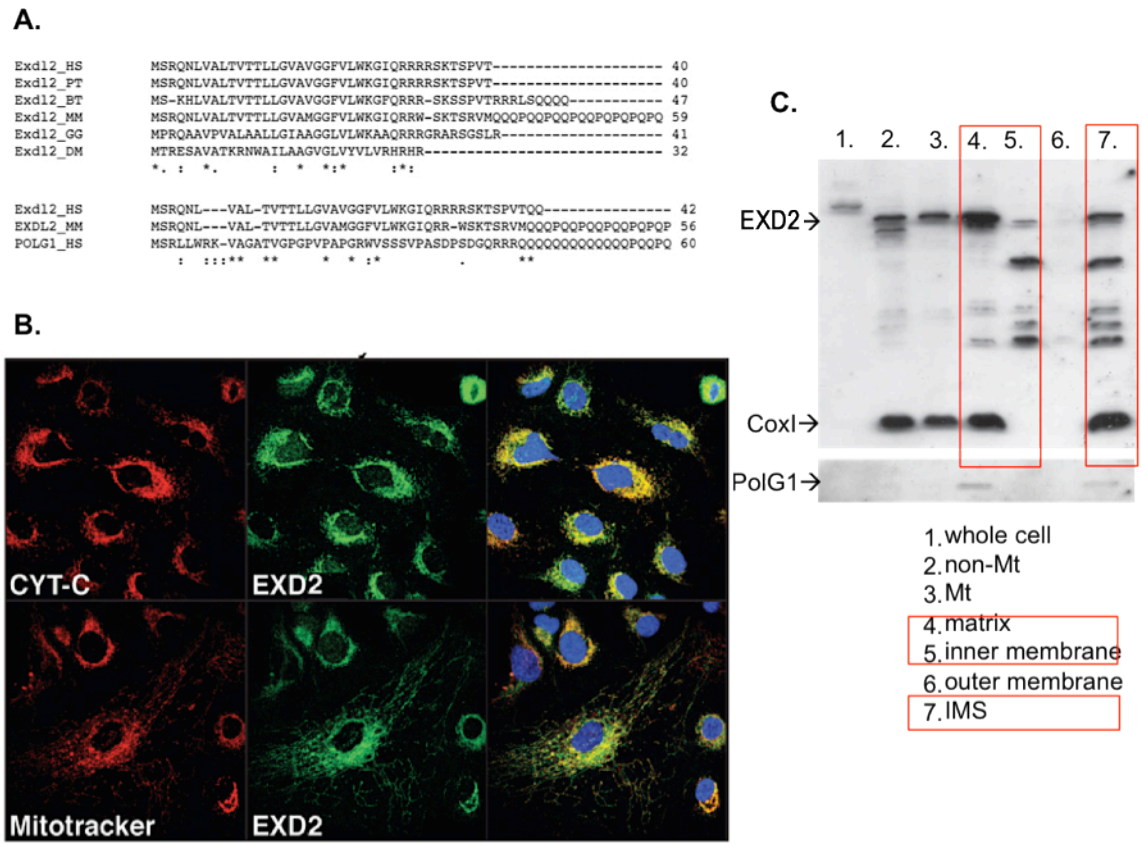

Figure 12. Endogenous EXD2 localizes to the mitochondrial inner membrane and matrix. A) Homology of the MLS across species and comparison to POLG1. B) Endogenous EXD2 co-localizes with mitochondrial markers Cyt-C and mitotracker. C) Western blot against EXD2 after cellular and mitochondrial fractionation. HS=*Homo sapiens*, PT=*Pan troglodytes* (chimpanzee), BT=*Bos taurus*, MM=*Mus musculus*, GG=*Gallus gallus*, DM=*Drorophila melanogaster*.

To establish with higher resolution the intermitochondrial localization of EXD2, we performed mitochondrial fractionation and electron microscopy. Mitochondrial fractionation allowed the enrichment of the OM, IM, IMS and matrix and subsequent WB showed that EXD2 localized in the matrix, as well as in the IM and IMS.

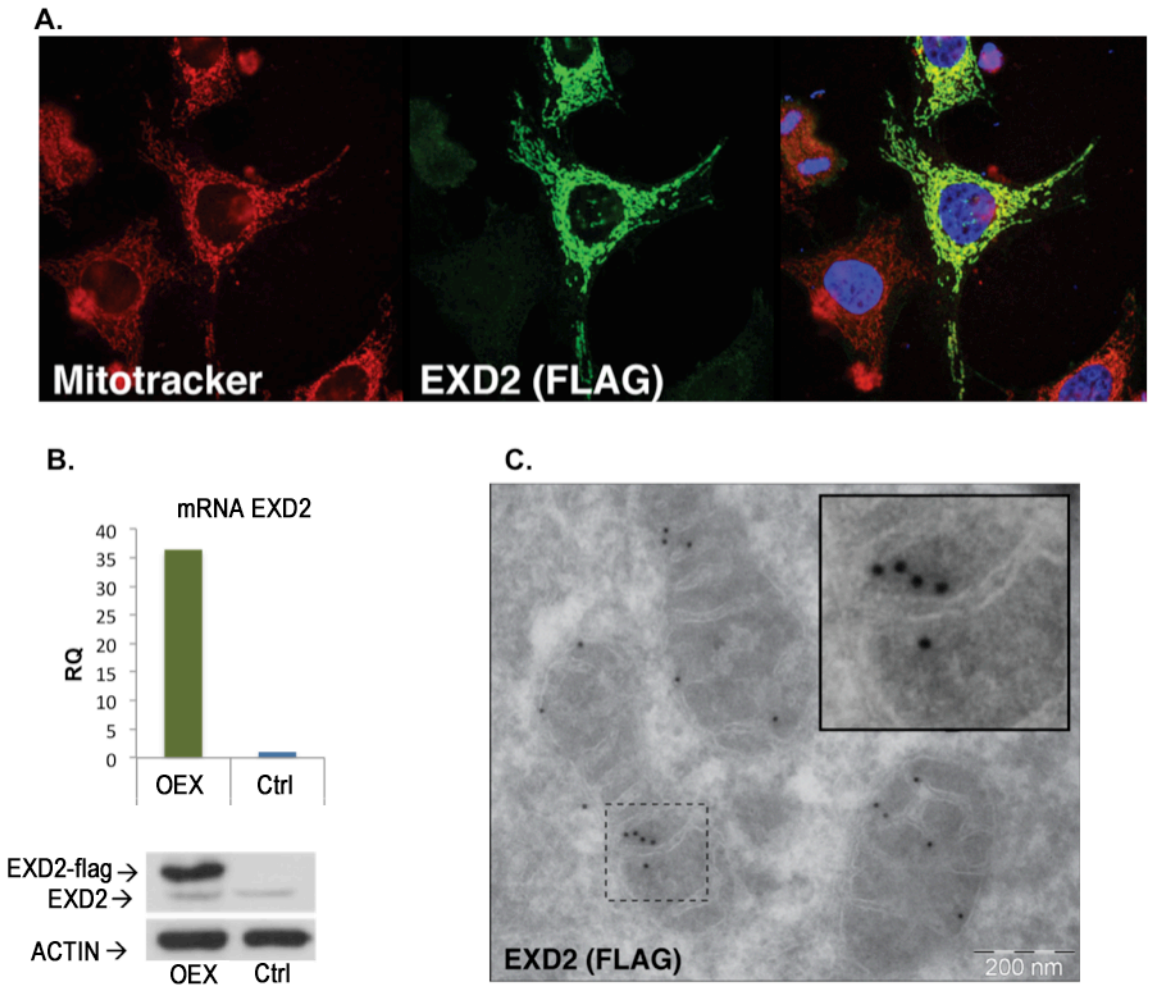

Figure 13. Overexpressed EXD2-SF localizes to the mitochondria. A) IF against Mitotracker and Flag in U2OS-cells stably overexpressing EXD2-SF. B) mRNA and protein levels of EXD2 in the U2OS cells overexpressing EXD2-SF (OEX). C) EM imaging of 293T-cells stably overexpressing EXD2-SF-TAP, conjugation with 17nm gold particles (EXD2-FLAG).

Due to the low endogenous levels of EXD2 we created stable cell lines overexpressing a Strep-Flag tagged EXD2 fusion protein (EXD2-SF) to better address its subcellular localization (Figure 13). Retroviral overexpression of EXD2-SF in U2OS and 293T-cells resulted in an over 30-fold increase in the transcript levels that was maintainable for many subsequent passages, suggesting that high protein levels of EXD2 were not harmful to the cells (Figure 13B). IF against overexpressed EXD2-SF showed strong mitochondrial pattern with little nuclear

signal (Figure 13A). This was confirmed by electron microscopy (EM) that the majority of the EXD2-SF signal localized to the mitochondria (Figure 13C). The quantification of EXD2-SF labeling revealed equal unspecific binding of the gold particles in the cytosolic and nuclear compartments in the EXD2-SF and Ctrl cells (Figure 14).

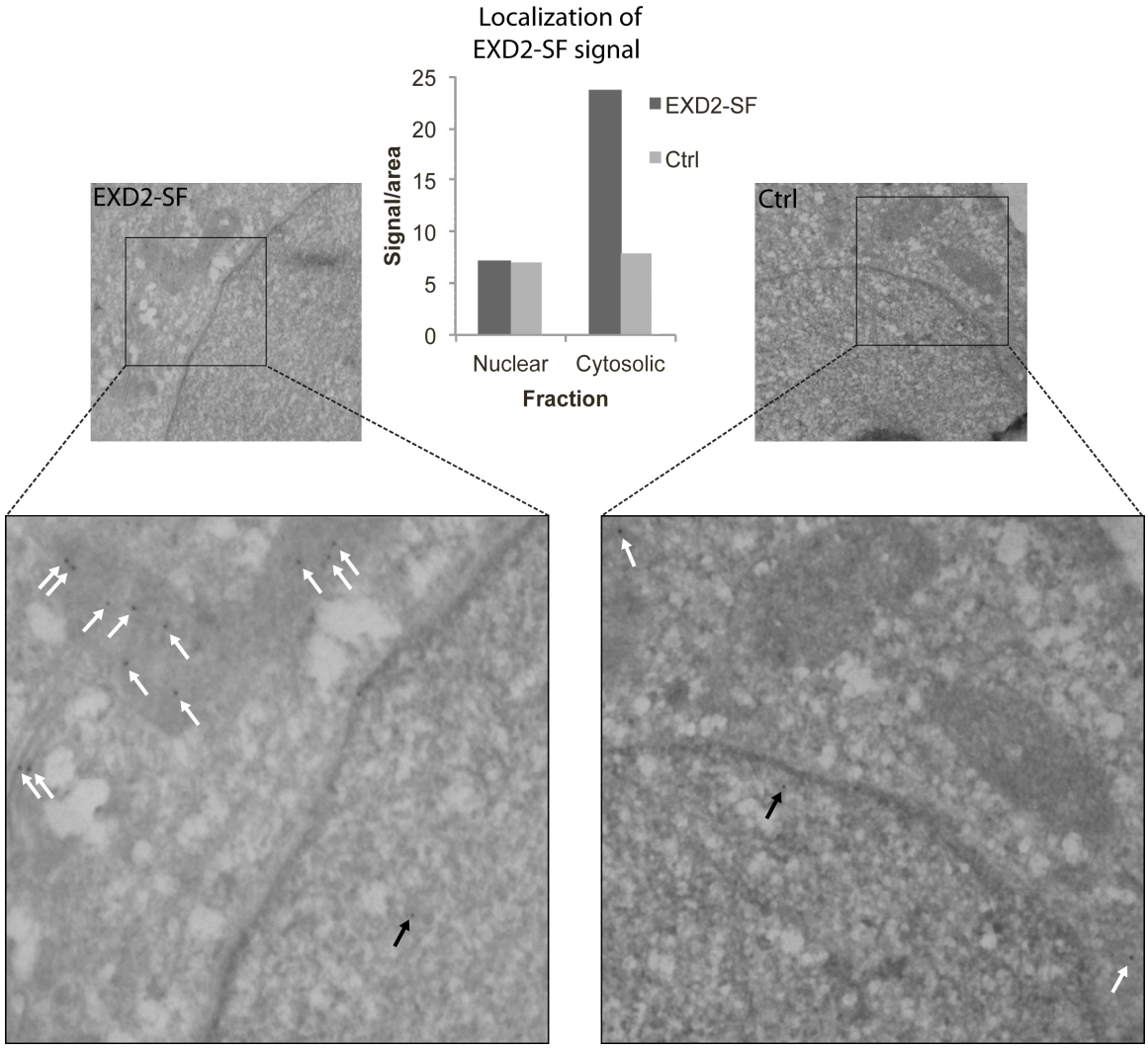

Figure 14. Localization of EXD2-SF in different cellular compartments. Gold particles conjugated with EXD2-Flag were quantified in the nucleus and cytosol of the EXD2-SF expressing cells and cells expressing Ctrl plasmid. 50 photos were analyzed and amount of label was normalized to the total area of the analyzed cellular compartment (nuclear label/nuclear area, cytosolic label/cytosol area).

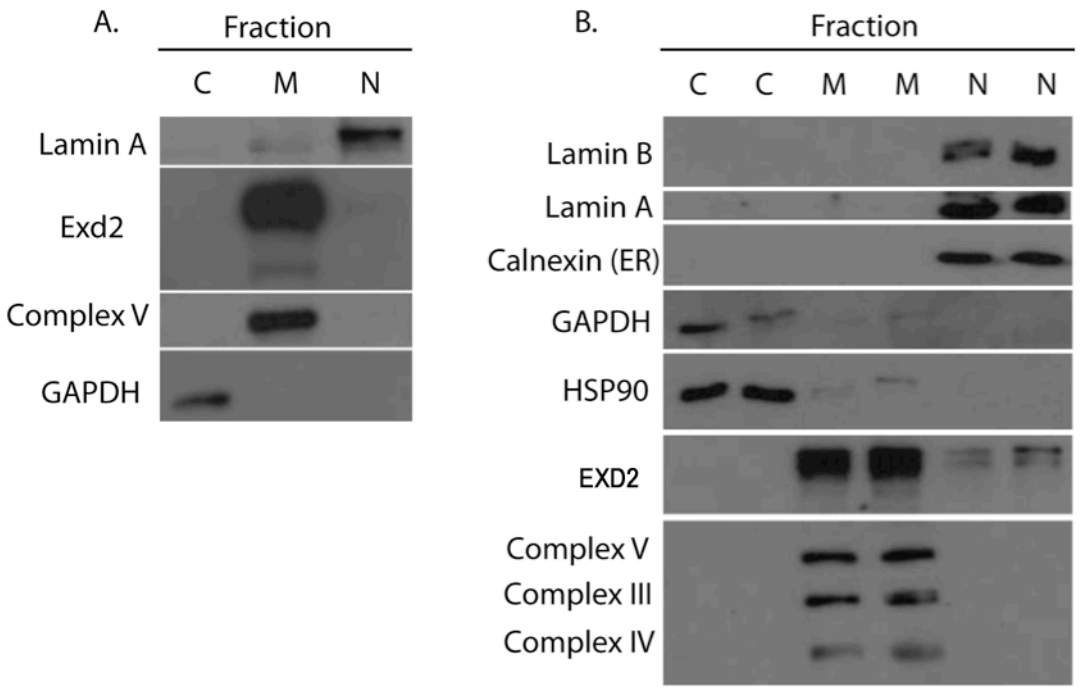

Figure 15. Cellular fractionation of 293T-cells. A) 293T-cells were fractionated and WB was done with 5% of the fractions (C=9ng, M=6ng, N=5ng). **B)** Duplicates of 293T-cells were fractionated and WB was done with 10ng of protein per fraction. C, Cytosolic; M, Mitochondrial; N, Nuclear; ER, endoplasmic reticulum.

To verify the absence of EXD2 in the nucleus, we performed cellular fractionation. The separation of cytosolic, mitochondrial and nuclear fractions indicated, that there was a very small portion of EXD2 residing in the nucleus (Figure 15) However, when we blotted the membranes against ER-specific antibody, we confirmed the presence of ER-residing proteins in the nuclear fraction. As we believe EXD2 to be secreted from the ER, this would explain its presence in the nuclear fraction of the 293T-cells (Figure 15B)

As EXD2 labeling for EM was successful, we wanted to use it to address whether EXD2 localizes close to the mtDNA that is known to be localized to discrete nucleoids. For this purpose we performed EM with double labeling against EXD2-FLAG (8um gold particles) and DNA (17nm particles). We found no significant correlation between the localization of the two signals (Figure 16). This might signify that EXD2 does not, under steady state conditions, associate with the

mtDNA, but instead may relocalize there in response to stress. Another possibility is that it targets various mitochondrial RNA species that localize in the matrix.

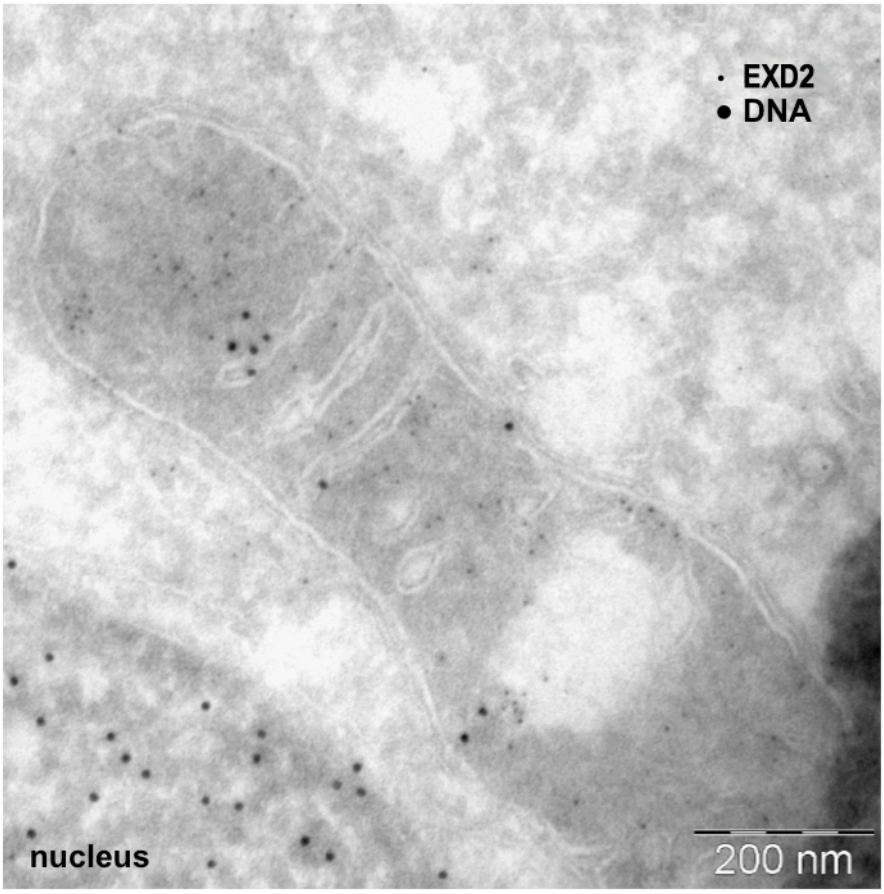

Figure 16. EM image of mitochondrial localization of EXD2 and DNA. 293T-cells stably overexpressing EXD2-SF labeled with 8 nm gold particles (EXD2-Flag) and 12 nm gold particles (DNA).

According to the Mitoprot prediction, the first 60 aa of EXD2 represent a MLS. To find out if it was required for transporting EXD2 to the mitochondria, we removed the stop codon and fused the full length EXD2 to a C-terminal GFP-tag. We also created a GFP fusion-protein consisting of the MLS alone, or a full length protein lacking the MLS, to determine if it was necessary and sufficient to target the protein into the organelle. An alternative EXD2 mRNA has been reported in humans that

would be predicted to lack the MLS and motif I of the exonuclease domain, suggesting that it could localize independently of the MLS. The results showed that EXD2 lacking the MLS was not able to localize to the mitochondrion and was rapidly degraded. In contrast, the full-length EXD2-GFP was efficiently transported to the mitochondrion and the MLS alone was sufficient to transport GFP to the organelle (Figure 17.). This confirmed that the N-terminus of EXD2 is a *bona fide* MLS and necessary and sufficient for mitochondrial targeting of the protein.

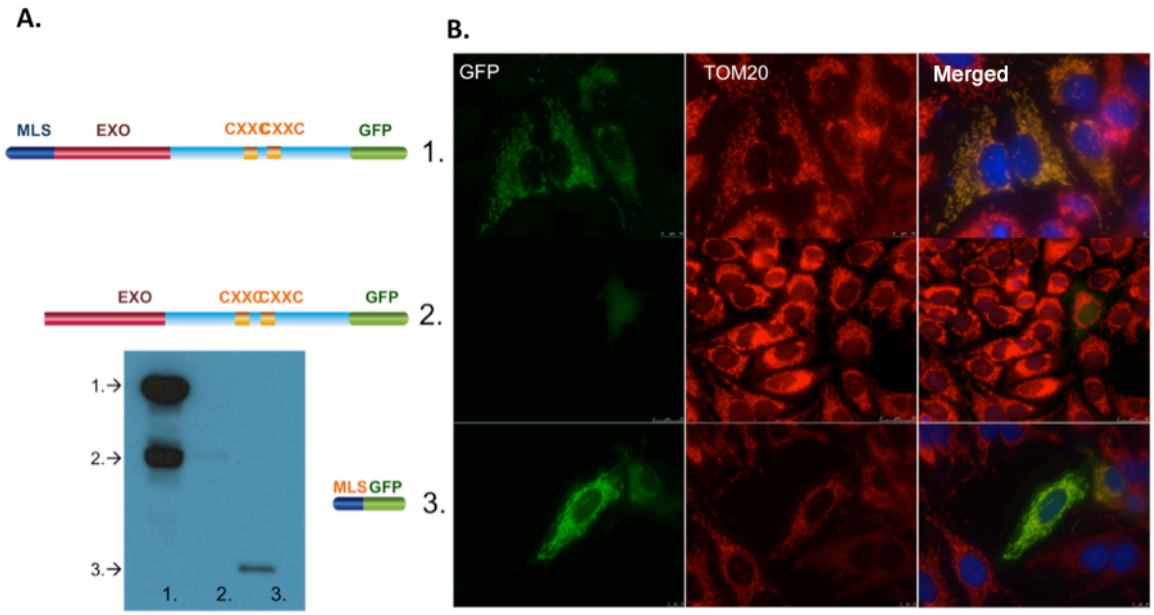

Figure 17. The MLS is necessary and sufficient to transport EXD2 to the mitochondria. A) Structure and localization of EXD2-GFP fusion protein constructs with (1.) and without the MLS (2.) or the MLS alone (3.) fused with GFP. **B)** Transiently overexpressed GFP fusion proteins in HEK293-cells. TOM20 is used as mitochondrial marker.

