Identification of Genes Modulating Mitochondrial Biogenesis in *Caenorhabditis elegans*

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Table of Contents

List of Figures

List of Tables

Abbreviations

%	percent
°C	degrees Celsius
~	approximately
2D AGE	two-dimensional agarose gel electrophoresis
3'	three prime end of nucleotide sequence
3D	three-dimensional
4E-BP	eukaryotic translation initiation factor 4E binding protein
5'	five prime end of nucleotide sequence
A	adenine
acetyl-CoA	acetyl coenzyme A
AD	Alzheimer's disease
ADP	adenosine dihosphate
α-KG	alpha ketoglutarate
AMP	adenosine monophosphate
AMPK	AMP activated kinase
ANT1	adenine nucleotide translocase 1
ARE	antioxidant response elements
ATFS-1	bZip activating transcription factor associated with stress 1
ATP	adenosine triphosphate
bp	base pair
bZip	basic leucin zipper
C	cytosine
C-terminus	carboxyl terminus
C. elegans	*Caenorhabditis elegans*
C/EBP	CAAT/enhancer-binding protein
CaMK IV	calcium/calmodulin-dependent protein kinase IV
cAMP	cyclic AMP
cDNA	complementary DNA
*Ce*TOR	*C. elegans* TOR
ChIP-seq	chromatin immunoprecipitation followed by deep sequencing
CHOP	C/EBP homologous protein
CI/II/III/IV/V	complex I/II/III/IV/V
CIT1	mitochondrial citrate synthase
CIT2	peroxisomal citrate synthase
CoQ	Coenzyme Q10
COX	cytochrome c oxidase
CR	caloric restriction
CREB	cAMP response element-binding protein
CytC	cytochrome c
DNA	deoxyribonucleic acid
DVE-1	homolog of Defective proVEntriculus

e.g.	*exempli gratia*
eIF2α	eukaryotic initiation factor 2 alpha
eIF4E	eukaryotic initiation factor 4E
ER	endoplasmatic reticulum
ERR	estrogen related receptor
ETC	electron transport chain
$FADH_2$	flavin adenine dinucleotide, hydroquinone form
FAO	fatty acid oxidation
Fe/S	iron-sulfur
G	guanine
g	gram
g	gravitational force
GABP	GA-binding protein
GCN-2	general control non-derepressible-2 kinase
GFP	green fluorescent protein
GST-4	Glutathione S-Transferase
H-strand	heavy strand
H^+	proton
H_20	water
HMG	high mobility group
HSF-1	heat shock factor 1
HSP	heavy strand promoter
HSP	heat shock protein
IDH	isocitrate dehydrogenase
IGF	insulin-like growth factor
IMM	inner mitochondrial membrane
IMS	intermembrane space
IRE-1	inositol-requiring 1 protein kinase (homolog)
IRES	internal ribosome entry site
kb	kilo base pairs
KLF	Kruppel-like factor
L-strand	light strand
L1/2/3/4	larval stage 1/2/3/4
LSP	light strand promoter
mAAA	matrix ATPases Associated with diverse cellular Activities
MAPK	mitogen activated protein kinase
Mb	mega base pairs
MEF	mouse embryonic fibroblast
MFRTA	mitochondrial free radical theory of aging
mm	millimeter
mRNA	messenger RNA
MSR	mitochondrial stress responses
mtDNA	mitochondrial DNA
MTERF	mitochondrial transcription termination factor
mTOR	mammalian TOR