Collectively, our results based on cellular imaging and subcellular fractionation indicated that EXD2 is a resident mitochondrial protein with no significant localization to the nucleus. This suggested that if its functions were similar to that of WRN, it would be restricted to the RNA and DNA resident to the mitochondria.

Work from Smogorzewska *et al.* suggested EXD2 to be involved in cross-link repair as part of the Fanconi Anemia (FA) complex based on their results from an

shRNA-screen against over 30,000 transcripts[384]. However, we failed to detect any sensitivity to the crosslinking agent MMC and alkylating agent MMS in cells with reduced levels of EXD2(Figure 18).

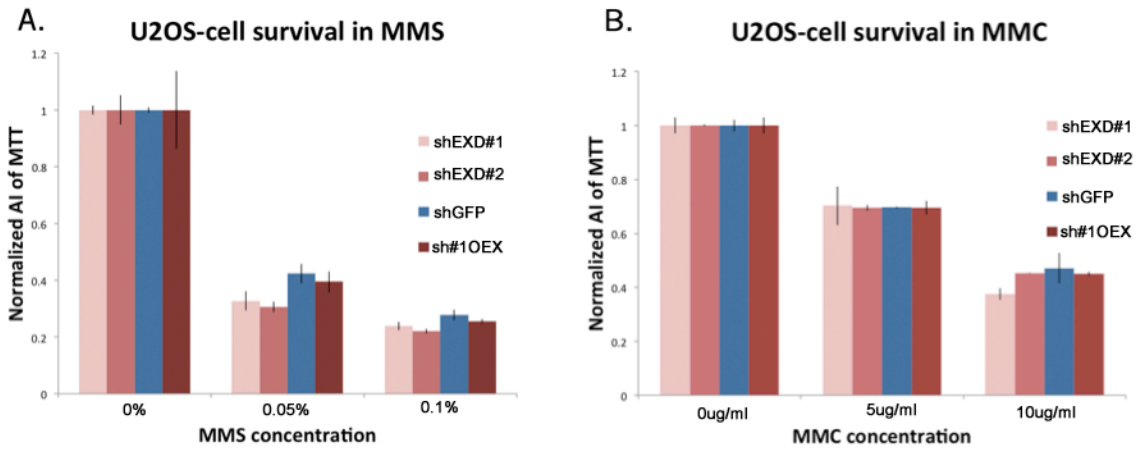

Figure 18. EXD2-levels and sensitivity to genotoxic agents. U2OS-cells were incubated for 24h in the presence of A) MMS or B) MMC to induce alkylating or crosslink-damage, respectively. Survival was measured by MTT-assay and normalized to absorbance of untreated cells. sh#1OEX, shExd#1-cells overexpressing WT EXD2 insensitive to the shRNA (rescue).

Given this results and the fact that EXD2 does not localize to the nucleus, our results are inconsistent with this proposal and suggest that EXD2 is a mitochondrial protein with no direct role in DNA damage response in the nucleus.

EXD2 is an active 3'-5' exonuclease

Next we wanted to confirm that EXD2 was an active exonuclease and determine if it had substrate preference that may give insight into its biological functions in the cell. The full-length protein was insoluble when produced in bacteria. To generate soluble protein, we removed part of its C-terminus as well as N-terminal MLS. For controls and domain mapping, we generated several mutant proteins lacking key residues in the exonuclease-domain and CXXC-CXXC motif (Figure 19A). Close to full length EXD2 (61-470) was incubated with ssDNA, dsDNA, and templates with 3' and 5' overhangs in the presence of ATP and the divalent metal cofactor (Mn+). In all cases, a degradation pattern indicative of exonuclease activity was observed

(Figure 19B). As nuclease contaminants are often present in bacterial extracts, an exonuclease mutant (DE>AA) of EXD2 was created to ensure that observed activity was not due to bacterial contaminants (Figure 19B). As expected, the EXD2-DEAA was inactive in all of the assays testes (Figure 19B and 19D). We tested the dependence of EXD2 activity on metal binding, and found both Mn+, and to a lesser extent, Zn+ supported its activation, in contrast to Mg+ or Ca+ that did not (Figure 19C).

As many exonucleases, including WRN, are needed to facilitate normal DNA replication or DNA repair, we tested the activity of EXD2 against various cruciform structures generated as replication intermediates (Figure 19B). All templates tested were degraded by the exonuclease activity of EXD2, suggesting that it a very unselective DNA-nuclease *in vitro*. Interestingly, we also found that EXD2 was able to robustly degrade both ssRNA and RNA/DNA hybrids, suggesting a potential role in RNA metabolism (Figure 19D).

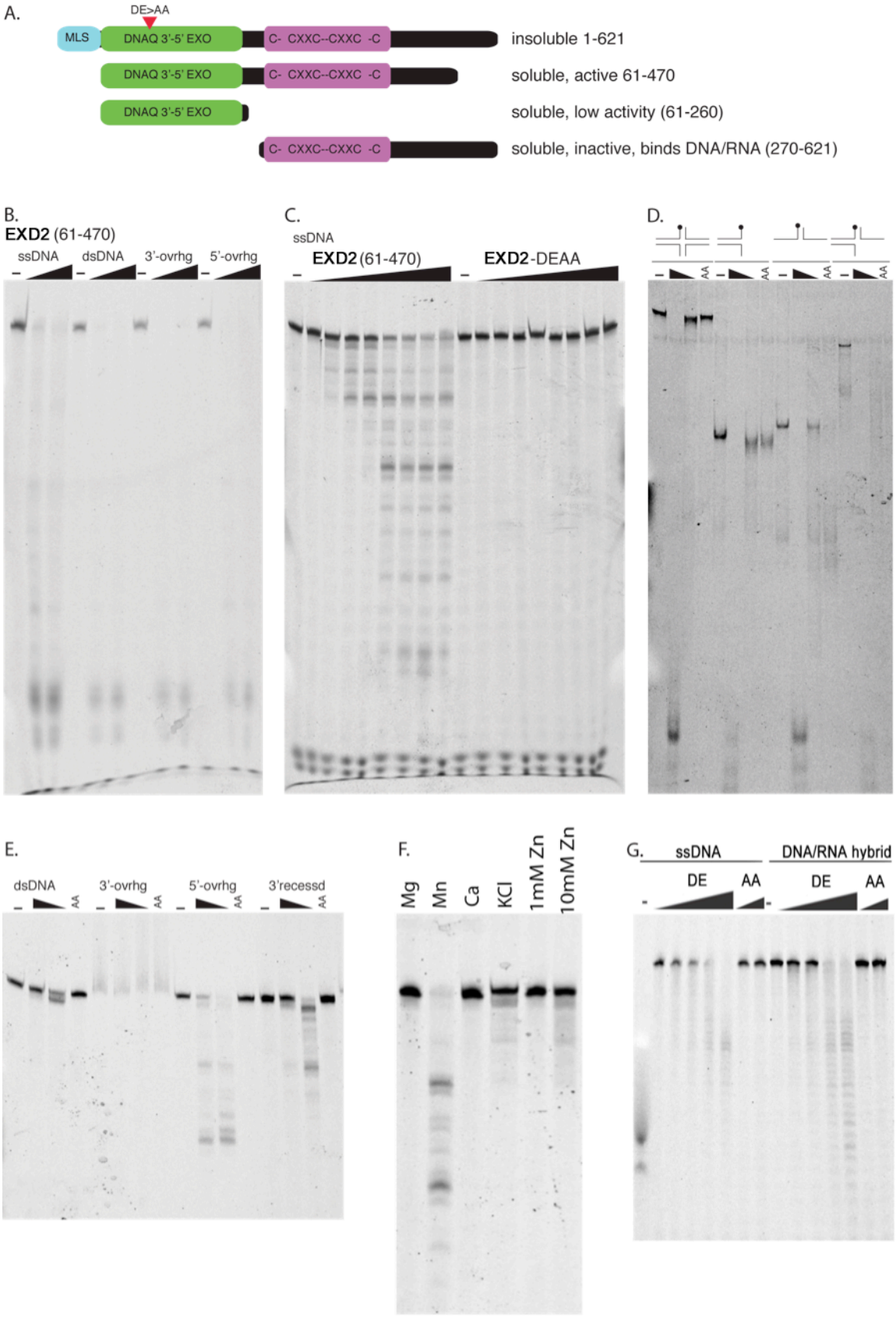
A.
DE>AA
MLS
DNAQ 3'-5' EXO
C- CXXC--CXXC -C
insoluble 1-621
soluble, active 61-470
soluble, low activity (61-260)
soluble, inactive, binds DNA/RNA (270-621)
B.
EXD2 (61-470)
ssDNA
dsDNA
3'-ovrhg
5'-ovrhg
C.
ssDNA
EXD2 (61-470)
EXD2-DEAA
D.
AA
E.
dsDNA
3'-ovrhg
5'-ovrhg
3'recessd
F.
Mg
Mn
Ca
KCl
1mM Zn
10mM Zn
G.
ssDNA
DNA/RNA hybrid
DE
AA

Figure 19 (previous page). EXD2 is an active exonuclease with little specificity for structure or template. A) Graphical summary of bacterially produced EXD2-proteins. **B)** Exonuclease activity of EXD2 against ssDNA, dsDNA and 3' and 5' overhangs. **C)** Nuclease activity of nuclease active EXD2 and nuclease mutant EXD2 on ssDNA substrate **D)** DNA structure-specific exonuclease activity of nuclease active EXD2 and nuclease mutant EXD2. **E)** Exonulcease activity of nuclease active EXD2 and nuclease mutant EXD2 against dsDNA and overhangs. **F)** Metal-dependence of exonuclease activity of EXD2. **G)** EXD2 exonuclease activity on ssRNA-substrates and RNA/DNA hybrids.

According to our EM-analysis EXD2 did not clearly co-localize with mtDNA. However, biochemical characterization revealed DNA-binding and exonuclease activity on DNA templates. To further study this discrepancy between the results from cellular and *in vitro* and biochemical analysis, we decided to perform chromatin immunoprecipitation (ChIP)- expreriment with the EXD2-SF-TAP overexpressing cells. Interestingly, we found that in 293T-cells overexpressing WT and nuclease-dead (DEAA) EXD2 does co-precipitate with mtDNA, suggesting a physical interaction between them (Figure 20). This interaction was absent in cells overexpressing EXD2 with mutations in the first CXXC-domain, suggesting it to be responsible for the DNA-binding of EXD2, a possibility we are currently testing *in vitro.*

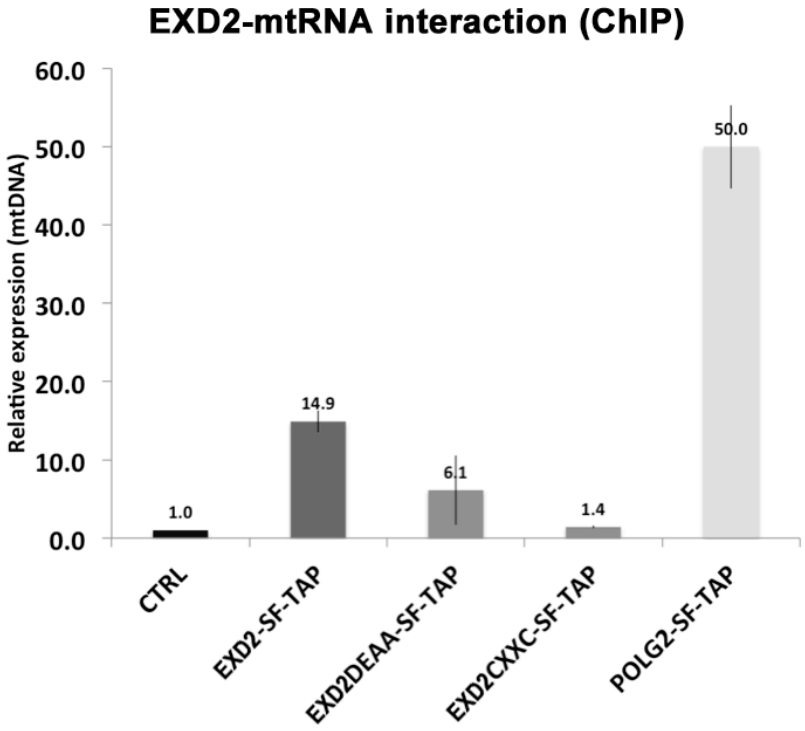

Figure 20. Binding of EXD2 to the mtDNA. 293T-cells overexpressing EXD2-SF-TAP, negative control vector (CTRL), and positive control vector (POLG2-SF-TAP) were crosslinked, ChIP´d and nucleic acids were affinity purified**.** Q-PCR against the Cytochrome C oxidase subunit 2 (mtCO2)-gene was run to detect presence of mtDNA and normalized to the CTRL-vector sample.

As WRN is implicated in DNA replication, we wanted to determine if EXD2 had a similar role in mtDNA replication. To address this, we created cells with a stable known down (KD) of EXD2 using three different short hairpin RNAs (shRNAs) targeting different parts of the mRNA. Each one resulted in an over 80% reduction in mRNA expression of EXD2 in U2OS cells. (Figure 21A). Next we performed 2D-age of purified nucleic acids from these cells and Southern blotting to detect DNA and RNA mtDNA replication intermediates. Our results showed that the progression of the mitochondrial replication fork is not affected by EXD2 silencing in either of the cell lines analyzed (Figure 21B, C). These results indicated that EXD2 is not essential for mtDNA replication under normal conditions, consistent with the lack of specificity in the *in vitro* assays.

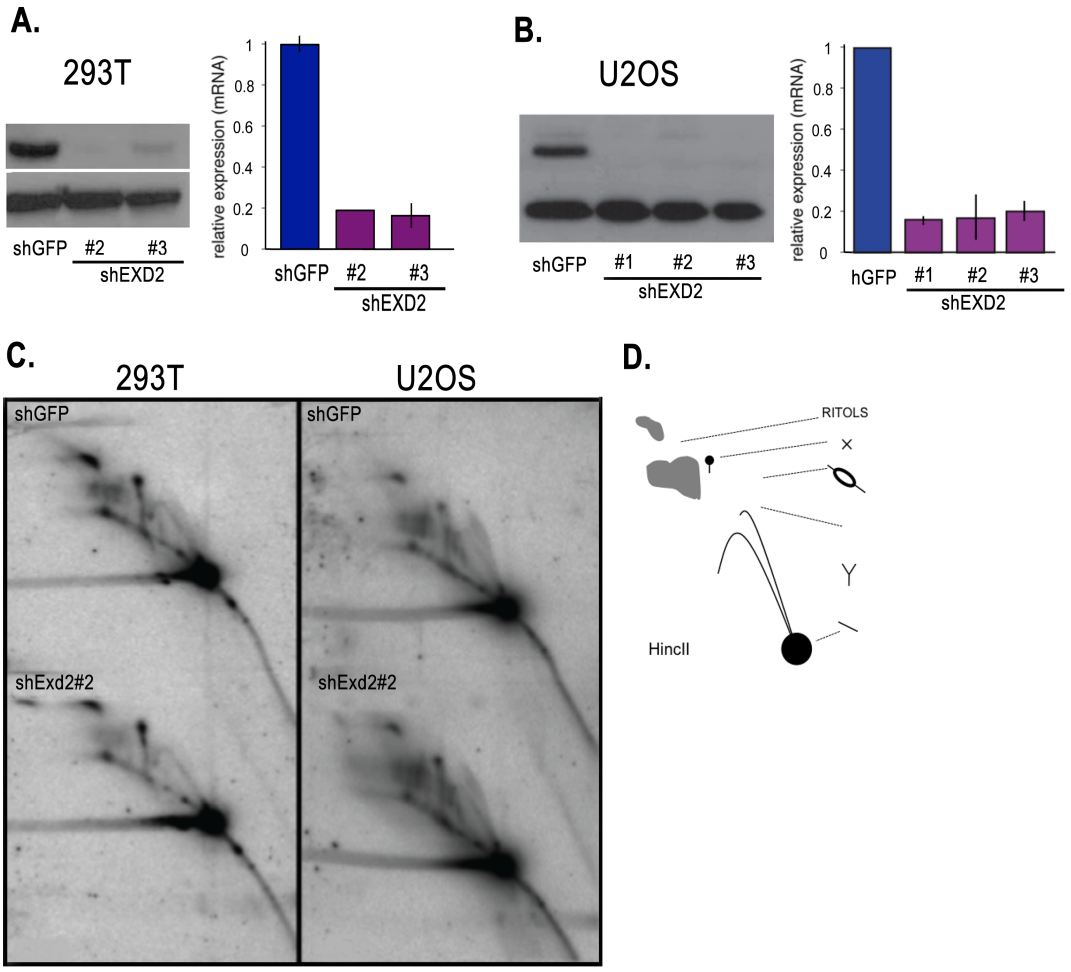

Figure 21. EXD2 is not required for mtDNA replication. A) mRNA and protein levels of EXD2 in U2OS-cells stably expressing control shRNA (shGFP) and shRNAs against EXD2 (shEXD2#1-3). B) 2D gel images of mtDNA replication fork progression in 293T and U2OS-cells expressing shGFP and shRNA-EXD2#2. C) Model for interpretation of 2D-gels.

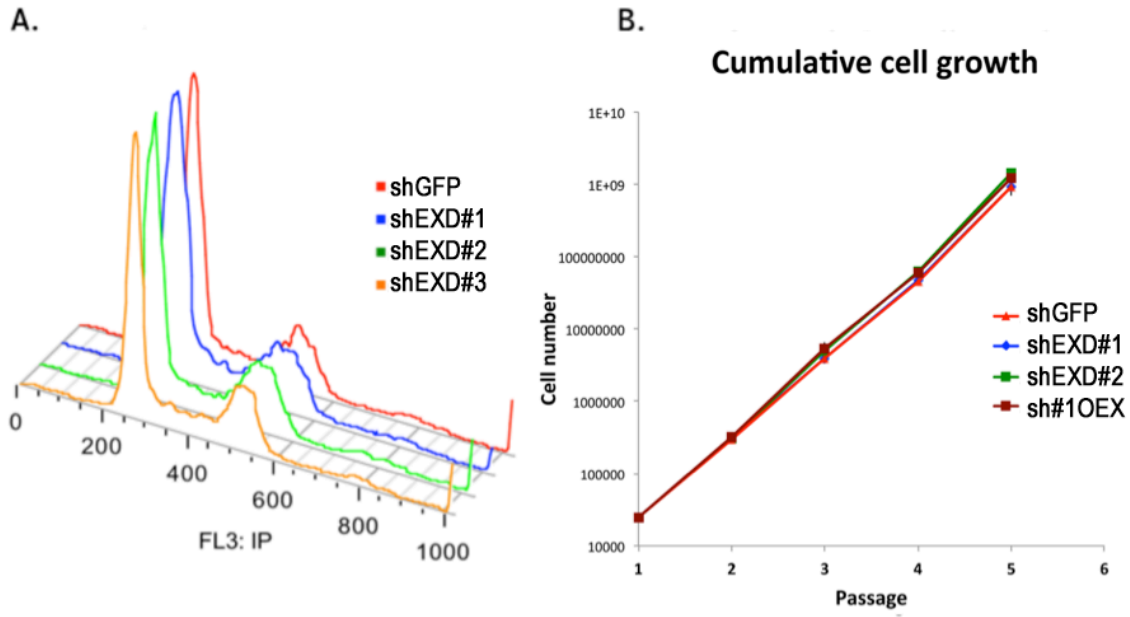

Figure 22. Analysis of cell cycle and cell growth in shEXD2-cells. A) Histograms of cell cycle of U2OS-cell lines stably expressing shEXD2s or shGFP. Cells were fixed and labeled with propodium iodide (PI). Cell cycle profiles were analyzed by flow cytometry and plotted as cell count vs DNA content. B) A representative U2OS shEXD2-cell line was selected for the cell growth assay. 100,000 cells were plated and counted every four days.

Cell cycle defects commonly occur in cells with impaired DNA repair and replication machinery, and it has been shown in *Drosophila* that mitochondrial defects can trigger the cell cycle checkpoint[385 386 387]. To investigate whether the loss of EXD2 led to cell cycle defects or affected cellular proliferation, we analyzed the cell-cycle and performed a 3T3 growth assay on shEXD2 U2OS-cells (Figure 22). Results indicated that under normal conditions, EXD2-loss does not alter cell cycle progression or cell proliferation significantly.

Taken together, we have confirmed that EXD2 is an active 3'-5' exonuclease able to degrade double and single strand DNA or RNA and DNA/RNA hybrids, and that loss of EXD2 does not lead to obvious defects in the replication of mtDNA or the nuclear genome under normal cellular conditions.

Loss of EXD2 leads to mitochondrial dysfunction

As defects in the maintenance of mtDNA and RNA can cause dysfunction of the organelle without affecting cellular proliferation, we wanted to see if this was the case in EXD2-KD cells. Consistent with its hypothesized role in nucleic acid homeostasis, we observed a 10-20% reduction in mitochondrial mass following EXD2 silencing (Figure 23A), and a 30-40% decrease in mtDNA copynumber in various tested shEXD2-cell lines (Figure 23B). These together suggested that EXD2 was potentially involved in regulating mitochondrial replication or turnover. However, despite these differences, we did not observe increased levels of ROS when measured with 2-7DCFH. 2-7DCFH becomes fluorescent when oxidized by intracellular ROS or peroxides and is commonly used to detect changes in the overall oxidative state of the cell, but does not differentiate between mitochondrial and cytosolic ROS, or whether the oxygen species are in the form of superoxide, peroxide or hydroxyl radicals[388 389]. Therefore we cannot rule out possible changes in the levels of individual ROS species that may arise from the mitochondria or elsewhere. We also measured the mRNA-levels of the master regulators of mt biogenesis, TFAM and PGC-1α, but found no consistent changes upon depletion of Exd2 in 293T or U2OS-cells (Figure 23D, E).

To assess the role of EXD2 in mtDNA degradation, we cultured U2OS-cells with a low dose of EtBr to deplete cells of mtDNA. EtBr inhibits thymidine-incorporation into the mtDNA and inhibits its replication, leading eventually to a total absence of mtDNA[390 391]. If cultured in media supplemented with uracil (synthesis of uracil requires functional mtDNA) and pyruvate, the cells without mtDNA are viable, although not able to perform OXPHOS. In addition, they maintain their ΔΨ and participate in anti-oxidant regulation and apoptosis to some extent[392 393].

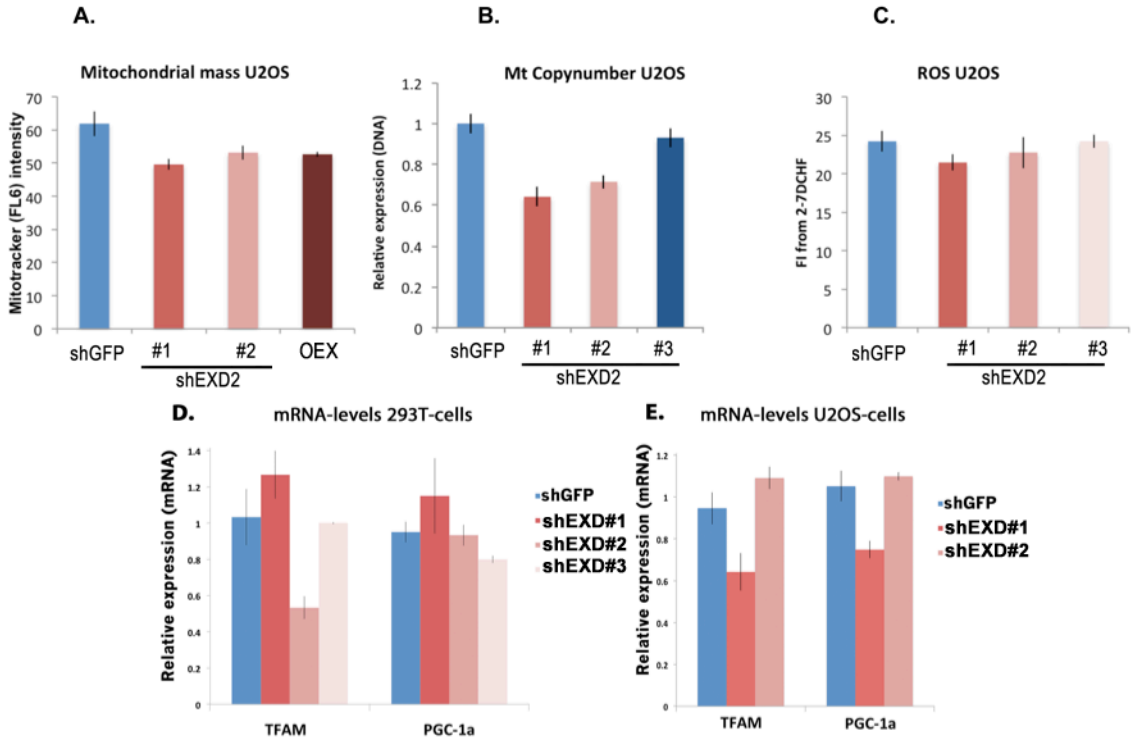

Figure 23. Loss of EXD2 leads to a decrease in mtDNA-mass and mtDNA copy number in U2OS-cells. A) Mitochondrial mass measured as Mitotracker Deep Red-signal with FACS. B) Mitochondrial copynumber measured with Q-PCR, COII normalized to GAPDH and ACTB. C) ROS measured with 2-7DCHF –probe with flow cytometry. D) TFAM and PGC-1α mRNA-levels in 293T-cells and E) U2OS-cells. OEX; EXD2 overexpression, WT; wild type cells

Under normal physiological conditions mtDNA copynumber remains relatively stable[394], but in pathologies such as cancer both increased and decreased mtDNA levels have been reported[395 396 397]. Whereas increased mtDNA copynumbers have been associated with a compensatory response to deficient cellular respiration, a decrease in mtDNA levels has been associated with metabolic changes, such as the Warburg effect (increased glycolysis and decreased mitochondrial respiration), that become more severe during cancer progression.

As the changes in copynumber can also reflect poor quality of the mtDNA, we wanted next to assess the integrity of mtDNA molecule in shEXD2-cells. For this

purpose, we performed long PCR against 9kbp of mtDNA on both U2OS and MDA231-cell lines. Both of the cell lines show an increase in mtDNA lesions in response to lower EXD2-levels (Figure 24A, B).

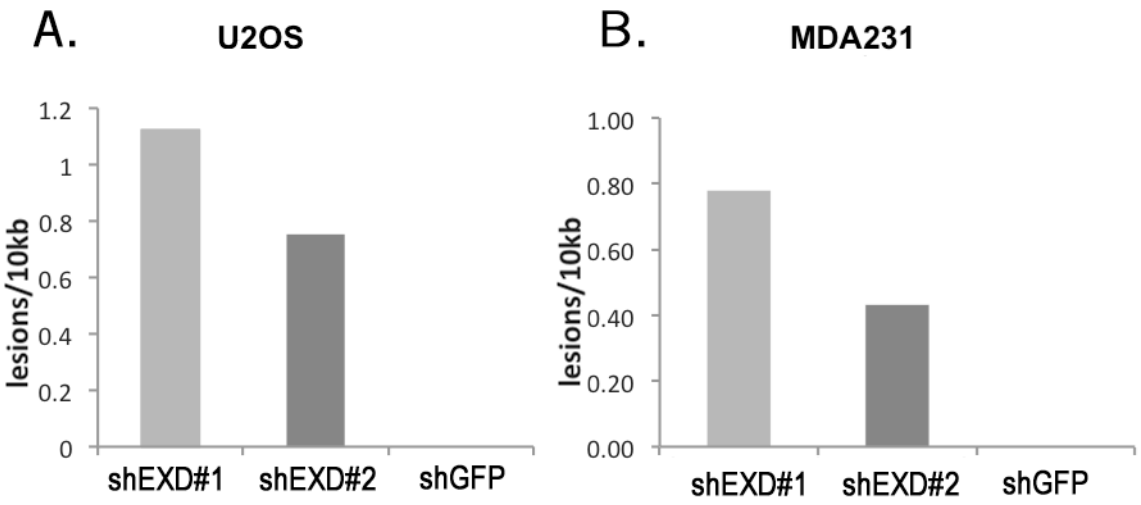

Figure 24. Frequency of lesions in the mtDNA in U2OS and MDA231-cells. 9kbp of mtDNA was amplified by long PCR and the frequency of lesions was calculated based on the concentration of amplified end-product quantified by PicoGreen® signal in A) U2OS-cells and B) MDA231-cells.

To determine if the lesions and decrease in mtDNA levels affect the transcription of mt mRNAs, we performed Q-PCR against protein-coding mRNAs in the mt. Since the mtDNA lacks introns, it was not possible to exclude unspecific amplification of the mtDNA by designing primers on exon-exon junctions as for the nuclear genes. Therefore, to eliminate mtDNA contamination, the RNA-prep was DNAse treated and the absence of mtDNA confirmed prior to the PCR to determine mRNA-levels of mitochondrial transcripts. According to our results, loss of EXD2 decreased the transcription of tested genes from 50 to 70% in breast cancer cell lines MCF7 and MDA231. Contrary to the nuclear encoded rRNAs, mitochondrial rRNA-genes are polyadenylated and thus their transcript levels can be determined by Oligo(dT)-based amplification used in our assay. Measuring the levels of mitochondrial rRNAs revealed contradictory results as mtS16 was downregulated but only a mild decrease in the ribosomal mtS12 was observed (Figure 25A, B). This might reflect the longer half-life and more stable nature of the rRNAs, but it does not explain

absence of similar effect in the mtS16 transcript. Since mechanisms for mitochondrial rRNA degradation are poorly characterized, it is difficult to speculate what would cause stability of a single rRNA-transcript to be affected. Anything from its close proximity to the replication and transcription start site, to changes in various destabilizing and stabilizing polyadenylation sites in the transcript, could have a role in affecting its stability.

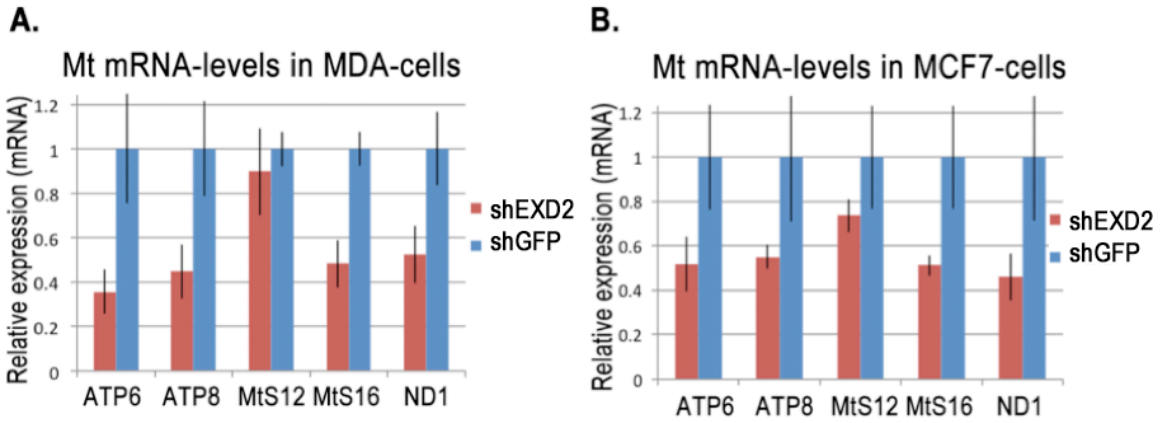

Figure 25. mRNA-levels of mitochondrial transcripts in MDA231 and MCF7-cells. mRNA-levels of the indicated gene were measured by real time PCR and normalized to GAPDH in shExd#2 and shGFP cells. Values are normalized to shGFP in each case.

Since the reduction in mtDNA copynumber and transcription could have a direct impact on mitochondrial respiration and induce a metabolic switch towards glycolysis, we wanted to see if this was occurring when EXD2 levels were reduced. For this purpose we measured the clonogenic survival and cellular proliferation of U2OS and MCF7 cells under decreasing glucose and glutamine concentrations. Interestingly, whereas the response of shEXD2-cells did not differ from control cells under glucose-depletion, we observed shEXD2-cells to require glutamine for their survival and cell proliferation (Figure 26A, B). This suggested to us that whereas glycolysis was not compromised, EXD2-loss affected some essential rate-limiting part of the glutaminolytic pathway.

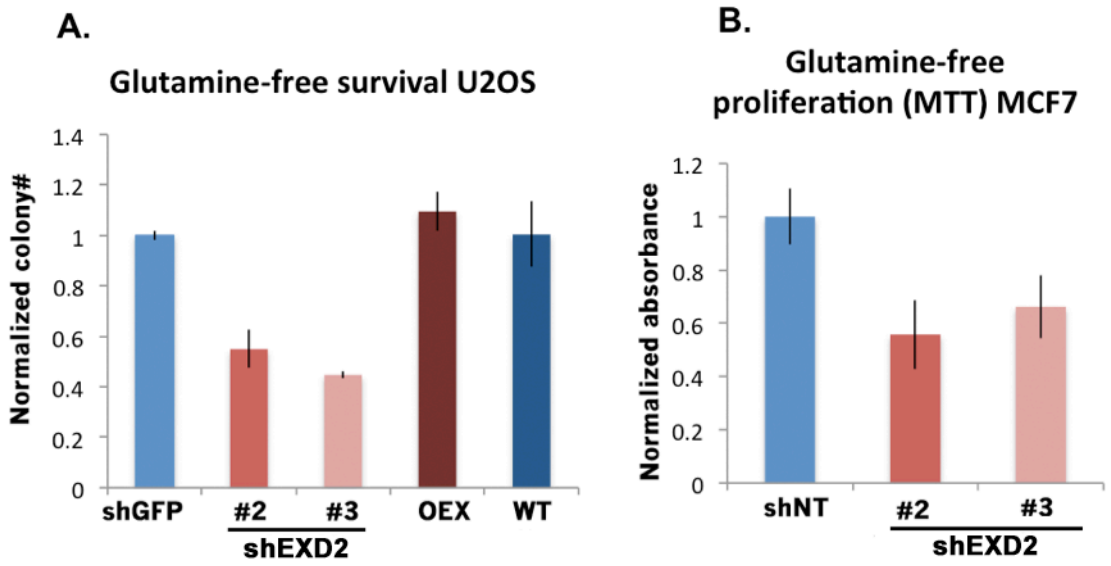

Figure 26. Clonogenic and cellular proliferation assays with U2OS and MCF7 shEXD2-cells. A) Clonogenic survival of U2OS-cells stably expressing shGFP, shEXD#1 and #2, overexpressing (OEX) and wild type (WT) EXD2 measured by colony forming assay B) Cell proliferation of MCF7-cells stably expressing shEXD2s measured by MTT absorbance.

Since glutamine, in addition to glucose, is an essential source of carbon atoms and can directly fuel mitochondrial respiration, we next sought to investigate whether loss or overexpression of EXD2 would alter cellular respiration. For this purpose the shExd#1-3 and OEX –cells lines were analyzed using with the Seahorse bioassay. The Seahorse XL Analyzer is able to measure both glycolysis and mitochondrial respiration simultaneously, and gives information about the basal respiration vs maximal respiration rate as well as ATP production and proton leakage occurring during energy metabolism (Figure 27A). The oxygen consumption rate (OCR) of shEXD2-cells was approximately 50% to that of control cells, indicating a severe defect in aerobic respiration upon EXD2-loss (27C, D). Although the ATP-production in the shEXD2-cells was only mildly affected by this decrease, we observed that after uncoupling ATP-synthase from the OXPHOS chain with FCCP to force the mitochondria to function at maximal capacity, the cells lacking EXD2 were not able to increase their respiration. In other words, this indicated that shEXD2-cells are constantly using their respiration at its maximum

capacity. Interestingly, EXD2 OEX was able to induce respiration two-fold, suggesting a direct correlation between the protein levels of EXD2 and aerobic respiration (Figure 27B).

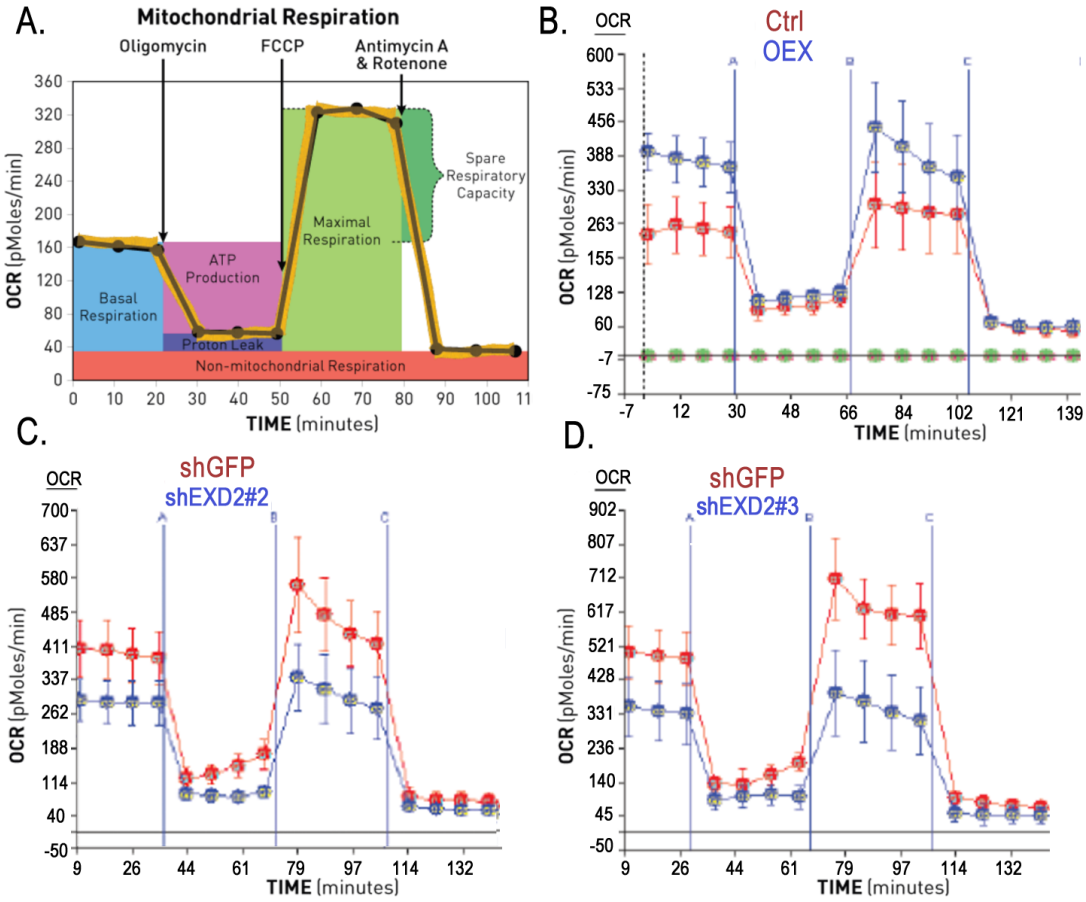

Figure 21. Effect of EXD2 levels on cellular respiration. A) Schematic presentation of the principal of the Seahorse assay. Basal respiration represents the OCR of the cells in normal conditions. Addition of oligomycin inhibits ATP-synthase, leading to drop in respiration. The remaining oxygen consumption is due to proton leakage through the IM. Addition of FCCP permeabilizes the IM, leading to free proton movement across the membrane leading to maximal OXPHOS complex function and maximal respiration. Antimycin A/Rotenone inhibits complex I/III respectively and inhibits OXPHOS. B) Mitochondrial function of the U2OS-cell lines, shExd#2, shExd#3 and OEX, measured by OCR.

Since a reduction in aerobic respiration could result from lower levels of OXPHOS complexes, we analyzed the protein levels of subunits from each complex (I-V).

Most of the antibodies we used were against nuclear-encoded protein subunits of OXPHOS-complexes, thus defects in the mitochondrial protein translation might not be directly reflected in their levels. Antibody for the only mt-encoded subunit is against cytochrome C oxidase 1 (mtCO1) (complex IV), and its levels most probably should be affected by alterations in mitochondrial translation, unless compensated by post-translational processing. Our results showed, that while there is a slight fluctuation in the protein levels between cell lines, that we also observe between experiments, there is no clear trend in the tested OXPHOS chain subunit protein levels that could be due to loss of EXD2 (Figure 28). Thus, the decrease in aerobic respiration in shEXD2-lines must be originating from other sources, such as defective function of the OXPHOS-complexes.

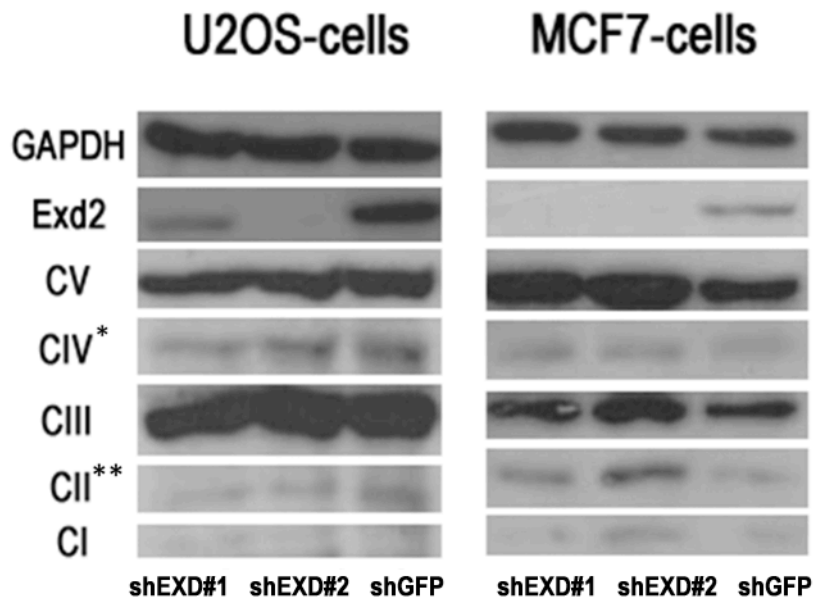

Figure 28. Protein levels of OXPHOS complex subunits. Protein expression of different subunits from OXPHOS complexes (CI-CV) in U2OS and MCF7-cell lines. The corresponding antibodies are: CV=ATP5A, CIV=MTCO1, CIII=UQCRC2, CII=5DH8, CI=NDUFB8. *Protein encoded by mtDNA, **Complex encoded totally by nuclear genome.

The shape of the cristae is one of the determining factors affecting aerobic respiration, and many mitochondrial pathologies arising from dysfunctional ETC

complexes or mitochondrial proteins are characterized by abnormalities in the morphology of the organelle[398 399]. Thus, we wanted to determine if mitochondrial morphology was affected by EXD2 depletion to determine if there was a connection to the lower respiration rate of the KD cells. We had observed no difference in the mitochondrial and cristae structure of the EXD2 OEX cells used in the EM studies and EM done with the shEXD2-cells revealed that the cells lacking EXD2 had intact cristae and showed no evident changes in mitochondrial structure (Figure 29A). The molecular mediators involved in mitochondrial fusion and fission have been recently connected to diseases and phenotypically lead to various fragmented or interconnected mitochondria[400]. To address this we quantified the size of the mitochondria in our EM-samples but found no differences in the size of the mitochondria between the cell lines expressing different levels of EXD2 (Figure 29B). Furthermore, when we studied the mitochondrial network by performing IF on cells lacking EXD2 we found that they had a normal organellar network, suggesting functional fusion and fission dynamics in the shEXD2-cells (Figure 29C).