MTS	mitochondria targeting sequence
MTSS-1	mitochondrial single-stranded DNA binding protein
n	sample size
N-terminal	amino terminus
N2	wild type Bristol *C. elegans* strain
NAD^+	nicotinamide adenine dinucleotide, oxidized
NADH	nicotinamide adenine dinucleotide, reduced
nDNA	nuclear DNA
NFE2L2	nuclear factor (erythroid-derived 2)-like 2
NGM	nematode growth medium
NLS	nuclear localization signal
nm	nanometer
NRF	nuclear respiratory factor
O_H	origin of heavy strand replication
O_L	origin of light strand replication
OMM	outer mitochondrial membrane
OXPHOS	oxidative phosphorylation
P-eIF2α	phosphorylated eIF2alpha
PCR	polymerase chain reaction
PD	Parkinson's disease
PDI-3	protein disulfide isomerase
PGC-1	PPARY coactivator 1
P_i	inorganic phosphate
POLγ	mitochondrial DNA polymerase Y
POLRMT	mitochondrial DNA-directed RNA polymerase
PPAR	peroxisome-proliferator activated receptor
PTM	post-translational modifications
qPCR	quantitative PCR
RC	respiratory chain
RFU	relative fluorescence units
RITOLS	RNA incorporation throughout the lagging strand
RNA	ribonucleic acid
RNA-seq	RNA sequencing
RNAi	RNA interference
ROS	reactive oxygen species
RPG	ribosomal protein gene
rRNA	ribosomal RNA
RTG	retrograde
RXR	retinoid X receptor
SEM	standard error of mean
SIRT1	sirtuin 1
SP	specificity protein
SPG7	spastic paraplegia 7
SSBP-1	mitochondrial single-stranded DNA binding protein 1
T	thymine

TCA cycle	tricarboxylic acid cycle
TFAM	mitochondrial transcription factor A
TFB1M/2M	mitochondrial transcription factor B 1/2
TIM	translocase of inner membrane
TOM	translocase of outer membrane
TOMM20L	translocase of outer mitochondrial membrane 20 homolog (yeast)-like
TOR	target of rapamycin
tRNA	transfer RNA
U	Unit
UBL-5	ubiquitin-like protein 5
UCP1	uncoupling protein 1
UPR^{er}	endoplasmatic reticulum unfolded protein response
UPR^{mt}	mitochondrial unfolded protein response
UV	ultraviolet
VDAC1	voltage-dependent anion channel 1
vs.	*versus*
YY1	Ying Yang 1
µm	micrometer

Abstract

Since Altmann recognized ubiquitously distributed "bioblasts" in 1890, understanding of mitochondria has evolved from "elementary organisms" living inside cells and carrying out vital functions, over the Harman's "free radical theory" in 1956, to one of the driving forces of aging and cause of multiple associated diseases impacting society today. While a tremendous amount of work has contributed to the understanding of mitochondrial biology in different model organisms, the precise molecular mechanisms of basic mitochondrial function have yet to be deciphered.

By employing an RNA interference mediated screen in *Caenorhabditis elegans*, we identified two transcription factors: SPTF-3, a member of Sp1 family, and an uncharacterized, nematode specific W04D2.4. We propose that both proteins modulate expression of many genes with regard to mitochondrial function including mitochondrial single-stranded binding protein encoded by *mtss-1*, whose promoter was used as transcriptional reporter in the screen. Further, RNA sequencing data indicate that W04D2.4 indirectly regulates expression of mitochondrial DNA via control of genes functionally related to mitochondrial replication and translation machineries. We also demonstrate that from all interventions targeting cytosolic translation, MTSS-1 levels are elevated only upon knockdown of genes encoding cytosolic ribosomal proteins. Reduction of ribosomes leads to increased *sptf-3* translation, most likely in an internal ribosome entry side (IRES) mediated manner, eventually inducing *mtss-1* expression. Moreover, we identify a novel role for SPTF-3 in the regulation of mitochondrial unfolded stress response (UPR^{mt}) activation, but not endoplasmatic reticulum or oxidative stress responses.

Taken together, this study identifies two transcription factors previously not associated with mitochondrial biogenesis and UPR^{mt} in *C. elegans*, establishing a basis for further investigation of mito-nuclear interactions.