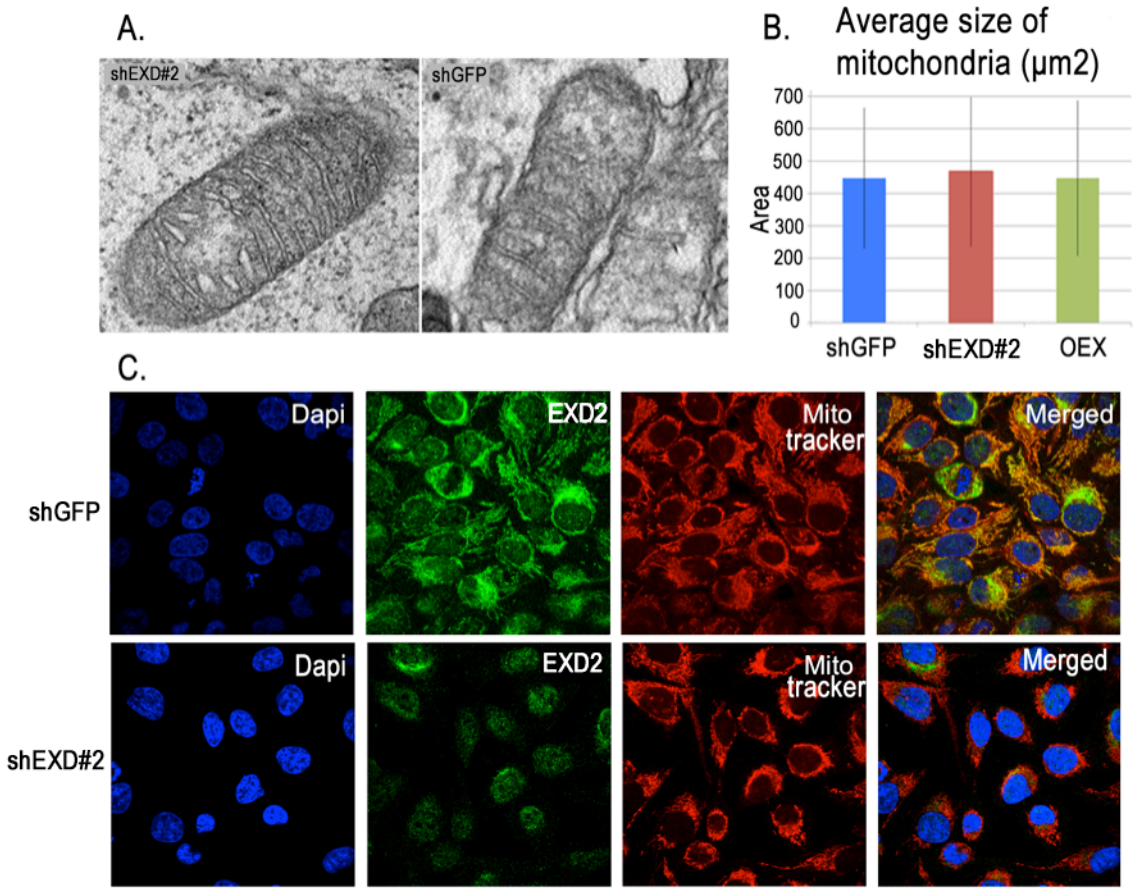

Figure 29. Mitochondrial morphology and size are unaffected by loss of EXD2. A) Representative EM pictures of shEXD2 and shGFP 293T-cells. B) Quantification of mitochondrial size in shGFP, shEXD2 and OEX HEK293-cell lines. Large standard deviations are indicative of the various shapes of the mitochondria observed in EM depending on how the sample was cut during preparation. C) IF performed on 293T-cells stably expressing shExd#2 and shGFP. EXD2 (green), Mitotracker (Red), Dapi (Blue). Nuclear staining pattern observed in the shExd#2-cells is due to unspecific binding of the secondary antibody and is absent when different fluorochromes are used.

Metabolic changes induced by loss of EXD2

It is becoming increasingly clear that changes in the protein and mRNA-expression do not always reflect the functional activity of a pathway or the observed cellular phenotypes. To get a better understanding of biological processes in a cellular context, we focused on the metabolomic profiling of the most downstream end products in the cellular metabolism. By using metabolic profiling of U2OS-cells we hoped to pinpoint the metabolic pathways that silencing of EXD2 affected and their potential relation to phenotype. Primarily we wanted to address whether EXD2-loss led to imbalances of cellular metabolites or to changes in their generation or usage. Of particular interest were the carbon donors glucose and glutamine.

To get an overall picture of how the silencing of EXD2 affected metabolic pathways, we extracted the metabolites from the cells cultured with normal glucose (C12) and analyzed them with mass-spectrometry and NMR. This resulted in a long list of metabolites with changes levels upon EXD2-levels, of which the most significantly altered ones are presented in Figure 24. As expected, we found that many intermediates of the TCA-cycle were decreased, including succinate, fumarate and aspartate, consistent with the observed reduction in cellular respiration (Figure 30). We also observed down-regulation of some of the members of the PPP, such as the ribonucleosides adenosine and cytidine. As our earlier results indicated that loss of EXD2 lead to dependence of glutamine, it was very interesting to notice that the glutamine-derived glutamate and the total levels of antioxidant glutathione were also down-regulated in shEXD2-cells. This would suggest that EXD2 participates in the activation of glutaminolytic pathways, and that the loss of EXD2 renders cells sensitive to glutamine deprivation, probably due to a drop in the level of glutamine-derivatives below the threshold necessary for cellular needs.

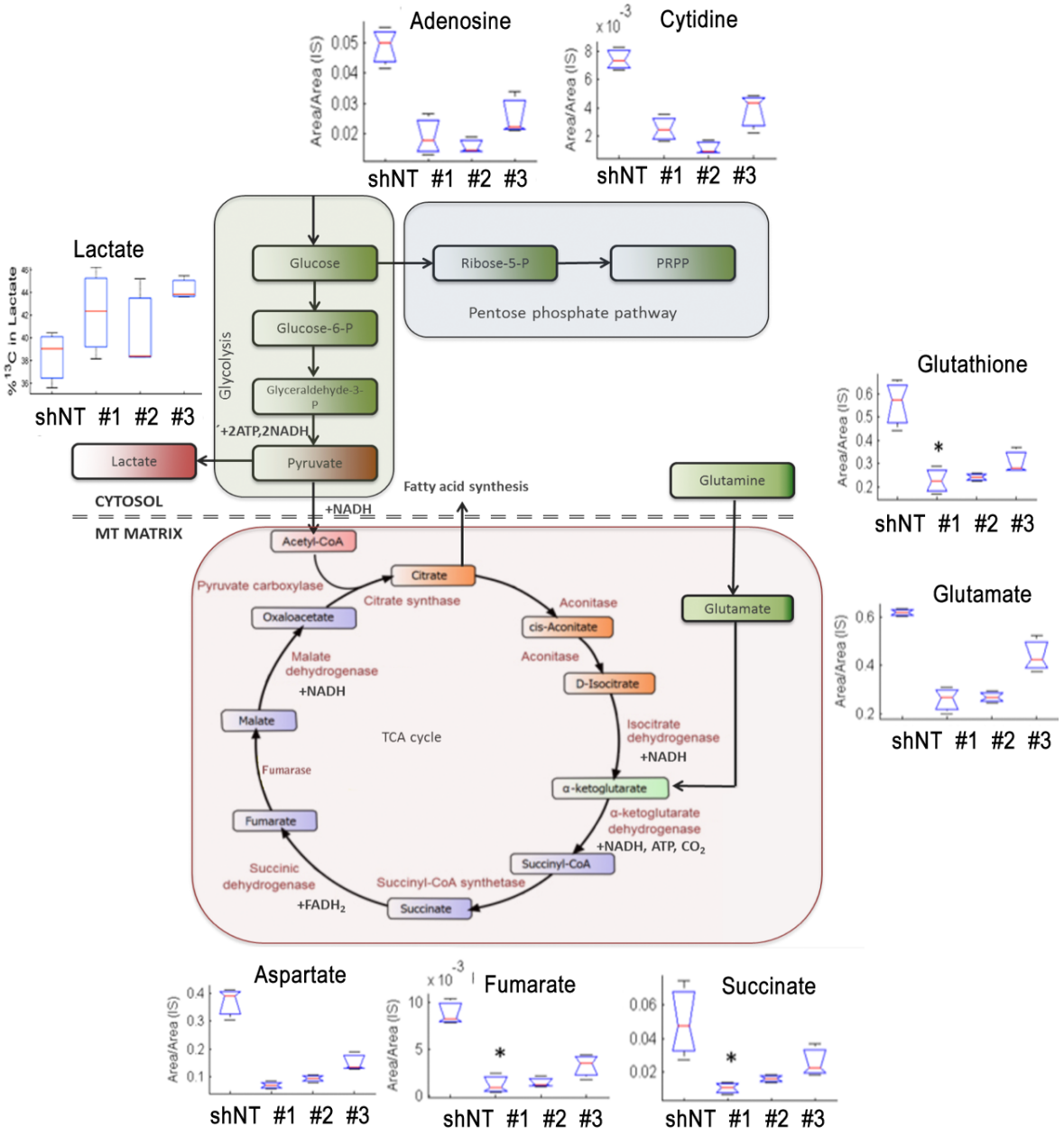

Figure 30. Schematic presentation of the effects of EXD2-silencing on cellular energy-pathways. Total metabolites in U2OS-cells expressing shGFP and shEXD#1-3 were analyzed by targeted (lactate) and non-targeted (the rest) metabolomics analysis. The most severely affected metabolites are shown.

As the incorporation of glutamine-derived carbon into the TCA occurs via its conversion into glutamate, and loss of EXD2 reduced the levels of glutamate, we deduced that the levels of glutamine-derived carbon atoms might be reduced in the mitochondrial intermediates of shEXD2-cells. Thus, we wanted to study if the cells were compensating this loss by producing more glucose-derived TCA-intermediates. To study this, we used stable isotope tracing. In short, we fed U2OS-cells with isotopically labeled glucose (C13) and measure the patterns of isotope incorporation that emerge in downstream metabolites by using mass-spectrometry and NMR. By systematically following the labeled carbon atoms we could reconstruct a comprehensive flux-map of how the cells incorporate glucose-derived carbon into various metabolic pathways. The comparison of flux maps from the control cell line (shGFP) with shEXD2-lines (shExd#1-3) provided us with a functional readout of the global impact that the loss of EXD2 had on cellular metabolism. In addition, by comparing the levels of C13 signal with the C12 signal, we could find see whether the metabolite was derived from glucose or from another source of carbon.

Strikingly, the metabolites that were down-regulated in the shEXD2-lines seemed to be solely composed of glucose-derived carbon, whereas the carbons in metabolites of shGFP-line were derived approximately 50% from glucose and 50% other sources such as glutamine and salvage pathways (Figure 31). In other words, during the 6-hour incubation with glucose-C13, all the tested cell lines incorporated heavy carbon equally. However, during that time shGFP-line additionally incorporated non-heavy carbon from other sources, producing a total of two times more metabolites. In shEXD2-lines these differences could be explained by a blockade in glutamine- or salvage pathway-derived carbon incorporation into cellular metabolites. We also observed an increase glucose-derived lactate-production in the shEXD2-cells, indicative of cellular acidification during which lactate acts as a proton- neutralizing buffer (Figure 30).

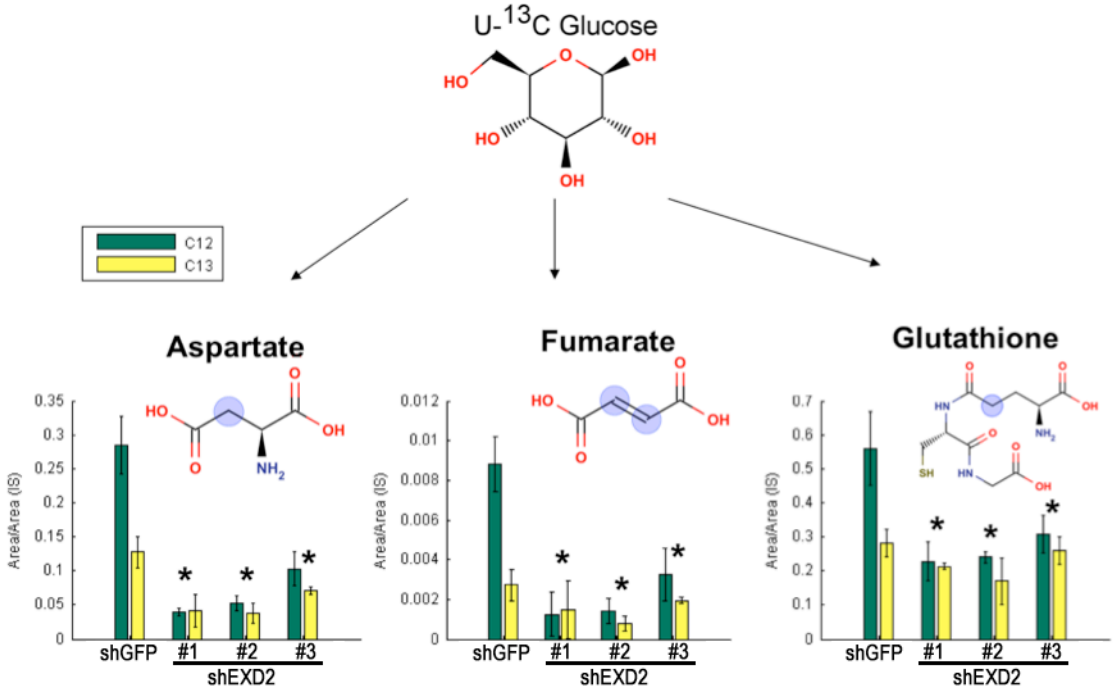

Figure 31. Glucose C13-isotope incorporation into mitochondrial metabolites upon silencing of EXD2. Glucose-derived carbon atoms (circled in blue) identified in Aspartate, Fumarate and Glutathione. Green bars represent the signal intensity coming from the total carbon atoms belonging to each metabolite (e.g. total level of each metabolite). Yellow bars represent the signal originating from glucose-derived carbon atoms. The difference between the green and yellow bars within each cell line represents the portion of metabolite originating from alternative carbon source.

In addition to the untargeted metabolomics assays, we also performed targeted analysis of metabolites in the U2OS-cells expressing shEXD2s and control shRNAs. This study confirmed some of our results from the untargeted assay and, in addition, provided us with an interesting piece of data about a new, relatively unknown metabolite present in cell lines lacking EXD2. It appeared that upon EXD2-silencing, cells used citrate to synthetize and accumulate 2-HG a product commonly found in gliomas and lymphomas with mutated IDH1 or IDH2 (Figure 32A)[401 402]. However, when we sequenced both IDH1 and IDH2 in the U2OS cells used for the metabolomics assays, we found no evidence of mutations in either of the enzymes. Interestingly, 2-HG accumulation was recently found in triple negative breast cancers with intact IDH1 and 2, and the investigation on its role as

an oncometabolite is currently under focus[403].

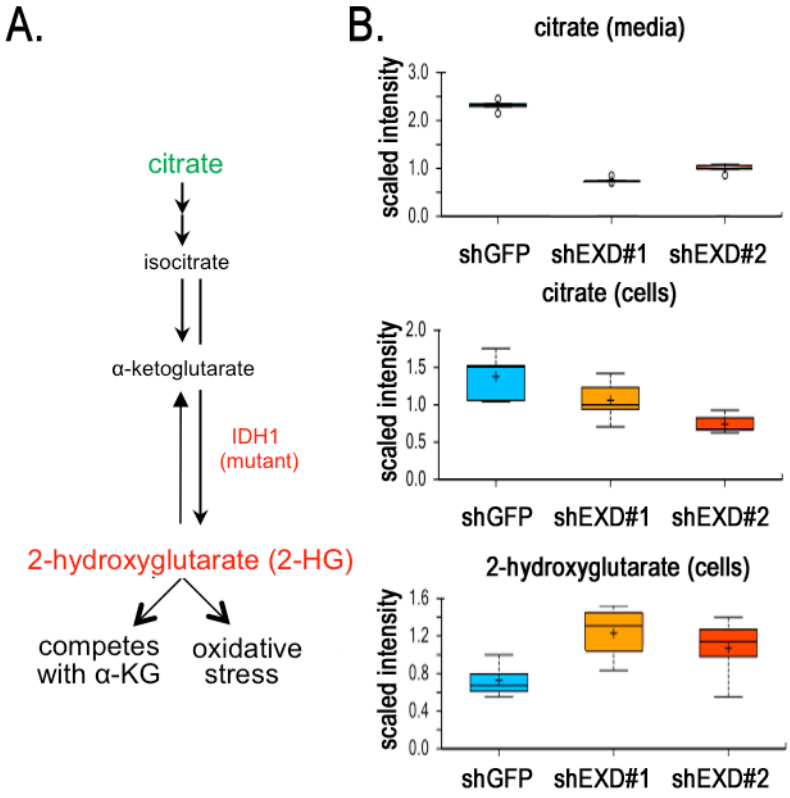

Figure 32. ShEXD2-cells use citrate to produce 2-HG. A) Citrate is converted into 2-HG that further complexes with a-KG and inhibits a-KG-mediated enzymatic reactions, leading to oxidative stress. This occurs if due to mutations in IDH1 and for reasons yet unknown. B) Levels of Citrate and 2-HG in U2OS-cells expressing shGFP, shExd#1 and 2 measured by targeted metabolomics analysis.

Taken together, the metabolomics analysis showed that loss of EXD2 resulted in various metabolic changes, including reduced TCA-cycle function, reduced synthesis of glutamine-derived intermediates and increased acidification and production of oncometabolite 2-HG.

Breast cancer models

Due to the widespread metabolic changes occurring in cells upon loss of EXD2, we wanted to assess its role in human cancers. For this purpose we analyzed expression analysis datasets from various cancer cohorts to determine if other genes shared similar expression patterns. Interestingly, when comparing the expression pattern of *EXD2* with the genes in various breast cancer cohorts, we noticed that it highly correlated with estrogen receptor 1 (ER1) expression (Figure 33). ER1 levels are used as a major prognostic marker in clinical assessment of breast cancer.

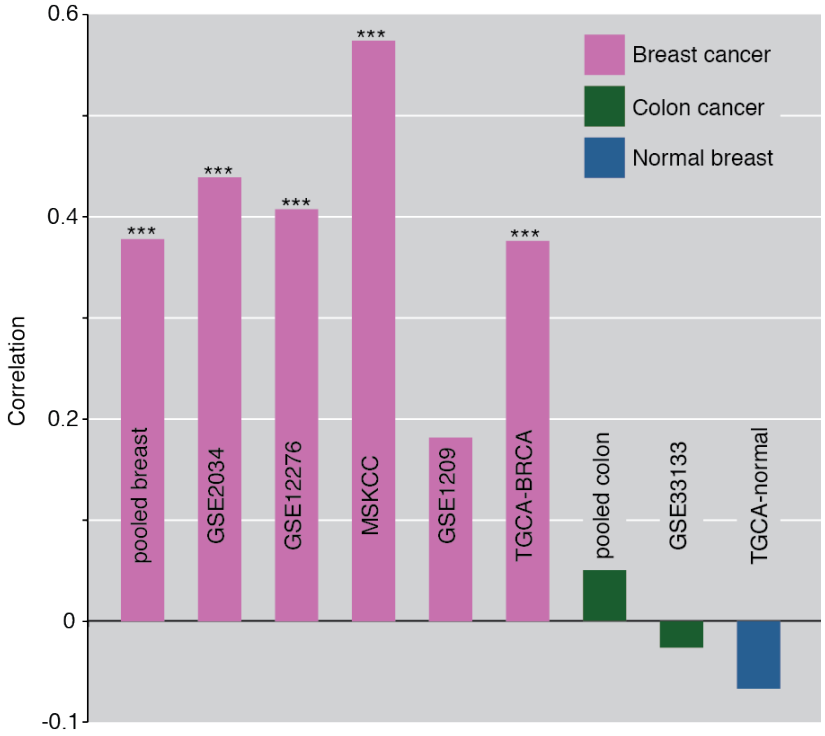

Figure 33. Correlation of EXD2 and ER1 in breast and colon cancer cohorts. five breast cancer cohorts; gse2034, gse12276, mskcc, gse1209 and tdmEXD2a_brca were analyzed for correlation of ER1 and EXD2. For negative control of correlation three cohorts of colon cancer were analyzed. Pooled colon includes gse17537 and gse14333. Pooled.colon includes all breast cancer cohorts shown in the figure. Cancer cohorts are available from Gene Expression Omnibus (GEO).

In tumors that had high expression of ER1, EXD2 mRNA-levels were also high, suggesting that it provides ER1 positive tumors a selective advantage. Comparing this with our data from Seahorse-assays, where low EXD2-levels decreased the rate of aerobic respiration, and over-expression of EXD2 increased respiration, we hypothesized that if both EXD2 and ER1 were under the same regulatory pathway inducing Warburg-effect, the tumors with high ER1 would similarly have increased rate of mitochondrial respiration.

To determine if EXD2 levels influenced estrogen signaling or ER1 levels, we examined its mRNA and protein levels in cells with reduced EXD2. Results showed a 20-30% decrease in ER1 mRNA following EXD2-depletion, whereas the protein levels of ER1 are only minimally affected by loss of EXD2 (Figure 34).

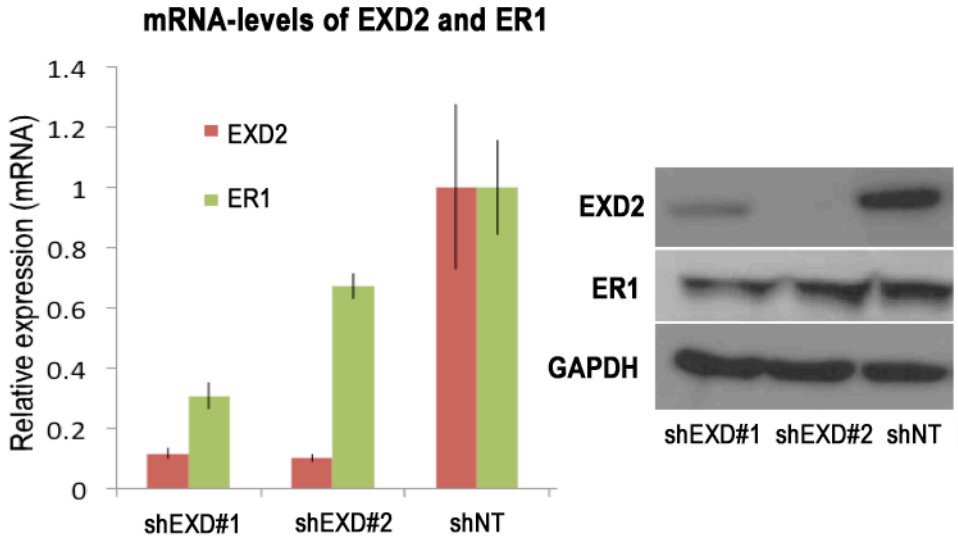

Figure 34. Correlation of ER1 mRNA and protein levels in shEXD2 MCF7-cells. MCF7-cells stably expressing shEXD#1, shEXD#2 and shNT were used to measure mRNA-levels of *EXD2* and *ER1* by Q-PCR and Western blot was used to detect their protein levels.

Database analysis on cells with silenced ER1 revealed no changes in EXD2-levels, indicating that the correlation between EXD2 and ER1 expression in breast cancer

might be part of a larger scale metabolic changes occurring in breast cancer, affecting expression of both genes.

ER1-antagonists, such as Tamoxifen, have been used to treat breast cancer for over thirty years, and it has been widely documented that inhibiting estrogen signaling reduces the growth of estrogen-sensitive breast cancers[404]. The mechanism of action of the ER1-antagonists is based on blockage of transcriptional activation of ER1-targets leading to cell cycle arrest and apoptosis of the tumor cells. Eventually, in many patients a subpopulation of tumor cells develop mechanisms to bypass the dependence of estrogen and the tumor becomes ER1 negative, leading to resistance to ER1-antagonists. Interestingly, this switch from ER1+ (luminal) to ER1- (basal) state is accompanied by a metabolic change from highly glycolytic tumors towards glucose-independent growth and a more aggressive phenotype[405]. To study the relationship between EXD2, ER1 and the metabolic changes in breast tumors *in vivo*, we used two breast cancer cell lines, ER1+ MCF7 (luminal) and ER1- MDA231 (basal) with stable down-regulation of EXD2. According to published microarray data (GSE26370), the MCF7-line has roughly 5-fold higher expression of EXD2 than the MDA231-line (Figure 35), supporting our notion that EXD2-levels correlate with ER1-expression. We decided to use these cells as a model to study the role of EXD2 and its relationship to estrogen signaling in breast carcinogenesis.

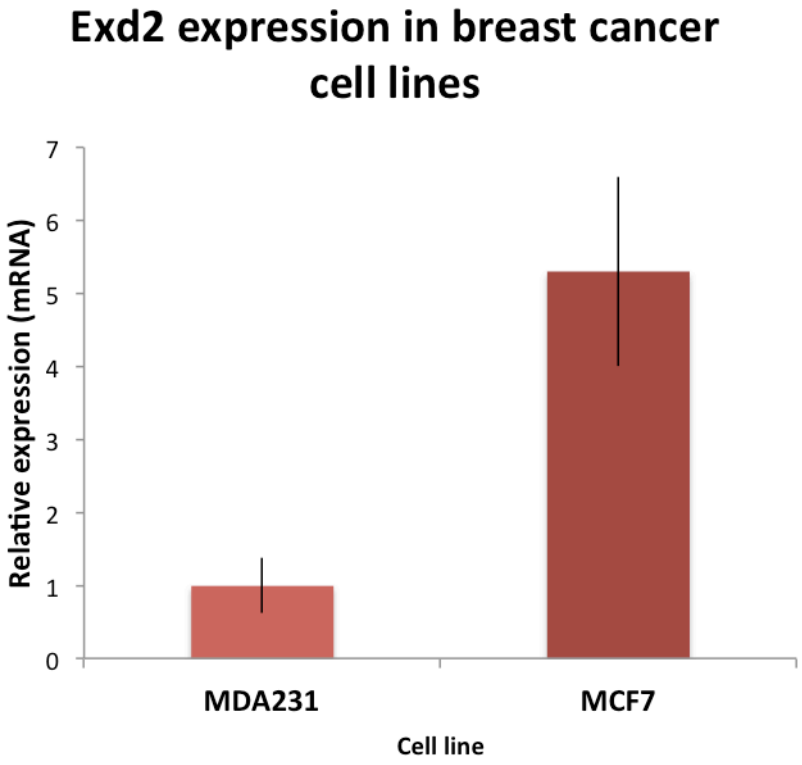

Figure 35. mRNA-levels of EXD2 in breast cancer cell lines MDA231 and MCF7. Levels of EXD2 mRNA expression measured by Q-PCR.

To assess breast cancer tumor growth upon EXD2-silencing, luciferase-expressing shExd#1, shExd#2 and shNT MCF7 and MDA231-cells were injected into the mammary fat pads of immunosuppressed BALB/c nude mice. The growth of tumors was monitored weekly by measuring the size and photon flux after luciferin-injection. Consistent with our hypothesis, 8 weeks after the injection, tumors from ER1-positive MCF7-cells expressing shEXD2 were noticeably smaller than the tumors originating from cells infected with shNT (Figure 36A, C). Also, the tumor growth measured by photon flux showed a decrease upon lower EXD2-levels (Figure 36B). To exclude the possibility of tumor outgrowth by clones expressing higher levels of EXD2, we confirmed the silencing of EXD2 was maintained during tumor growth by measuring mRNA-levels of EXD2 in the tumors. The results

confirm that the expression of EXD2 remained similar to the levels observed prior to xenograft injection (Figure 36D).

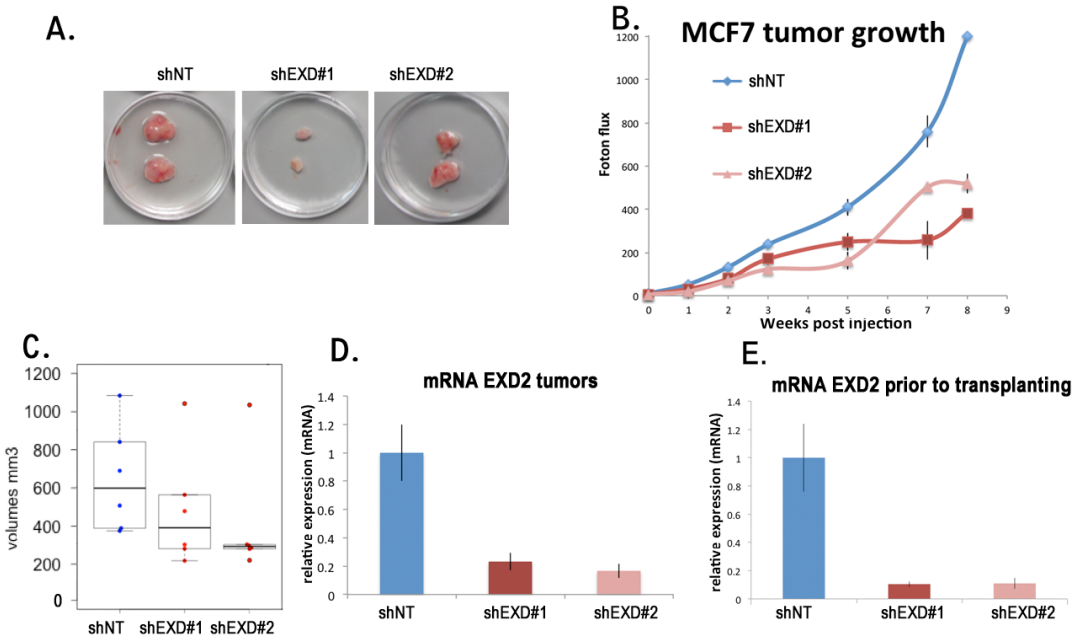

Figure 36. Size and growth of MCF7-xenografts transplanted in nude mice. A) Tumors dissected 8 weeks after transplantation of MCF7-cells expressing shNT, shEXD#1 or shEXD#2 into mammary fat pads of nude mice. B) Tumor growth measured by photon flux from the luciferase positive MCF7-cells. C) Tumor sizes at the end of the experiment on week 8. D) mRNA expression of EXD2 in tumors dissected from the mice and E) prior to transplanting.

These results agreed with previous reports indicating that oxidative phosphorylation directly correlates with aggressiveness of cancer[406 407]. Next, we hypothesized that if the EXD2-mediated reduction in the tumor growth was dependent on ER1, the growth of the ER1- MDA231-tumors should be unaffected upon EXD2-silencing. However, our results from MDA231-xenografts showed very similar results to the MCF7-derived tumors, with a consistent reduction in the mRNA-levels of EXD2 correlating with smaller size and growth of the tumors (Figure 37 A, B, C, D). This suggested to us that the metabolic changes induced by loss of EXD2 were affecting tumor growth independently from estrogen signaling.

Taken together, our data supports the possibility that EXD2 and ER1 function in the same metabolic pathway, downstream from events triggering metabolic changes leading to increased dependence on glutamine.

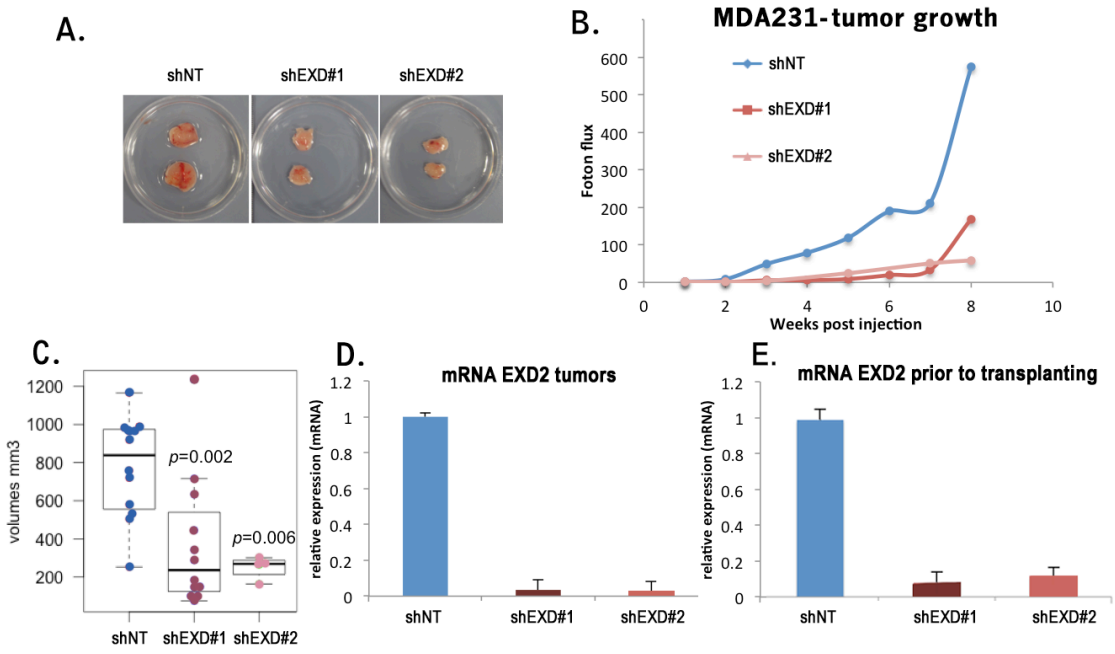

Figure 37. Size and growth of MDA231-xenografts transplanted in nude mice. A) Tumors dissected 8 weeks after transplantation of MDA231-cells expressing shNT, shEXD#1 or shEXD#2 into mammary fat pads of nude mice. B) Tumor growth measured as photon flux from the luciferase positive MDA231-cells. C) Tumor sizes at the end of the experiment on week 8. D) mRNA expression of EXD2 in tumors dissected from the mice and E) prior to transplanting.

In order to investigate the possible mechanisms by which EXD2 could affect the *in vivo* growth of both MCF7 and MDA231-cells, we wanted to find out if reduction in EXD2-levels was also affecting cell growth *in vitro.* Consistent with our previous results from U2OS-cells, we did not observe differences in cumulative cell growth of MCF7 and MDA231-cells with reduced EXD2-levels (Figure 38 A, C). To assimilate the *in vivo*- growth conditions of MCF7-xenografts, we cultured MCF7-cells *in vitro* in the presence of 4-hydroxytamoxifen (4-OH), an active derivative of 17-beta-estradiol –pellet that was administered subcutaneously into the nude mice prior to injection of MCF7-xenografts. The addition of 4-OH in the media had no

effect on the growth rate of the cells regardless of their EXD2-levels (Figure 32 B).

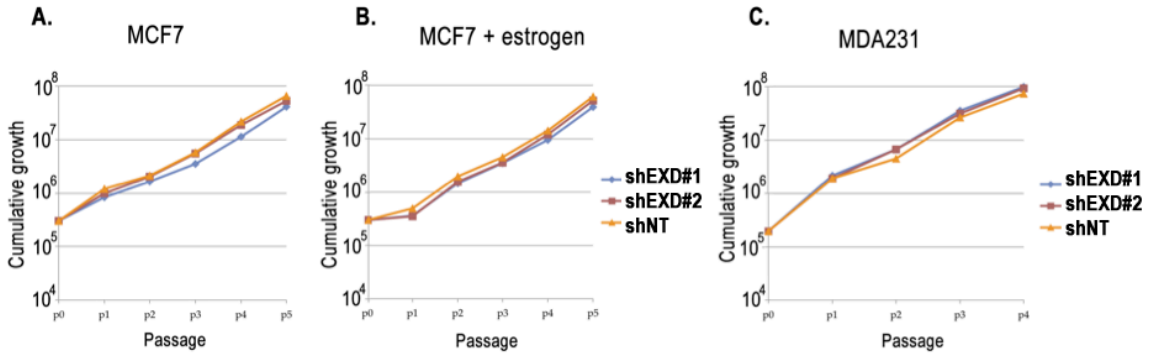

Figure 38. Cumulative growth of MCF7 and MDA231-cells *in vitro.* MCF7-cells expressing shExd#1, 2 and shNT were cultured in the absence A) and presence B) of estrogen in normal DMEM and accumulative growth was calculated during 5 passages. C) MDA231-cells expressing shExd#1, 2 and shNT were cultured in normal DMEM and accumulative growth was calculated during 4 passages.

As it has been shown that phenol red can act as a weak estrogen, we tested if this was the case in our MCF7-cells[408]. However, the proliferation of MCF7-cells was unaffected by both phenol red and addition of 4-OH (Figure 39). Whether the specific sera we used contains metabolites that are able to activate estrogen receptor or our MCF7-cells have become insensitive to estrogen remains to be studied.

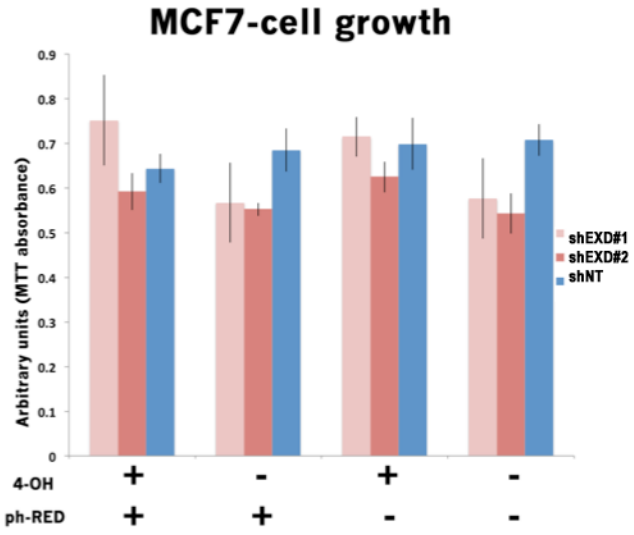

Figure 39. Effect of estrogen signaling on MCF7-cell growth. MCF7-cells were grown in the presence and absence of 4-OH and Phenol Red (ph-RED) and their proliferation was measured by MTT-assay.

However, our results do confirm that the inhibition of tumor growth upon EXD2-loss was independent of estrogen signaling and instead originated from other factors in the tumor environment shared between both MCF7 and MDA231-cells.

To find out the mechanism underlying diminished growth of shExd-tumors, we performed immunohistochemical (IHC) staining on selected MDA231-tumors. Staining and quantification of the proliferation marker Ki67 revealed that EXD2-loss led to slower proliferation, specifically in the center of the tumor, where the most robustly proliferating, highly glycolytic epithelial breast cancer cells are located (Figure 40 A-C)[409].

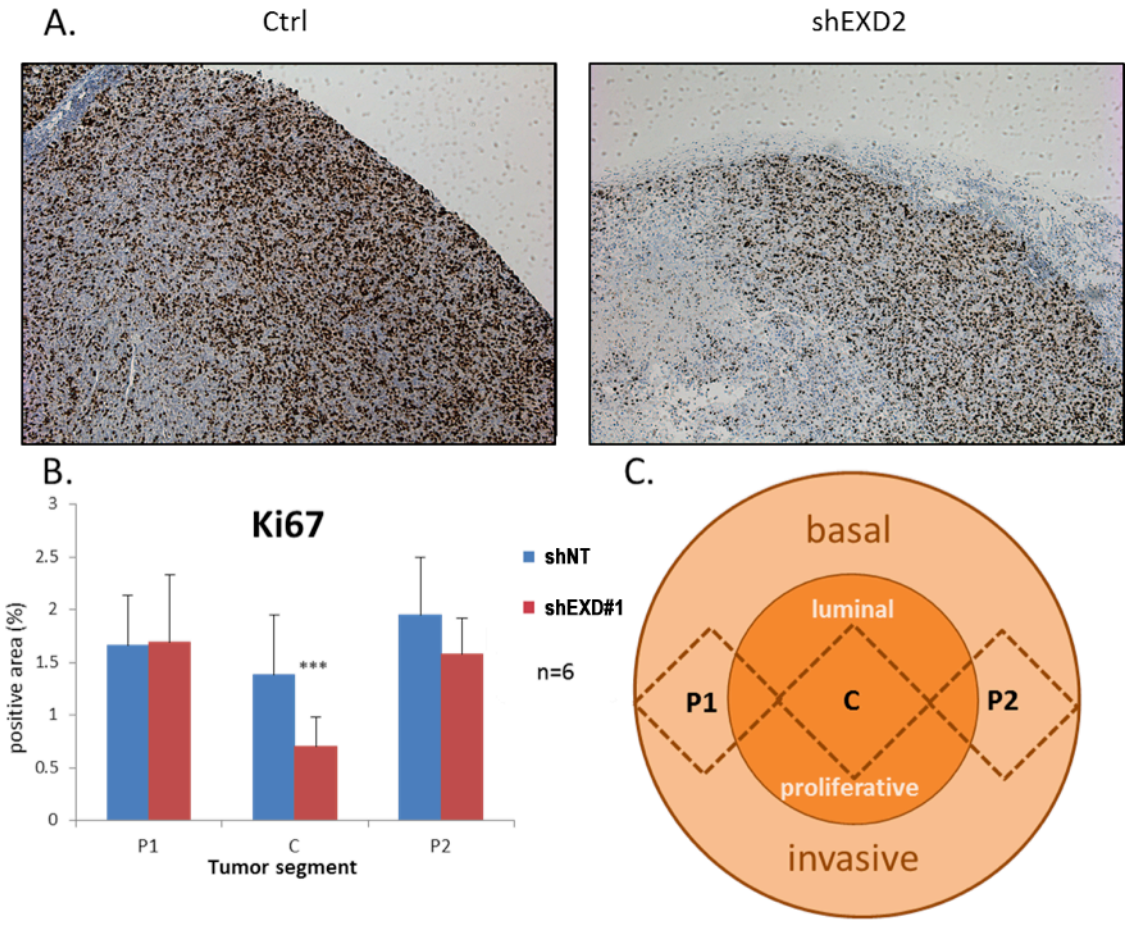

Figure 40. Effect of reduced EXD2-levels on proliferation of MDA231-tumors. A) IHC staining of shNT and shExd#1 MDA231-tumors with proliferation-marker Ki67. B) Quantification of Ki67-signal from different segments of the tumor (P1, C, P2). C) Schematic presentation of tumor

segments used for quantification of Ki67-signal. Tumor compartmentalization between Basal/invasive – Luminal/proliferative based on Liu et al[409].

As the development of intra-tumoral vasculature is one of the most significant factors contributing to the cell proliferation and tumor growth[410 411], we next analyzed the angiogenesis in the MDA231-cells by staining with vascular marker CD31. As shown in Figure 41, CD31-signal in the center of the shExd#1 tumor is nearly absent, suggesting that the impairment in tumor angiogenesis due to loss of EXD2 could be one of the contributing factors in the observed reduction in tumor growth.

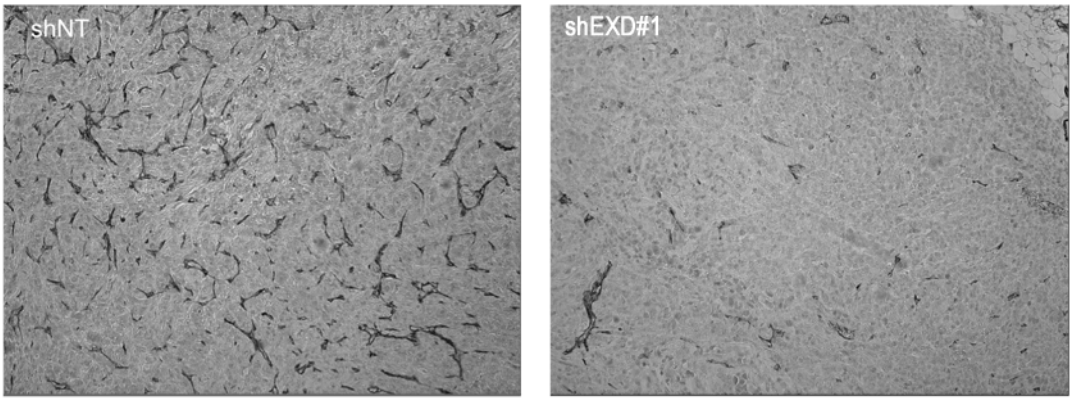

Figure 41. Immunohistochemical staining of MDA231-tumors with vascular marker CD31. Tumors expressing shNT and shExd#1 in MDA231 cells were fixed and stained with CD31 antibody against vascular endothelial cells. Dark areas represent blood vessels.