Zusammenfassung

Seit der deutsche Pathologe Richard Altmann im Jahr 1890 Mitochondrien fälschlicherweise als Elementarbauteile der Zelle bezeichnet hatte, trug der unaufhaltsame technische und wissenschaftliche Fortschritt dazu bei, dass den Mitochondrien heutzutage eine zentrale Rolle im Alterungsprozess und bei diversen Pathologien zugeschrieben wird. Trotz beachtlich vieler Erkenntnisse über die Funktionsweise und Aufbau der Mitochondrien, existieren immer noch Wissenslücken über grundlegende Funktionen des Organells. In der vorliegenden Arbeit konnten wir mit Hilfe von Screens, basierend auf dem RNA-Interferenz Mechanismus, zwei Transkriptionsfaktoren identifizieren: SPTF-3, Mitglied der Sp1-Familie und den bis dato nicht charakterisierten, nematodenspezifischen W04D2.4. Unsere Studie deutet darauf hin, dass beide Proteine die Expression von vielen Genen mit mitochondrialer Funktion regulieren. Dazu gehört auch das Gen *mtss-1*, welches das mitochondriale Einzelstrang-bindende Protein kodiert und dessen Promoterregion wir im Transkriptionsreporterkonstrukt verwendet haben. Außerdem deuten die Ergebnisse der RNA-Sequenzierung darauf hin, dass W04D2.4 die Expression der mitochondrialen DNA indirekt reguliert, indem es Transkription von Genen kontrolliert, welche Replikations- und Translationsvorgänge in Mitochondrien bestimmen. Des Weiteren konnten wir demonstrieren, dass ein Knockdown von ribosomkodierenden Genen zu erhöhter *sptf-3* Translation führt, voraussichtlich begünstigt durch interne ribosomale Eintrittsstelle in der RNA-Sequenz. Darauffolgend führen erhöhte SPTF-3-Proteinmenge zu verstärkter Expression von *mtss-1*, was anhand entsprechender RNA- und Proteinmengen festgestellt wurde. Darüberhinaus deuten unsere Ergebnisse an, dass SPTF-3 eine Funktion in der Regulierung der mitochondrialen Antwort auf ungefaltete Proteine (mitochondrial unfolded protein response, UPR^{mt}) erfüllt. Abschließend ist festzuhalten, dass wir zwei Transkriptionsfaktoren identifiziert haben, welche zuvor nicht mit der Regulation der mitochondrialen Biogenese und UPR^{mt} im Fadenwurm *Caenorhabditis elegans* assoziiert wurden und somit neue Möglichkeiten eröffnen, Interaktionen zwischen Mitochondrien und Zellkern zu untersuchen.

1 Introduction

1.1 Mitochondria

1.1.1 Discovering mitochondria

At the end of the 19th century, new histological methods and advancing microscopy allowed Richard Altmann to detect “granular bodies” within various types of plant and animal cells (Altmann, 1890). These granular bodies, initially-called “bioblasts” or life germs, displayed staining properties of bacteria. Therefore, these organelles (later termed “mitochondria") were interpreted as autonomous, bacteria like structures. Moreover, Altmann mistakenly assumed that bioblasts were elementary building blocks within eukaryotic cells, which was predominantly the reason why his observations were largely neglected. Fifteen years later, Russian botanist Constantin Mereschkowsky hypothesized that plastids were once free-living cyanobacteria, which were incorporated by heterotrophic cells during the course of evolution (Mereschkowsky, 1905). In his follow-up publications he suggested that plant cells are animal cells that incorporated cyanobacteria (for review, see (Kutschera and Niklas, 2005)). Interestingly, he accepted Altmann’s idea that the nuclei of cells are simply accumulations of mitochondrial “granules”, without considering their bacterial origin. However, scientists mainly rejected theories about the bacterial origin of plastids and mitochondria, favoring “autogenous” theory, implying that the organelles evolved *de novo* within eukaryotes. Though Mereschkowsky’s hypothesis provides the basis for the endosymbiotic theory, it was revived only when, in 1970, Lynn Margulis presented her theory on the origin of plastids and mitochondria in the context of arising cell and molecular biology in *Origin of Eukaryotic Cells*. She postulated that both organelles were at some point engulfed by a protoeukaryote host and reduced to ensure energy supply. The first experimental evidence supporting endosymbiotic theory was delivered by molecular phylogenetic analysis of ferredoxins, 5*S* ribosome RNAs and c-type cytochromes between diverse types of bacteria, green algae and organelles of eukaryotes (Schwartz and Dayhoff, 1978). That study revealed shared recent ancestry between chloroplasts and cyanobacteria, and between mitochondria and respiring photosynthetic bacteria, supporting bacterial origin