A major driving force behind tumor angiogenesis is hypoxia, and both the increased mRNA-levels and stabilization of HIF-1a have been shown to induce secretion of Vascular endothelial growth factor (VEGF) to enhance blood vessel development in tumors[249]. Overexpression of HIF-1α tends to correlate with poor prognosis in cancer and inhibition of hypoxia-signaling has been shown to reduce tumor growth[412 413]. However, inhibition of tumor-growth by targeting HIF-1α remains a challenge due to its central role in various pathways involved in cellular homeostasis. To determine if hypoxia-signaling was affected in the shEXD2-

derived tumors, we performed Western blotting on MDA231 cells grown in hypoxia *in vitro.*

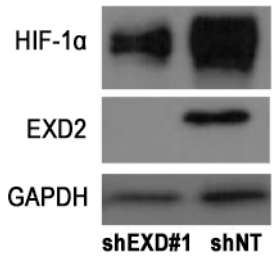

Figure 42. Expression of HIF-1α in MDA231-cells cultured in hypoxia. Protein levels of HIF-1α, EXD2 and GAPDH in MDA231-cells cultured in 1% oxygen

.

Consistent with the possibility that EXD2 regulates hypoxia induced signaling MDA231-cells lacking EXD2 showed lower stabilization of HIF-1α under hypoxia (Figure 42). We observed a similar effect in U2OS-cells, where cells lacking EXD2 were unable to stabilize HIF-1α in response to hypoxia (Figure 43).

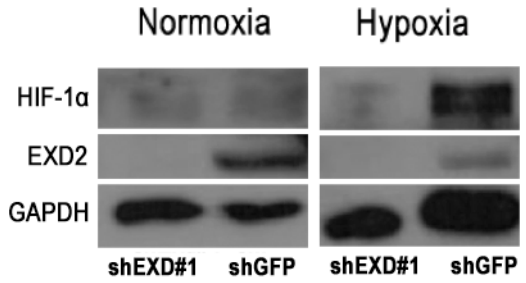

Figure 43. Expression of HIF-1α in U2OS-cells cultured in normoxia and hypoxia. Protein levels of HIF-1α, EXD2 and GAPDH in U2OS-cells cultured in 20% (normoxia) and 1% (hypoxia) oxygen.

Taken together, our results indicated that loss of EXD2 in cancer cells led to inhibition of tumor angiogenesis, potentially due to defective HIF-1α stabilization under hypoxia.

Next we wanted to study how the lack of a mitochondrial exonuclease could cause a reduction in HIF-1α stabilization under hypoxia. As many DNA-repair proteins are known to translocate to the nucleus or mitochondria upon specific triggers[414], we were interested in investigating if hypoxia would trigger the translocation of EXD2 from the mitochondria to another compartment where it could affect gene

regulation. However, IF against overexpressed EXD2 in U2OS-cells (Figure 44) and MCF7-cells (Figure 45) under normoxic and hypoxic conditions revealed that the mitochondrial localization of EXD2 is sustained even under hypoxia. This confirmed us that the reduction of HIF-1α stabilization in shEXD2-lines was likely due to a local effect of EXD2 in the mitochondria.

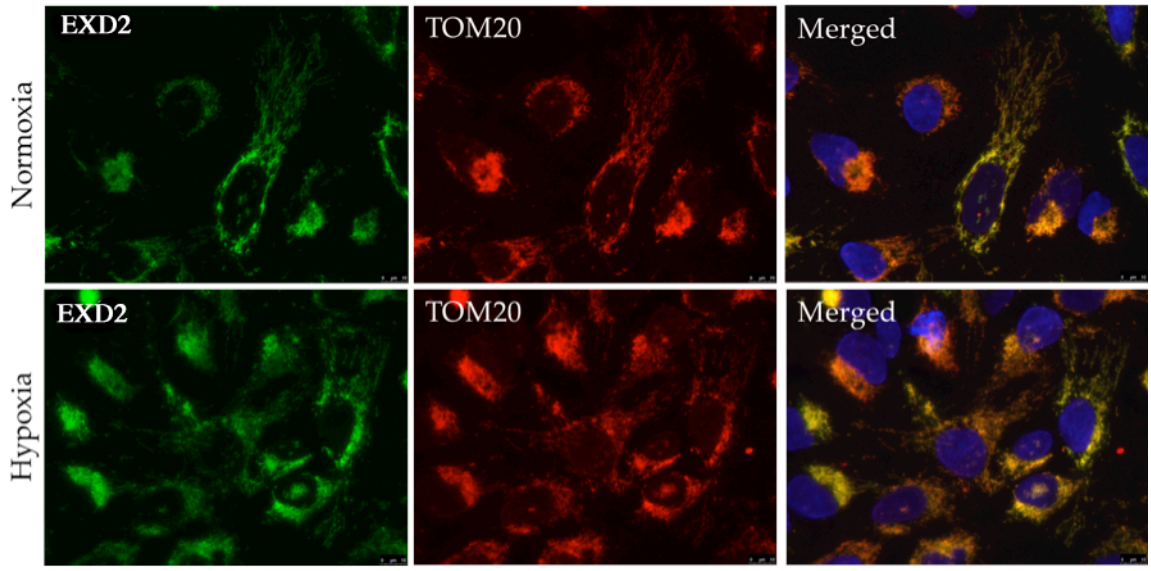

Figure 44. Localization of EXD2 under normoxia and hypoxia. IF on U2OS-cells overexpressing EXD2-SF-TAP and cultured under normoxic (21% O2) and hypoxic (1% O2) conditions prior to staining. TOM20 used as mitochondrial marker.

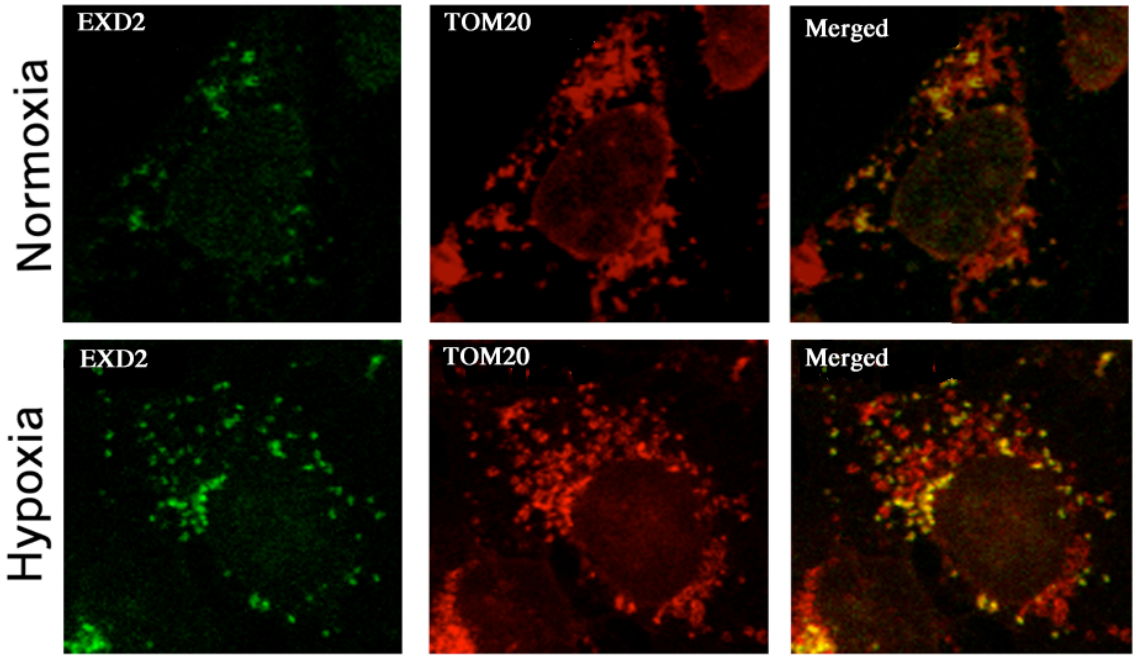

Figure 45. Localization of EXD2 under normoxia and hypoxia in MCF7-cells. IF on MCF7-cells overexpressing EXD2-SF-TAP and cultured under normoxic (21% O2) and hypoxic (1% O2) conditions prior to staining. TOM20 used as mitochondrial marker.

We had observed earlier that the protein levels of EXD2 decreased during hypoxia (Figure 43.) and wanted to find out if this was due to post-translational modifications affecting the stability of the protein or transcriptional regulation of EXD2 mRNA. Our Q-PCR results show that hypoxia reduced mRNA-levels of EXD2 by approximately 20% (Figure 46).

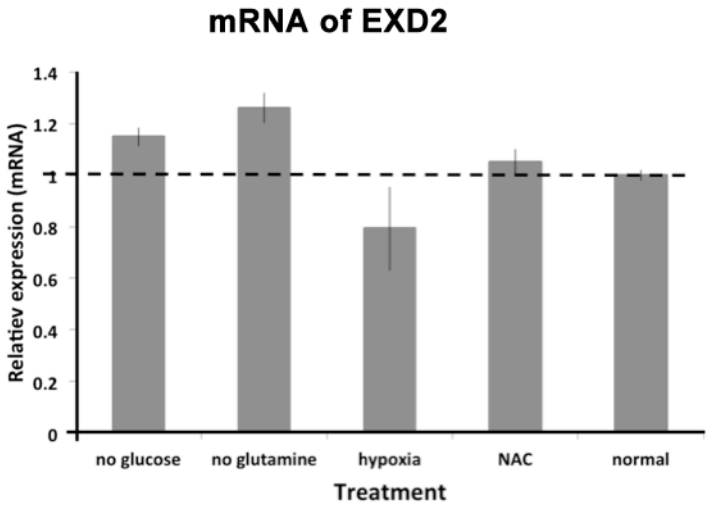

Figure 46. EXD2-mRNA-levels under hypoxia. Relative expression of EXD2-mRNA upon exposure to hypoxia in U2OS-cells, normalized to GAPDH mRNA.

Taken together, depending on the stimulus leading to the down-regulation of EXD2, HIF-1α is either stabilized or degraded in response to hypoxia (Figure 47). These contradictory results suggest that hypoxia-driven reduction in EXD2-levels is part of a complex network of tightly regulated events, whereas silencing of EXD2 by shRNA could lead to dysregulation of this network, and to an aberrant response to hypoxia.

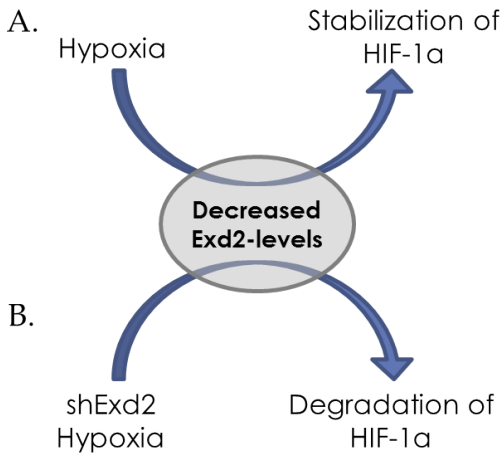

Figure 47. Model of the differential effects of decreased EXD2-expression in HIF-1α stabilization during hypoxia. A) Hypoxia leads to down-regulation of EXD2 and a normal hypoxic response with stabilization of HIF-1α. B) Hypoxia during down-regulation of EXD2 by shRNA leads to abnormal degradation of HIF-1α.

To shed some light into the mechanisms of how EXD2-loss could lead to such large-scale metabolic changes in tumor cells, we performed microarrays against the human genome (Affymetrix) on U2OS-cells expressing shExd#1, shExd#2 and shGFP. To our surprise we did not detect consistent changes in any known metabolic regulators or genes whose protein products are known to be targeted to the mitochondrion (Figure 48). Instead, many of the affected transcripts belonged to genes of unknown or poorly studied functions.

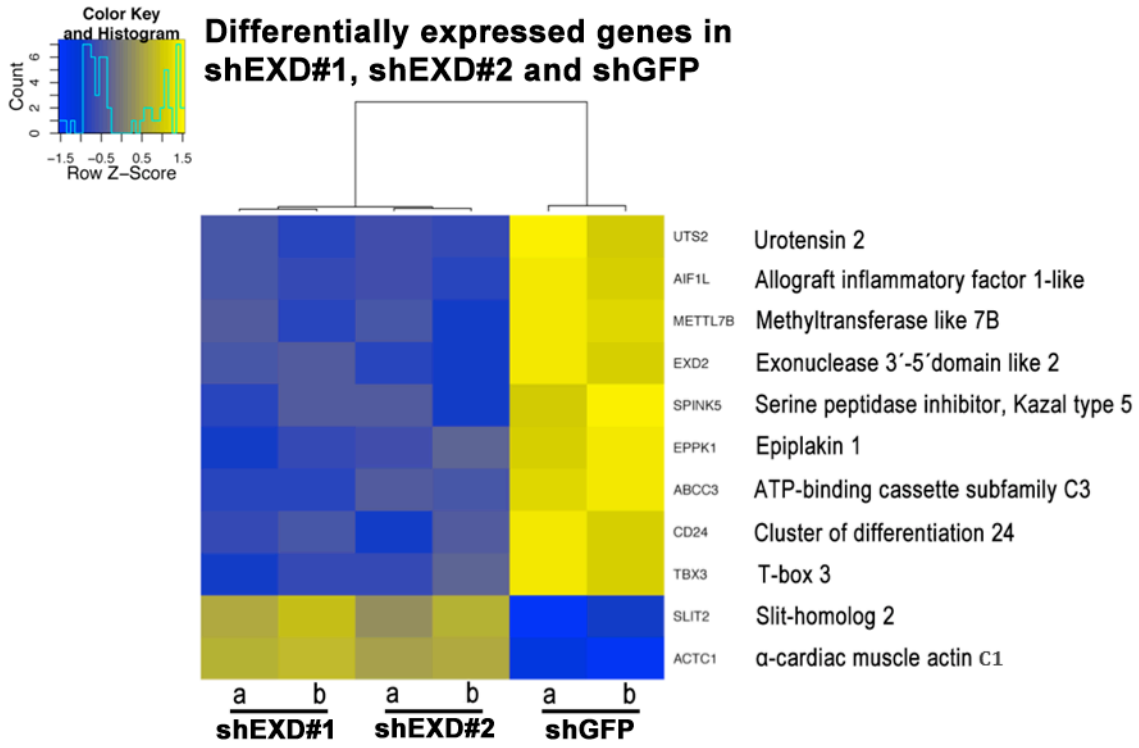

Figure 48. Differentially expressed genes in the shEXD2-cell lines. Heatmap of the most differentially expressed genes upon loss of EXD2 in two shEXD2-lines vs shGFP from two separate experiments. Blue (decreased expression), yellow (increased expression)

Among the more known down-regulated genes was CD24, a proposed prognostic marker for breast cancer stem cells and a known target of HIF-1 α [415] [416] Urotensin 2, a vasoconstrictor overexpressed in colon and prostate cancers is also a target of HIF-1α[417] [418]. T-Box 3 is a transcriptional repressor involved in mammary gland development and loss-of-function mutations have been observed in breast cancers (The Cancer Genome Atlas Network). Interestingly, only two genes were upregulated; SLIT2 (Slit-homologue 2) and ACTC1 (a-Cardiac muscle actin). SLIT2 is thought to be involved in cellular migration and development of vasculature, and its expression has been shown to be induced by hypoxia in human placenta[419] [420]. ACTC1 is an abundant extracellular matrix and cell-adhesion protein in the heart, but of its role in human disease is not clearly known. Together, the microarray data did not clearly uncover a gene network that would explain the previous results but did identify alterations in some HIF-1α targets.

To better understand the mechanisms of EXD2 function, we tried to identify its cellular binding partners. For this purpose we performed co-IP following a mitochondrial fractionation of cells expressing EXD2-SF-TAP in the C-terminus of the protein, allowing single- or double tag purification of native proteins[421]. We also overexpressed nuclease-dead EXD2-SF-TAP (EXD2DEAA-SF-TAP) to investigate if exonuclease-activity affected the binding of other proteins to EXD2, and a control SF-TAP construct (Figure 49).

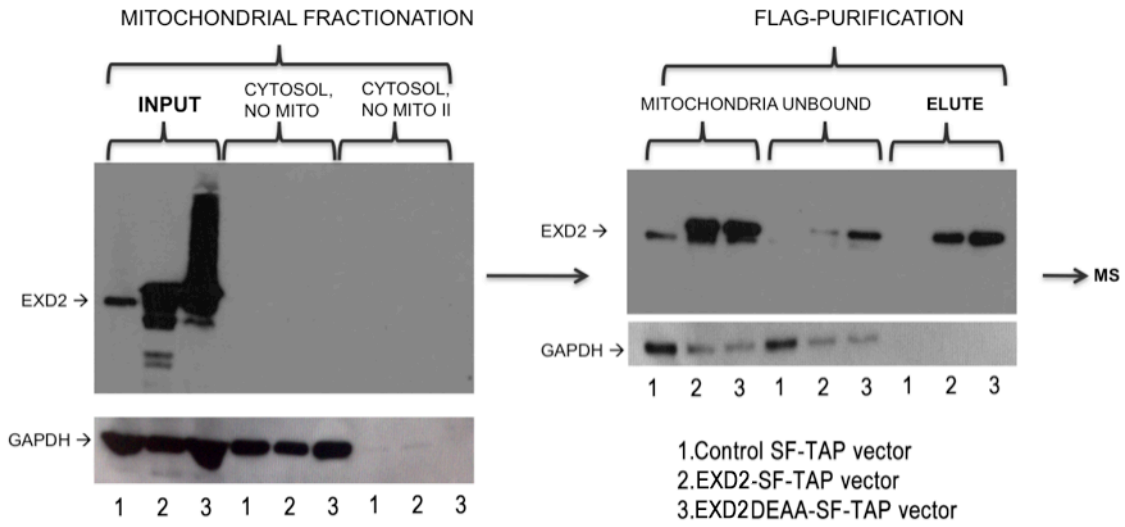

Figure 49. Co-immunoprecipitation of EXD2. EXD2-SF-TAP and EXD2DEAA-SF-TAP were affinity purified using Strep-Tactin resin from HEK293-cells. First the mitochondria were separated from the cytosolic fraction in two steps ("Cytosol, no mito") after which the mitochondrial fraction was incubated with flag-beads, washed and the flag-bound protein was eluted. The eluates were analyzed by mass spectrometry (LC-MS/MS) in the IRB Barcelona core facility.

The results from mass-spectrometry identified various proteins that co-purify with EXD2 (Figure 50). The enrichment score for EXD2 was 20% higher in the sample purified from EXD2DEAA than in the WT protein, suggesting that those cells either had a higher expression of EXD2 or the purification was more successful. Due to this the EXD2DEAA-purification potentially resulted in better resolution of possible interactors, with more proteins and a higher number of peptides identified. Also, we

noticed a strong smear in the blot on Exd2DEAA-SF-TAP –samples, suggestive of strong ubiquitynation of the protein.

Protein	Score EXD2	Score EXD2-DEAA	Function
EXD2	38.91	50.60	Exonuclease 3'-5' domain-containing protein 2
EEF1B2	10.67		Elongation factor 1B
COX5B	9.30	9.30	Cytochrome c oxidase subunit 5B
FAM211A	7.56		Leucine-rich repeat-containing protein FAM211A
MYL12A	5.85	5.85	Myosin regulatory light chain 12A
CALR	5.28	7.91	Calreticulin
TOMM70A	5.26	11.18	Mitochondrial import receptor subunit TOM70
CCT7	4.60	1.84	T-complex protein 1 subunit eta
STOML2	4.21	17.13	Stomatin-like protein 2
ATP1A1	4.11	10.07	Sodium/potassium-transporting ATPase subunit a-1
TOMM6		22.97	Mt import receptor subunit TOM6 homolog
PDIA3		21.98	Protein disulfide-isomerase A3
COX6A1		19.27	Cytochrome c oxidase subunit 6A1
RAP1B		13.59	Ras-related protein Rap-1b
ATP5H		11.68	Isoform 2 of ATP synthase subunit d
PTRH2		10.61	Peptidyl-tRNA hydrolase 2
PGAM5		10.59	Isoform 2 of Ser/thr-protein phosphatase PGAM5
ETFA		8.11	Electron transfer flavoprotein subunit alpha
ATP5O		7.98	ATP synthase subunit O
RHOC		7.77	Rho-related GTP-binding protein
UQCRFS1		7.66	Cytochrome b-c1 complex subunit Rieske
CYB5R3		6.83	Isoform 2 of NADH-cytochrome b5 reductase 3
HBD		6.80	Hemoglobin subunit delta
RAB7A		6.76	Ras-related protein Rab-7a
COX4I1		6.51	Cytochrome c oxidase subunit 4 isoform 1

Figure 50. The putative EXD2-interacting proteins identified by affinity purification. Proteins co-immunoprecipitating with EXD2-SF-TAP and EXD2DEAA-SF-TAP were affinity purified using Strep-Tactin resin from HEK293-cells and analyzed my mass spectrometry.

Among the putative EXD2 interactors, we found many known mitochondrial proteins, but also some that have no reported function in the mitochondria and are not predicted to be transported to the organelle. Especially interesting was the eukaryotic translation elongation factor 1B2, that is involved in the transfer of aminoacylated tRNAs to the ribosome. It could have a similar role in the mitochondrion and be closely associated with a mitochondrial exonuclease ready to degrade or modify tRNAs or mRNA released from the ribosomes. Many of the proteins identified have no known function, and more work needs to be done to confirm their interaction and role in regards to EXD2. Interestingly, several CytC oxidase (Complex IV) subunits immunoprecipitated with EXD2, suggestive of its possible interaction with the ETC complexes. Additionally, leucine-rich domain containing proteins like FAM211A purified only with active EXD2. Structural prediction programs (Phyre2) suggest it has a highly conserved fold common to many ribonuclease inhibitors, suggesting it might function as a regulator of EXD2-activity by directly binding it[422].

Drosophila melanogaster: a model to study the effects of EXD2 loss *in vivo*

Drosophila melanogaster (fruit fly) was introduced in research laboratories in the early 1900s[423]. Its genome is only 5% of the size of the human genome, but most important gene families and pathways, as well as many tissue and organ systems, are shared between the two species[424] [425]. Another advantage in working with the fruit fly is that large quantities of flies can be cultivated in small tubes, and their high fecundity allows large numbers of genetically homogenous flies to be produced rapidly. In the 1980s a library of genetically modified fruit flies was initiated to serve as a resource for facilitating the genetic and molecular analysis of various genes. Today the library consists of thousands of flies with genetically inserted single transposons called P-elements, that can disrupt the expression of the affected gene. As the P-elements are naturally occurring transposons with a heat-sensitive promoter, they can be induced to change genetic location by a brief heat shock. This “jump” of the P-element can re-establish the expression of the disrupted gene, to create a close to perfect genotypic control, or result in the deletion of nucleotides that can affect the coding region or gene expression.

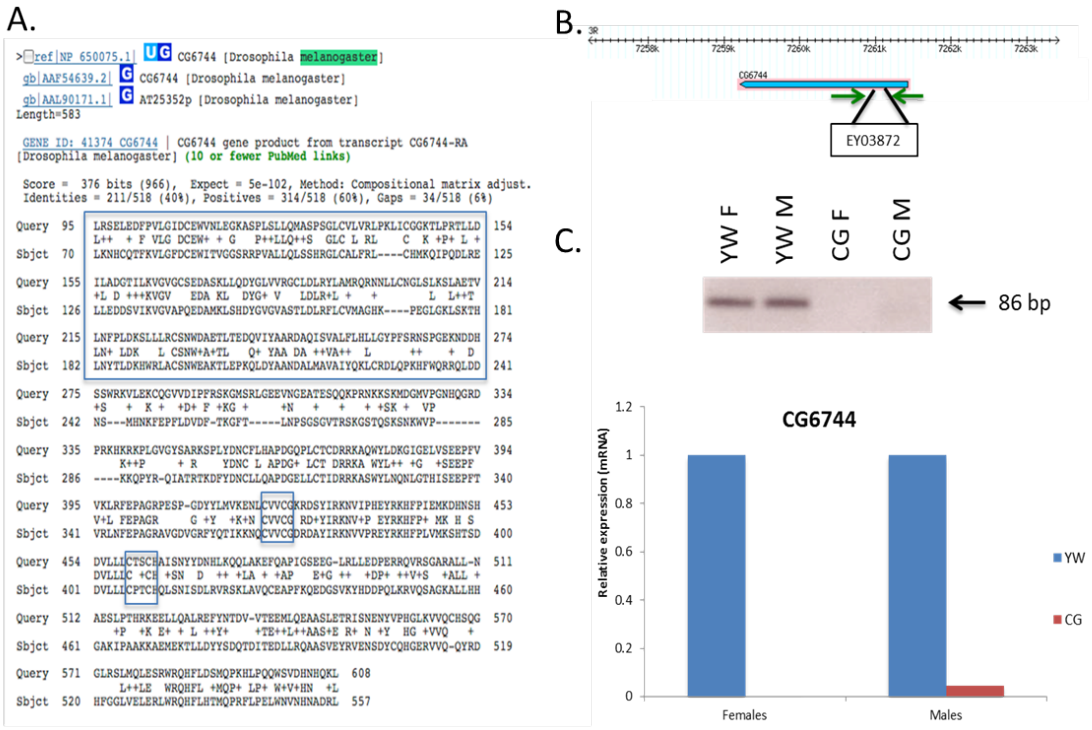

Figure 51. Drosophila melanogaster with disrupted expression of dmEXD2. A) Sequence similarity between human EXD2 and dmEXD2 with conserved domains (blue boxes). B) Position of the P-element insertion in dmEXD2. C) PCR with primers spanning the P-element, and Q-PCR-results of mRNA-levels of dmEXD2 in the flies with disrupted dmEXD2-expression compared with WT yellow whites (YW).

Since the putative functional domains of EXD2 are highly conserved between human and Drosophila, we decided to use the fruit fly to study the consequences of EXD2-loss *in vivo* (Figure 51A). First we verified the location of P-element in the gene for the fly orthologue of *EXD2*, dm6744 (dmEXD2), by PCR (Figure 51 B, C). Due to lack of antibodies against dmEXD2, we could not confirm the absence of protein with WB. However, as shown in Figure 43 C, little full length mRNA is being produced in the dmEXD2-mutant flies from the normal start codon, making it highly probable that they lack the protein product. In addition, no in frame ATG sequences are located within several hundred base pairs 3' of the transposon.

The dmEXD2-mutants were viable and did not bear any obvious differences in their behavior when compared to the Yellow White (YW) controls. However, when we

measured the lifespan of these flies, we noticed that the female mutants lived significantly longer than their YW counterparts (Figure 52). Many metabolic alterations are known to increase the lifespan of flies and the results might provide clues to the pathways that EXD2 affects in vivo. The most well-known factor extending lifespan is CR, and we wanted to study if loss of dmEXD2 led to a metabolic changes mimicking CR in the flies. For this purpose we put the flies on CR diet and analyzed their lifespan. However, an additional 25% increase of lifespan was observed even in the flies lacking dmEXD2-flies, suggesting that the lifespan extension induced by CR and EXD2 work at least partially through different pathways. As a similar life-extending effect was not seen in the mutant male flies, it suggested pathways involved specifically in female hormonal regulation could be involved.

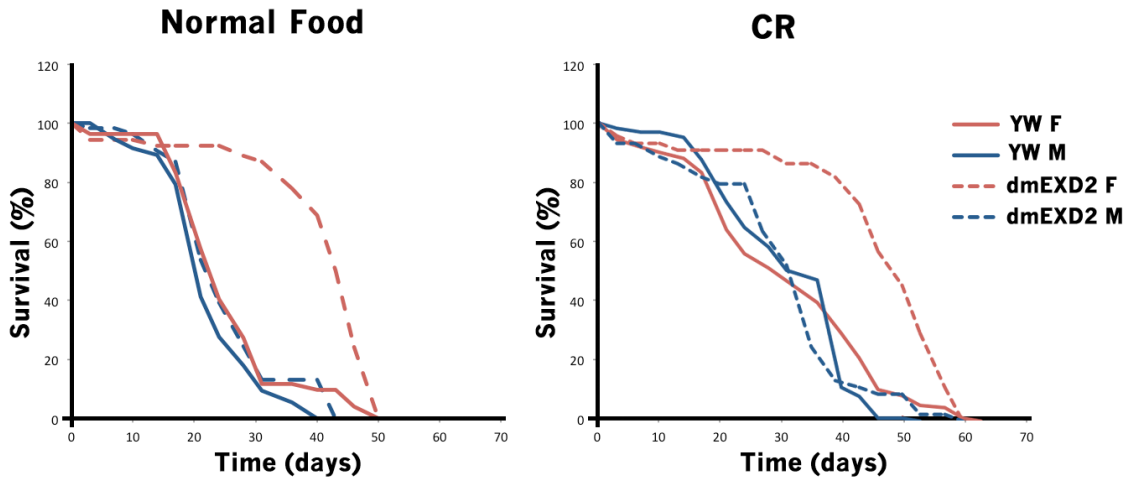

Figure 52. Lifespan of dmEXD2 flies in normal and calorie restricted food. Virgin YW and dmEXD2-mutant –flies were left to age in normal conditions and the number of surviving flies was counted every week during a change of tubes to fresh media. The total numbers of flies are indicated and the statistical analysis was performed using the Wilcoxon Rank Sum test in MStat. A) Flies fed with normal food, B) Flies fed with CR-diet. F= female, M= male

It has been documented that flies with a longer lifespan are often more resistant to various stresses than their shorter-lived counterparts, and indeed we observed that

both, the dmEXD2-females and males had higher resistance to environmental stressors such as exposure to cold. However, when we placed the flies in hypoxic conditions, the flies lacking dmEXD2 were much slower to recover when normal environmental oxygen levels were restored than the YW controls (Figure 53A). As the hypoxic response is thought to be partially regulated by increased ROS, and antioxidants have been implicated in lifespan extension, we wanted to see if feeding the flies with the antioxidant NAC would normalize the life-span of the dmEXD2-mutant flies. Female fruit flies are known to be more sensitive to the addition of antioxidants to their diet, causing a slight reduction in their lifespan, as occurred also in our experiments. Moreover, the antioxidant-diet restored the lifespan of dmEXD2-mutant females to the normal levels (Figure 53B), confirming that the increase in female lifespan was due to ROS-mediated mechanisms.

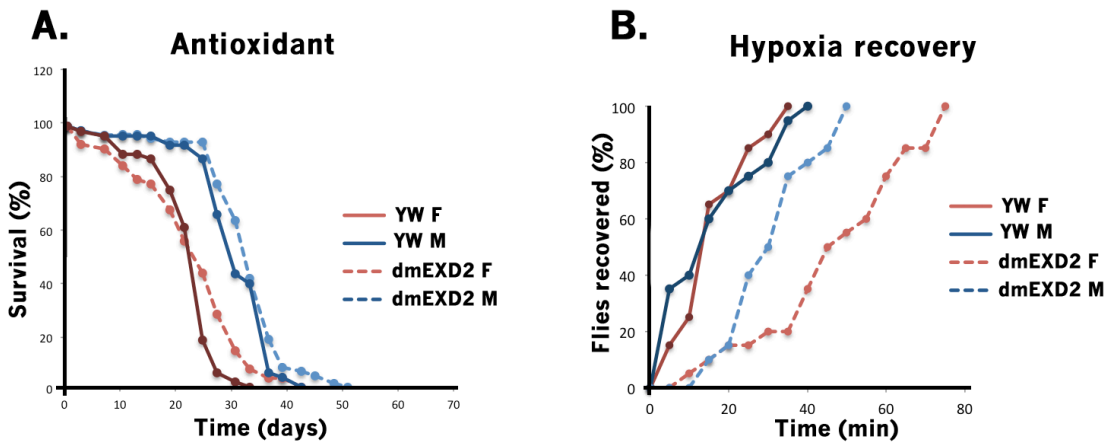

Figure 53. Sensitivity of dmEXD2-mutants to hypoxia and antioxidants. A) Flies were placed in hypoxia for 30 minutes and the time of recovery was recorded according to their ability to stand up. B) Flies were fed with food including NAC and the number of surviving flies was counted every week during change of tubes.

Impaired insulin-signaling has been implicated in ROS-mediated lifespan extension in *C.elegans*[426]*. Drosophila* Insulin-like peptides (dILPs) are able to directly activate the sole insulin receptor of the fruit fly and altered expression of dILPs 2, 3, and 5 have been shown to result in modulated insulin signaling and profound metabolic and longevity consequences[427] [428]. Studies on dIPLs 6 and 7 have resulted in

controversial results of their ability to modulate metabolism and therefore were not used in our study[429]. To determine if insulin signaling was affected in our mutant-flies we performed Q-PCR against dILPs 2, 3 and 5. According to the results, both female and male dmEXD2-mutants have alterations in the levels of genes involved in insulin signaling (Figure 54A). Interestingly, the activity of the insulin-pathway seems to be dependent on the sex of the flies with the dmEXD2-mutant background. Whereas males have overexpression of all tested dILPs, the female mutants have strongly decreased expression of dILPs, as well as lower expression of the insulin receptor (InR) itself. It has been documented that InR-mutant flies have reduced synthesis of the streroid hormone ecdysone from their ovaries[430], which prompted us to investigate the status of the closest fruit-fly homolog of estrogen receptor, the ecdysone receptor (EcR). Insect ecdysone receptor is activated by ecdysteroids, that are insect sex hormones secreted by both the male and female fruit flies and thus participates in hormonal regulation of both sexes. There is evidence that the regulation and downstream effects of EcR and insulin pathways are interconnected[431] [432] and consistent with this we observed a similar expression pattern to the DILPs in EcR of dmEXD2-mutant flies (Figure 54B). It seemed that loss of EXD2 resulted in two-fold increase in the levels of EcR in males, and in an opposite, 2-fold decrease in females. We also measured the expression of Sir2 due to its link to lifespan extension in *C.elegans* and *Drosophila*, but found no differences in its mRNA levels between the YW and dmEXD2-mutants (figure 54C).

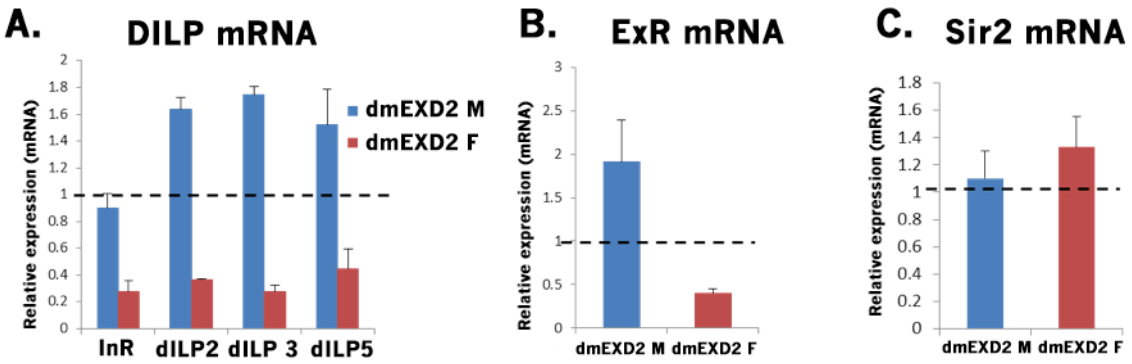

Figure 54. Dysregulation of longevity pathways in dmEXD2-mutant flies. A) mRNA-levels of InR and DILPs in dmEXD2-mutant male and female fruit flies. B) mRNA-levels of EcR in dmEXD2-mutant male and female flies. C) mRNA-levels of Sirt2 in dmEXD2-mutant male and female fruit flies. All values are normalized to GAPDH and to the values of YW males/females (dashed line).

As alterations in insulin signaling have been reported to result in smaller body size, we measured the length of the pupae and adult flies. Consistent with the changes in the DILP-levels, we found that the size of the dmEXD2-mutant pupae was much smaller than the YWs (Figure 55A). In pupae this might reflect a developmental delay that has been observed previously in EcR-mutant flies, and when we measured the time to pupation, we indeed found a delay in the development of dmEXD2-mutant animals (Figrue 55B). Consistent with this and the lower EcR-levels of the dmEXD2-mutant females, we also recorded an early onset decrease in fecundity. Lower fecundity has been shown to contribute to the longevity of female flies, which might partially explain the lifespan phenotype of dmEXD2-female flies[433].

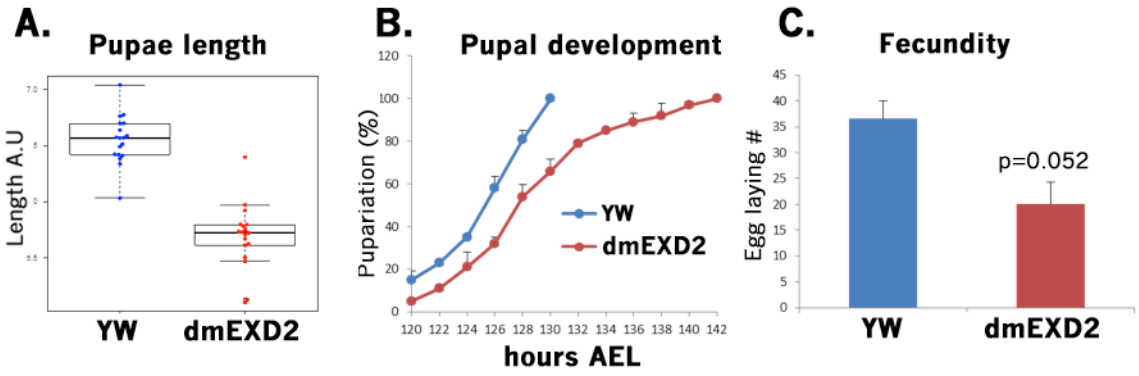

Figure 55. Developmental effects resulting from loss of dmEXD2. A) Length of the pupae of YW and dmEXD2-mutant animals. B) Embryonic development of the YW and dmEXD2-mutants measured as percentage of animals pupariating. C) Fecundity of the 4-weeks old adult flies measured as numbers of eggs laid.

In summary, our *in vivo* results using flies lacking expression of the EXD2-fly-orthologue suggest that EXD2 is necessary for regulation of various pathways implicated in lifespan regulation of *D.melanogaster*. Loss of dmEXD2 leads to

dysregulation of these pathways and cause hormonal changes in insulin and ecdysone pathways, leading to longer lifespan of females and altered intra-cellular oxygen-signaling.

Discussion

The importance of metabolism to cancer became apparent already in the 1960's when Warburg observed increased glucose uptake in tumor cells. Decades later, it was realized that mutations in DNA-repair genes are a major factor in cancer development and only recently have we begun to understand the coordination between these two processes, and how their co-operation creates a pro-tumorigenic environment.

The aim of this project was to characterize the putative exonuclease, EXD2, and determine if it influenced cancer progression. Its structural similarity to the exonuclease-domain of the well-studied WRN-protein suggested to us that it might have a similar role in repairing DNA-damage in the nucleus. As the exonuclease activity of WRN has ben shown to be necessary for telomere integrity[308] we thought that EXD2 may also act on particular structures that would impair the standard DNA replication machinery. In addition, Elledge and colleagues reported that EXD2 was involved in the repair of DNA crosslinking, suggesting a possible role of EXD2 in the maintenance of nuclear DNA through the FA complex[434]. However, as we observed that EXD2 localized to the mt, we considered that an association with the FA complex was unlikely. As the mt DNA-damage response is poorly understood and many key proteins are yet to be determined, we aimed to study the role of EXD2 in the maintenance of mt genome and understand the implications of its loss at the cellular and organismal level. The data presented in this thesis are the first to link EXD2 to the maintenance of the mt nucleic acids and demonstrate its ability to influence cancer growth and organismal aging.

EXD2 is an active 3'-5' exonuclease that localizes to the mitochondrion

My results suggest that EXD2 is primarily localized to the mitochondria with a small fraction localizing the ER where EXD2 is likely translated. Many DDR-proteins localize to both the nucleus and mitochondria and we cannot exclude the possibility that a small fraction of EXD2 resides in the nucleus, or that it translocates there upon certain stimulus. To date we have examined DNA damage and hypoxia carefully and not seen any evidence of this in primary and cancer cells (U2OS, 293T, MCF7). This was also supported by our notion that the cells deficient in EXD2 were not sensitive to agents inducing nuclear DNA cross-links or alkylating damage. Using single cell imaging, our collaborators found that EXD2 depleted cells died more slowly than control cells treated with camptothecin. We believe that the assay used by the Elledges group to identify EXD2 in crosslink sensitivity may reflect this alteration in the early response rather than long term survival that is more relevant to sensitivity.

It has been shown that WRN influences the replication of CG-rich substrates such as telomeres. Thus, it seemed logical that the CG-rich mtDNA could be a target of the exonuclease activity of EXD2. By using bacterially purified WT EXD2 and EXD2 with inactivating mutations in the putative exonuclease domain we examined the substrates and 3'-5' exonuclease activity of the protein. Interestingly, whereas other known mitochondrial exonucleases are able to degrade solely ssDNA, the substrates of EXD2 included both ss and dsDNA as well as ssRNA. In addition to this, EXD2 was able to degrade all of the tested replication intermediate structures that we had available, including DNA-RNA hybrids that occur during mtDNA replication, making its activity unique among the mitochondrial nucleases examined to date. Similarly to its nuclear homologue WRN, the exonuclease activity of EXD2 is regulated by Mn(II) and Zn(II), however WRN has not been

shown to degrade or bind RNA, despite its RNaseD-like domain, making EXD2 a more versatile nuclease in terms of substrates[435 436 437].

Role of EXD2 in the mtDNA replication and/or repair

The mitochondrial exonucleases, DNA2 and POLG, are necessary for the replication of mtDNA, whereas DDK1 is involved in the degradation of ssDNA in the control-region of mtDNA[438 48 439]. Unlike nuclear replication, mitochondrial replication occurs continuously throughout the cell cycle and is closely connected to the translation of mitochondrially encoded proteins[440 92]. Thus, the complex of proteins involved in replication, transcription and translation of the mitochondrial genes, as well as the mtDNA repair proteins, are assumed to remain in close proximity to the mtDNA[57 92]. However, using EM, we did not observe colocalization of EXD2 with the mtDNA, but instead, the EXD2-labeling seemed to be dispersed throughout the mitochondrial matrix. However, with ChIP we did observe EXD2-mtDNA interaction in cells overexpressing EXD2. This might be due to the fact that EM is less sensitive than ChIP or that the particles we used for labeling the DNA and EXD2 for EM are too large for detecting colocalization. Also, the samples for EM are cut extremely thin, to about 100nm, allowing labeling in a single plane, and narrowing its power in finding colocalization on a larger molecular scale. This is also supported by the fact that there are no examples in the literature showing colocalization of any mt proteins with the mtDNA by EM. We cannot exclude that the binding we observe in ChIP is non-specific. We have seen that EXD2 binds nuclear DNA or RNA in lysates leading to its entrapment in the well if samples are not boiled before SDS-PAGE electrophoresis. However, as we have identified a specific mutation that ablates mtDNA binding in ChIP and also fails to rescue mtDNA stability, we believe that the interaction we see represents a biologically relevant event.

As WRN is crucial in the response to nuclear replication stress, one of our first questions was whether EXD2 has a similar role in mtDNA replication. As our 2D-gel analysis showed no changes in mitochondrial replication after loss of EXD2, this suggested that EXD2 is not involved in the stabilization or repair of stalled replication forks during mtDNA replication under normal conditions. However, it is important to consider that although we analyzed the ability of EXD2 to degrade a wide array of substrates *in vitro*, its activity *in vivo* might be more specific and require a certain trigger or be regulated by post-translational modifications, interacting proteins or even some domains of EXD2. This could include the C-terminus of EXD2 that was deleted for the *in vitro* purification from bacteria. In addition, the CXXC-motifs in the Zinc-finger like domain could act as regulators involved in proper folding and activation of the protein, as is the case with some redox-regulated CXXC-domain containing mitochondrial proteins[441] [442]. Another possibility supported by *in silico* structural predictions is that EXD2 is a cytochrome and binds to heme through the second CXXC domain and that this potentially regulates its activity. We are currently working to purify the full length protein from human cells to examine these possibilities. In addition, the decrease of EXD2 mRNA-levels following hypoxia and their increase in response to glucose withdrawal might suggest that these stressors are involved in the enzymatic activation of EXD2, and it would be interesting to test if they affect the mt replication fork progress in shEXD2-cells using 2D gel analysis.

Although we did not observe a clear defect in mtDNA replication in the shEXD2-cells, we did find a higher incidence of DNA lesions in the tested cell lines using the real time PCR assay. This, together with the observation that silencing of EXD2 resulted in decreased mtDNA content in osteosarcoma, breast cancer and non-tumorous cell lines, as well as in the fruitfly lacking the EXD2 orthologue, suggests a mtDNA genome maintenance function for EXD2. In order to study the nature of the DNA-lesions in the shEXD2-cell lines we are planning to use probes designed

to detect specific DNA-lesions in the mtDNA by ELISA or mass spectrometry approaches.