of eukaryotic organelles. Moreover, temporal mapping of these events suggested that mitochondria and chloroplasts became endosymbionts at two distinct stages around 2 and 1.1 billion years ago, respectively. Since these discoveries, an ever expanding field of genomic sequencing and computational methods of phylogenetic inference have been shaping the evolutionary tree, generating models for eukaryotic origins (for review, see (Embley and Martin, 2006)). More recent analyses supporting endosymbiotic theory suggest that mitochondria evolved from free-living ancestors of the *Alphaproteobacteria* SAR11 clade, sister order to the *Rickettsiales* (Thrash et al., 2011).

Though being engulfed, mitochondria retained its genome. Mitochondrial DNA (mtDNA) was observed for the first time in chick embryo in 1963 (Nass and Nass, 1963). Two decades later, mitochondrial genome sequencing began with samples from human and murine cells and to this day mtDNA from over 6.000 organisms (http://www.ncbi.nlm.nih.gov/genome) has been successfully sequenced (Anderson et al., 1981; Bibb et al., 1981). This massive data flood revealed that mtDNA is highly diverse not only in the number and size of chromosomes but also in the number of protein-coding genes: whereas mammalian mtDNA is around 16 kb and harbors 13 protein-coding genes, the size of mtDNA from *Silene conica*, flowering plant, is around 11 Mb, encoding 25 proteins (Sloan et al., 2012). Notably, the mitochondrial genome is highly reduced compared to extant *Alphaproteobacteria*. Computational inference studies of *Alphaproteobacteria* genome evolution suggest that their ancestors contained 3.000-5.000 genes indicating endosymbiotic gene transfer from the protomitochondrial genome to the nucleus (Boussau et al., 2004). Remarkably, out of 800 human genes with *Alphaproteobacteria* signature, only 200 are found in the mitochondrial proteome, suggesting that eukaryotic cell evolution was affected by proto-mitochondrial genes beyond mitochondrial function (for review, see (Gray, 2012)).

1.1.2 Mitochondrial architecture and function

Mitochondria are organized in a highly dynamic, readily growing, dividing and fusing network, which cannot be synthesized *de novo* and therefore must be distributed between dividing cells. Mitochondria are composed of two lipid bilayers that subdivide the organelle into two aqueous compartments. Mammalian mitochondria contain

around 3.200 predicted, with currently 1.098 experimentally validated proteins, but mtDNA harbors only 13 protein-coding genes (Pagliarini et al., 2008; Richly et al., 2003). Therefore, the vast majority of mitochondrial proteins are nuclear DNA (nDNA) -encoded, synthesized in the cytosol and eventually transported into the organelle. The outer (OMM) and the inner mitochondrial membranes (IMM) possess distinct permeability, ensuring compartment specific content of intermembrane space (IMS) and matrix (Figure 1.1). Low molecular weight molecules can passively cross OMM through transmembrane channels called porins, thus IMS content is comparable to that of the cytosol. By contrast, protein rich IMM composition is characterized by four times higher cardiolipin content, a mitochondria specific lipid, which makes it highly impermeable to ions and solutes (Gebert et al., 2009; Zinser et al., 1991). Therefore, sophisticated transport machineries, translocase of outer (TOM) and translocase of inner membrane (TIM) complexes, actively facilitate movement of proteins into OMM, IMS, IMM and matrix. Many mitochondrial proteins contain mitochondrial targeting signals that can be present as cleavable N-terminal matrix targeting sequence (MTS) or an internal targeting signal, which direct proteins to the IMS or the IMM (Neupert and Herrmann, 2007). The TOM complex is considered to be the entry gate for virtually all mitochondrial proteins of cytosolic origin.