EXD2 and mitochondrial RNAs

Both our biochemical assays and Co-IP that were done on mitochondrial fractions suggested that EXD2 could be involved in RNA metabolism. Based on our affinity purification results, EXD2 physically interacts with a group of proteins involved in mt translocation and some subunits of OXPHOS complexes, particularly those in the CytC oxidase (complex IV). In addition to these, the interaction of EXD2 with two proteins was only observed with the wild type protein, not the nuclease dead. The first is the Elongation Factor 1B that is involved in the transfer of tRNAs to the ribosome. The second is an uncharacterized protein Leucine-rich repeat-containing protein or FAM211A. Some known mitochondrial proteins with leucine-rich repeat motifs are involved in stabilizing mt mRNAs and coordinating translation and antiviral responses[443] [444]. An alignment of FAM211A with known structures suggests that it is a ribonuclease inhibitor, and could be a negative regulator of EXD2. These interactions need to be confirmed further by pull-down assays, and the functions of both proteins need to be studied in order to understand the relevance of their interaction with EXD2.

One of the few (3) papers describing EXD2 identified it as a host factor for *Dengue*-virus replication. *Dengue* is an ssRNA virus and loss of EXD2 protected the cells from viral accumulation, making it possible that the nuclease activity of EXD2 plays a role in the replication of this or other human pathogens[445]. EXD2 may directly affect Dengue replication by acting on the ssRNA or some replication intermediate but as EXD2 is mainly localized to the mitochondria, it seems more likely that it plays an indirect role, potentially modulating innate immune responses. Another possibility is that it is the ER fraction of EXD2 that is relevant. Dengue is endocytosed and its translation and assembly take place in the ER and induce a

large-scale rearrangement of cellular membranes. The depletion of mitochondria from cells with control or EXD2 shRNAs would allow this possibility to be tested.

Another protein identified as a potential interactor involved in nucleic acid metabolism was Peptidyl-tRNA hydrolase 2 (PTRH2). PTRH2 is predicted to regulate transcriptional activity of the mitochondrion by releasing the peptide in the event of stalling of the ribosome during translation[446]. As the Co-IPs did not identify any core replication or DNA-interacting proteins, it could indicate a stronger role of EXD2 in RNA-maintenance. However, these results have to be confirmed by pull-down assays and Western blotting before any conclusions can be drawn.

According to the RITOLS-theory, mtDNA replication involves an RNA-strand that is synthetized and incorporated with the lagging DNA-strand. The enzymes involved in the degradation of this RNA-strand are yet unknown. If EXD2 was the enzyme responsible, the cells depleted for EXD2 should have an accumulation of this complementary RNA. Whether a defect in the degradation this complementary RNA could lead to the DNA-lesions we observed in our cells is not known, but it would undoubtedly increase the overall size of mtRNA-pool in the cells. The accumulation of mtRNA could act as a signal for feedback inhibition to mtDNA machinery to reduce the replication rate and result in a decrease in the mtDNA content.

Another RNA-degrading enzyme yet to be identified is the ribonuclease- component of the mammalian mRNA-specific mt degradosome. In yeast the degradosome is the main general ribonuclease and is responsible for the bulk of RNA turnover and surveillance activity and the mammalian degradasome is proposed to serve similar role. If EXD2 were to function in concert with the hSuv3 helicaseto degrade mt mRNAs, its loss would lead to the increase in the levels of mt transcripts. As mt transcription is regulated separately from protein translation, even cells expressing high levels of mt mRNAs can maintain their normal levels of protein expression. The shEXD2-cells seem to express lower levels of gene-encoding mt mRNAs, suggesting EXD2 is not a missing part of the mammalian degradasome. However, although published in various papers as a valid method, analyzing mt mRNA-levels

by PCR has many challenges: 1) The synthesis of mt cDNA by oligo(d)T, primers relies on polyadenylation of the mRNA-molecules. It has been shown that many, but not all of the mitochondrial mRNAs are polyadenylated, and in addition, each transcript has various internal polyadenylation sites able to bind the oligo(d)T primer and enhance amplification of the mRNA. 2) MtDNA lacks introns, which prevents designing primers that span the exon-junction. Therefore even a minor mt DNA-carry over from the RNA extraction will be amplified together with the cDNA. To avoid these pitfalls, the use of random hexamers instead of oligo(dT) primer can be used, together with a stringent DNase-treatment of the RNA. However, despite various DNase-treatments, we continued to detect small amounts of mtDNA in our RNA. For these reasons we will use Northern blotting in the future to detect mt mRNA-species until a reliable method for purifying RNA cleanly from mtDNA is found.

Yet another species of mtRNAs have been shown to been involved in decreased mtDNA-levels: Myopathy Encephalopathy with Lactic Acidosis and Stroke-like episodes (MELAS) is a syndrome that is caused by mutations in mt tRNA-genes in 80% of cases[447]. Depending on the mutation, these patients have downregulation of mtDNA copynumber due to accumulation of mRNAs and consequent feedback inhibition[448]. In order to determine the level of tRNAs in the shEXD2-cells we are also in the process of conducting a Northern blot-assay.

Taken together, we have direct and indirect evidence that EXD2 can act on mt DNA and RNA species, but the details of these interactions remain to be elucidated. It is very likely that EXD2 functions in highly conserved pathway, since there are no known eukaryotic exonucleases that can process both DNA and RNA and EXD2 is highly conserved in many organisms down to Drosophila.

EXD2 and the metabolic regulation of cancer

In addition to the substrates, activity, interaction partners and intracellular localization, we assessed the consequences of EXD2-loss on the metabolism of cancer cells since there is increasing evidence that mitochondrial abnormalities are an important influence on many types of cancer. However, it is not always clear if these alterations contribute to carcinogenesis or whether they arise as secondary effects during cancer progression[449]. Also, despite the vast effort of many research groups, the question of whether alterations in mtDNA cause cancer lacks conclusive results.

However, mutations in several nuclear genes affecting mitochondrial function are associated with cancer onset and progression. It is also shown that impaired mitochondrial respiration leads to a dependence on glycolysis for ATP production and a concomitant increase in lactic acid production, a phenomenon associated with oncogenesis known as the Warburg effect. Although we have observed this upon loss of EXD2, it did not enhance, but rather impaired tumor progression. It is worth noting that this was done in cells that were already highly glycolytic and it may suggest that EXD2 is important to maintain some basal mt function that is important for other aspects of tumor growth. Strikingly, we also observed an accumulation of 2-HG accompanied by a reduction in various TCA cycle intermediates following the step of a-KG synthesis. Accumulation of 2-HG has been observed in many gliomas with mutations in IDH1/IDH2 and recently also been linked to breast cancer and mutations in citrate carrier, both expressing intact IDH1/IDH2. Expression of 2-HG promotes global hypomethylation[450] by preventing a-KG from binding histone demethylases[451]. The mechanism by which 2-HG is synthetized in these cells in unclear, but involves increased glutaminase activity and usage of a glutaminolytic pathway. The glutaminolytic pathway is also known to be the main source of a-KG during hypoxia, when OXPHOS is shut down and

cells use almost solely the isocitrate pathway to create lipids *de novo*. It also seems that the accumulated 2-HG can interfere with hypoxia signaling by sequestering the prolyl hydroxylases responsible for stabilizing HIF-1a, and leading to blunted cellular response to hypoxia. Taken together, it appears that loss of EXD2 switches the cells towards reductive carboxylation, where glutamine is required for synthesis of a-KG and citrate to provide macromolecules for *de novo* synthesis of lipids for the needs of cellular proliferation (Figure 56). However, for reasons yet to be studied, the a-KG does not get converted to isocitrate, but is used for synthesis of 2-HG, leading to the observed decrease in metabolites (succinate, fumarate) later in the TCA-cycle. This accumulation of 2-HG, together with insufficient succinate that is a co-factor in Hif-1α stabilization, lead to decreases in the stability of HIF-1α in these cells.

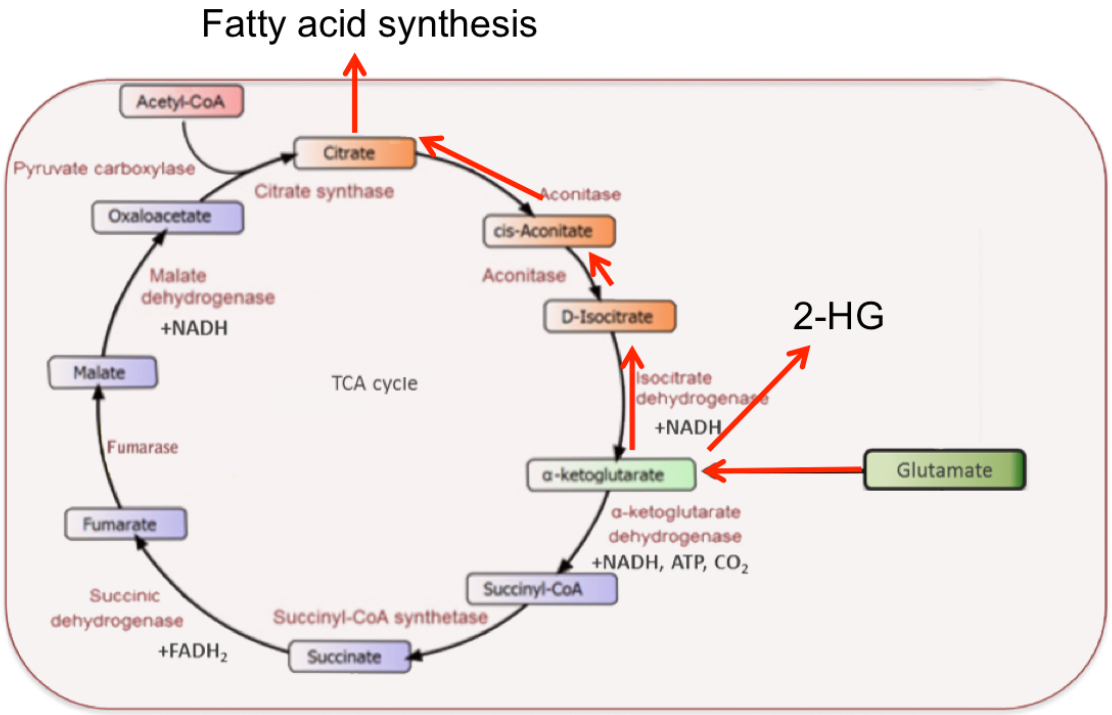

Figure 56. Loss of Exd2 leads to increased in reductive carboxylation. During reductive carboxylation, glutamine becomes indispensable for creating molecular precursors for lipid synthesis via reversed TCA-cycle function. This reversion is also required for α-KG-conversion to oncometabolite 2-HG observed in various cancers and shExd2-cells.

Enigmatically, the decrease in succinate, fumarate and increased 2-HG observed in various cancers seem to be mainly due to mutations in the nuclear encoded synthetases, and no connection between mtDNA or mtRNA quality and changes in the levels of these metabolites have been published to date. The intriguing question remains, how does a 3'-5' exonuclease illicit such a large scale metabolic alteration in these cells?

One possible explanation is, that the changes in mt metabolites are secondary to increased lactic acid production in the shEXD2 cells. Lactic acidosis has been described in various cancers and mitochondrial diseases due to mutations in the mt tRNAs[452] and mtDNA[453 454]. In both cases the protein synthesis of the mt encoded gene(s) is perturbed leading to dysfunction of the affected OXPHOS complex(es) and reduced ATP synthesis. This slows down the whole machinery of mitochondrial respiration, and leads to accumulation of cytosolic pyruvate, and further, lactic acid. The extent to which the basic cellular functions are affected by these changes has been shown to be highly tissue-specific, the most strongly affected ones being metabolically very active muscle and brain tissues[452]. This might explain why we do not see any growth defects *in vitro* in the shEXD2-cells in any tested cell lines (U2OS, MCF7 and MDA231).

Since the metabolic profile of shEXD2-cells mimicked metabolic changes reported in various cancers, we were surprised to observe that the tumor growth of breast cancer xenografts was strongly reduced after loss of EXD2. This was especially puzzling since the shEXD2-cells were highly overexpressing ACTC1, a member of actin-family of proteins, that are known to be crucial for forming the tumor-supporting scaffold and supporting tumor cell migration[455]. Since the *in vitro*-growth of the breast cancer cell lines is not affected after loss of EXD2, the growth-inhibitory signals must be dependent on the tumor niche. Indeed, it seems that the inability of the shEXD2-cells to stabilize HIF-1a in the hypoxic environment inside a tumor leads to insufficient development of vasculature followed by reduced growth. Even though our data-analysis revealed a high correlation between the expression

of *EXD2* and *ESR1* in breast cancer tumor cohorts, loss of EXD2 led to an equal reduction in tumor growth in both cell lines; the ERa-positive MCF7 and ERα-negative MDA231 despite our observation that *in vitro* shEXD2 cells exhibited lower expression of ESR1 mRNA. Thus, it seems that in the tumor environment EXD2 functions independently of an estrogen-dependent pathway.

When extrapolating results between cell lines we must consider the cell line-specific differences in metabolism. The metabolomics and microarray analysis were performed in U2OS-cells that are epithelial, chromosomally highly unstable cell line, whereas the breast cancer lines used for the xenograft-experiments are endothelial and express the oncogene WNT7B. However, decreases in mtDNA, increased glutamine sensitivity and aberrant hypoxia signaling seem to be consistent phenomena in response to loss of EXD2 between all of the cell lines tested.

Despite the expression of the same oncogene, MCF7 and MDA231 cell are metabolically very different. The basal type MDA231 exhibits high dependence of glutamine for their ATP-production, whereas glutamine withdrawal has only minor effect on MCF7-cells[456]. Thus, we do not believe the anti-tumorigenic effect of EXD2-loss in these cells is, at least completely, due to glutamine-related metabolic changes. However, it has been shown that MDA231-cells have aberrant HIF-1α signaling due to a constitutively active unfolded protein response (UPR) in the endoplasmic reticulum[457]. Only one publication describes mtUPR in MCF7-cells, and more solid evidence whether these cells are also under mtUPR stress remains to be found[458]. Why we believe that EXD2 might be involved in triggering the mtUPR is discussed further in the next chapter.

Taken together, we have shown that EXD2 is involved in mtDNA and/or RNA maintenance and when downregulated, leads to destabilization of HIF-1 α and poor growth of breast cancer tumors. Many of the phenotypes we observed (decreased succinate and fumarate, expression of 2-HG) could be the primary cause of the

aberrant regulation of HIF-1α, and more research has to be done to determine the specific effect of EXD2 and identify the origin of these phenomena.

Functions of EXD2 at the organismal level

As mentioned previously, the cellular response to hypoxia includes production of intracellular misfolded proteins followed by induction of the UPR[459]. This leads to suppression of translation as an adaptive mechanism for reducing the load of newly synthetized and unfolded proteins, especially in the context of cancer cells. It has been shown that in *C.elegans,* inactivation of any of the 23 (including 6 mitochondrial) aminoacyl-tRNA transferases (aaRS) leads to increased hypoxia tolerance via decreased translation and UPR[460], coupling tRNA quantity to hypoxia signaling. Chloramphenicol (CA) has emerged recently as a potential anti-proliferative drug for cancer treatment. Its antiproliferative effect is based on its ability to stall the mitoribosomes and prohibiting mt translation. It would be interesting to test if the shEXD2-cells are more sensitive to CA-induced inhibition in cell proliferation. Another way to study mt translation would be to use radiolabeled precursors, although this method has only been applied in yeast, and has no yet been described in human cells.

Since EXD2 is conserved in Drosophila, we were able to create a strain with highly reduced expression of the Drosophila-homologue of EXD2. These flies have a significantly prolonged lifespan, which is more pronounced in female flies, and they are strongly sensitive to hypoxia. Various studies in worms, flies and mice show, that mutations that cause ETC dysfunction extend lifespan up to 50% due to activation of the mtUPR [461 462 463 464]. If loss of EXD2 leads to an increase in some mt tRNA-species the cells and flies lacking EXD2 would be under constant mtUPR activation conferring sensitivity to hypoxia, and leading to lifespan extension due to a hormesis effect. Our observation that the expression of HSP90 (dmHSP83) is

increased in flies lacking EXD2, is consistent with a constant upregulation of mtUPR in the female flies during the first four weeks of their lives [465]. Although the verification of mtUPR-asociated gene upregulation remains to be done, the fact that lifespan extension was fully reversible with addition of antioxidant, strongly suggests that the mitohormesis-effect is due to mtUPR based on the previous reports in the literature[466].

Summary and model

We have shown biochemically, that EXD2 is an active mitochondrial 3'-5' exonuclease able to degrade various DNA structures, as well as DNA/RNA hybrids. *In vitro*, evidence shows that EXD2 binds mtDNA, and the loss of EXD2 leads to decreases in mtDNA copynumber, mtDNA lesions and large-scale metabolic reprogramming of the cell. These metabolic changes include increased conversion of glucose into lactate, reduced mitochondrial respiration and synthesis of TCA intermediates. We have also observed a mild accumulation of the oncometabolite 2-HG, and an increased dependence on glutamine in these cells, probably due to reductive carboxylation. Downregulation of EXD2 in two metabolically distinct breast cancer cell lines led to highly reduced tumor growth in both lines, correlating with reduced intratumoral vasculature likely resulting from aberrant hypoxia-signaling. Drosophila lacking dm*EXD2* are sensitive to hypoxia and the females exhibit significant lifespan extension that is reversed by antioxidants and independent of calorie restriction. Based on these data, we propose the following model.

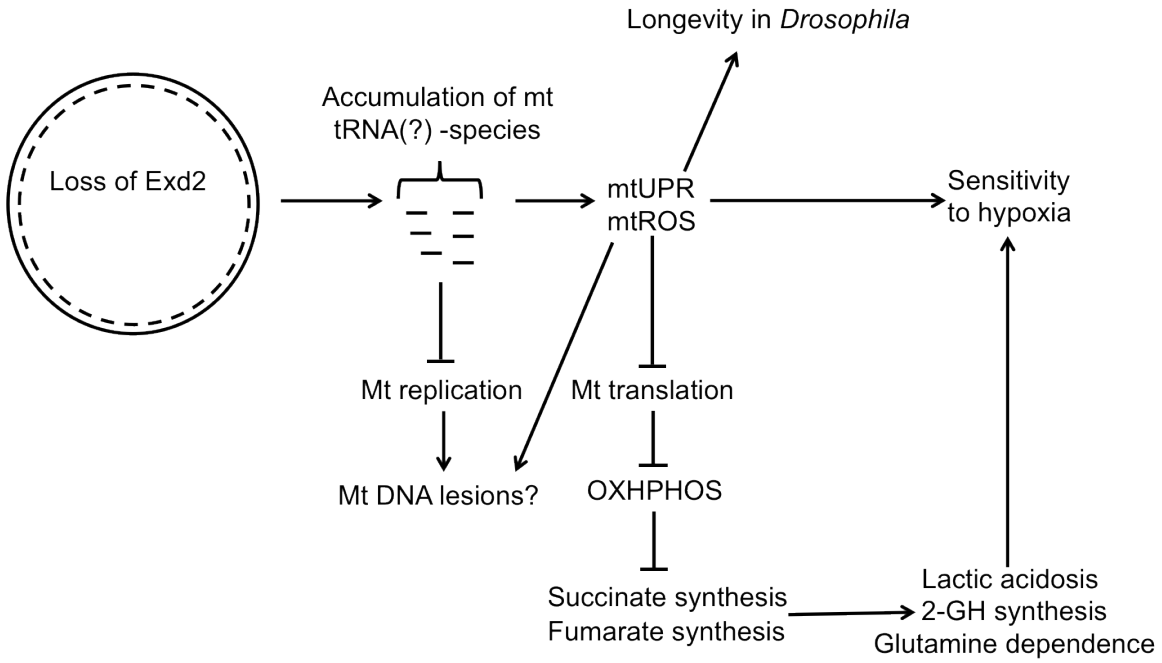

Figure 57. Proposed model for the mechanism of metabolic changes induced by loss of EXD2. The loss of the enzymatic activity of EXD2 leads to accumulation of mt tRNA species. The consequent increase in mt protein translation leads to increase in mtROS and mtUPR. The mtUPR iniciates a signaling cascade leading to repression of translation resulting in decrease in OXPHOS and TCA intermediates. This leads to increased requirement of glutamine for lipid synthesis (reductive carboxylation), accumulation of 2-HG in the mt and lactic acid in the cytosol. The lack of co-subsrates (succinate, fumarate) and high levels of competitive inhibitor (2-HG) of HIF-1a lead to sensitivity to hypoxia.

The future studies will focus on deciphering the molecular mechanism by which Exd2 affects the nucleic acid homeostasis. Does it bind the mtDNA in order to degrade the short RNA-templates during or after mtDNA replication? Or does it have a role in post-transcriptional modifications or degradation of tRNAs or in transcription of other mtRNA-species from the mtDNA, like short and long ncRNAs complementary to the mtDNA? Since the machinery for processing of mt sRNAs in mammalian cells is completely unknown, EXD2 could be also involved in the

editing sRNAs from the mtDNA in response to certain stressors in a manner similar to epigenetic regulation of metabolism by sRNAs in the bacteria.

Conclusions

1. EXD2 localizes to the mitochondrion.
2. EXD2 is an active 3´-5´exonuclease able to degrade ds and ssDNA and dsDNA, RNA, DNA/RNA hybrids and various DNA structures *in vitro.*
3. EXD2 binds mitochondrial DNA.
4. EXD2 is not necessary for mitochondrial replication fork progression.
5. Loss of EXD2 does not sensitize cells to DNA crosslink damage.
6. Loss of EXD2 leads to a decrease in mtDNA levels and increases number of mtDNA lesions
7. Loss of EXD2 leads to a metabolic phenotype resembling reductive carboxylation that leads to glutamine-dependence.
8. Loss of EXD2 in ER positive and negative breast cancer cell lines impairs tumor growth in xenografts.
9. Loss of EXD2 results in defective angiogenesis and hypoxic signaling in cells and tumors.
10. Loss of dmEXD2 extends lifespan in female *D. melanogaster* and this is reversible with an antioxidant-diet.
11. Loss of dmEXD2 sensitizes flies to hypoxia.
12. Loss of EXD2 leads to aberrant insulin and ecdysone signaling in fruitflies.

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