Mitochondria are broadly known as the power plants of the cell, supplying them with energy in the form of adenosine triphosphate (ATP) generated by oxidative phosphorylation (OXPHOS). On a regular day humans use the amount of ATP equal to their own weight, largely due to brain and muscle activity. Since the human body contains only around 250 g of ATP at a time, a highly efficient ATP recovery system is a prerequisite. Nevertheless, in addition to supplying energy via oxidative phosphorylation, β-oxidation (catabolism of fatty acids) and tricarboxylic acid cycle (TCA cycle, also known as Krebs cycle), mitochondria are important for calcium homeostasis, apoptosis, cellular differentiation, and heme and iron-sulfur (Fe/S) cluster synthesis (Alberts et al., 2002). Notably, Fe/S proteins of mitochondrial origin are indispensible for nDNA maintenance and cytosolic translation, emphasizing the importance of mitochondria for cell survival (Stehling and Lill, 2013).

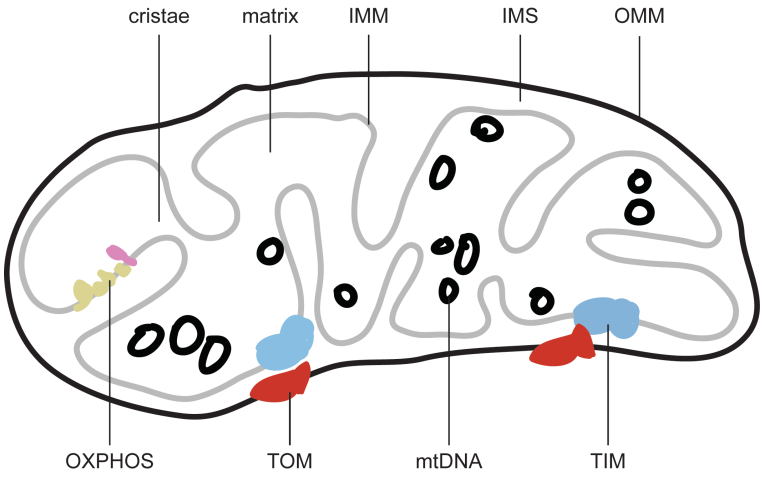

Figure 1.1 Mitochondrial architecture
Schematic representation of mitochondria, enveloped by two separate lipid bilayers: inner (IMM) and outer mitochondrial membranes (OMM). Thus, two distinct compartments are formed, each containing specific set of molecules. Mitochondrial DNA (mtDNA) resides in the matrix; translocase of outer (TOM) and translocase of inner membrane (TIM) complexes facilitate movement of proteins from cytosol into mitochondria. Cristae are invaginations of IMM into the matrix, that increase total surface of IMM and serve as primary site of action for oxidative phosphorylation (OXPHOS) complexes (Vogel et al., 2006).

The mitochondrial OXPHOS system is unique as all five multiprotein complexes of the OXPHOS system reside in the IMM, whereas its around 90 components are encoded by two distinct genomes, nuclear and mitochondrial DNA. Thirteen mtDNA-encoded proteins are translated in the matrix in close proximity to the IMM, where they are assembled into functional complexes together with around 80 imported nDNA-encoded proteins. The electron transport chain (ETC) consists of four complexes: complex I (CI, NADH:ubiquinone oxidoreductase, EC 1.6.5.3), II (CII, succinate:ubiquinone oxidoreductase, EC 1.3.5.1), III (CIII, ubiquinol:ferricytochrome *c* oxidoreductase, EC 1.10.2.2), and IV (CIV, cytochrome *c* oxidase, COX, EC 1.9.3.1) (for review, see (Fernandez-Vizarra et al., 2009)) (Figure 1.2). ETC receives electrons from two donors: NADH and $FADH_2$. Whereas NADH is produced in the course of glycolysis, TCA cycle and β-oxidation, $FADH_2$ stores the energy from oxidation of succinate to fumarate, two intermediates in the TCA cycle. This redox-reaction is facilitated by complex II, functionally connecting TCA cycle with ETC. The donated electrons enter the ETC at complexes I and II, facilitating sequential oxidoreductase reactions that culminate in the reduction of molecular oxygen to water by complex IV. Electron flow through the ETC provides energy, enabling complexes I, III and IV to pump H^+ ions across the IMM into the IMS. (for review,

see (Lodish et al., 2000)). The difference in H^+ concentration between the matrix and IMS builds up a proton-motive force across the IMS. Eventually complex V exploits flow back of protons into the matrix to generate ATP from adenosine diphosphate (ADP) and phosphate group (P_i).

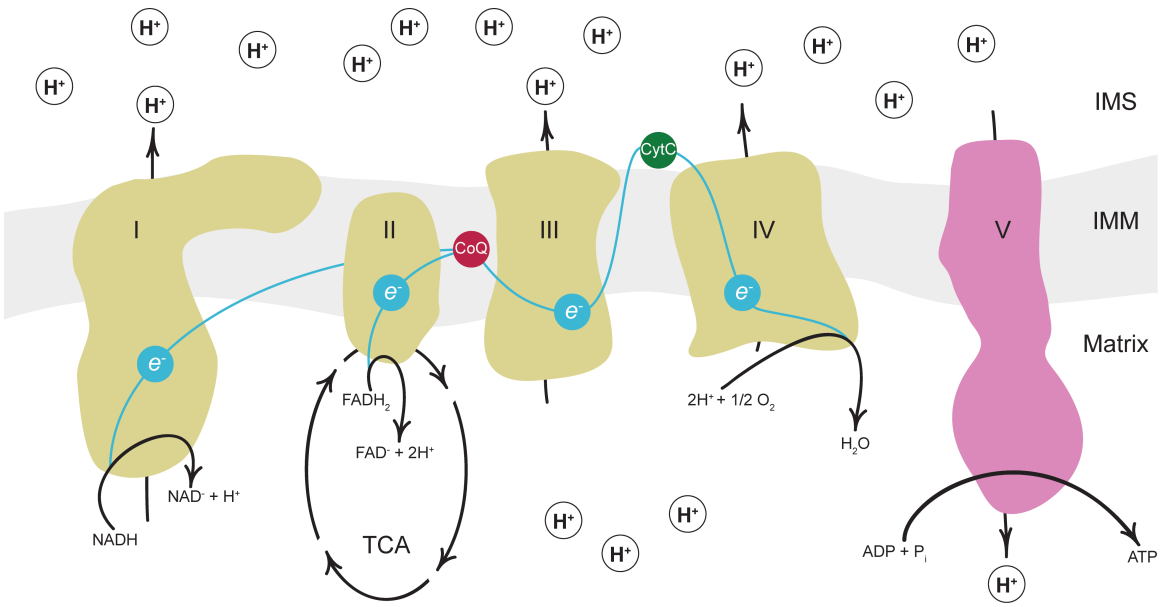

Figure 1.2 Respiratory chain
Schematic representation of oxidative phosphorylation, with complexes I-IV functionally grouped into electron transport chain (ETC). Electrons from NADH and $FADH_2$ are passed from complexes I and II by Coenzyme Q_{10} (CoQ) to complex III, further by cytochrome c (CytC) to complex IV resulting in reduction of molecular oxygen to water. The energy from the electron flow is driving proton pumping activity of complexes I, III and IV, generating a proton gradient across the IMM. Flowing back into matrix, protons are exploited by complex V to generate ATP from ADP and P_i.

The efficiency of the OXPHOS system depends on many factors, one of which is the spatial distribution of single complexes, as electrons are passed down from CI and CII, via CIII to CIV. Though for the sake of clarity, OXPHOS is presented as strung-together complexes. There are two distinct models explaining organization of complexes in the IMM (Figure 1.3).

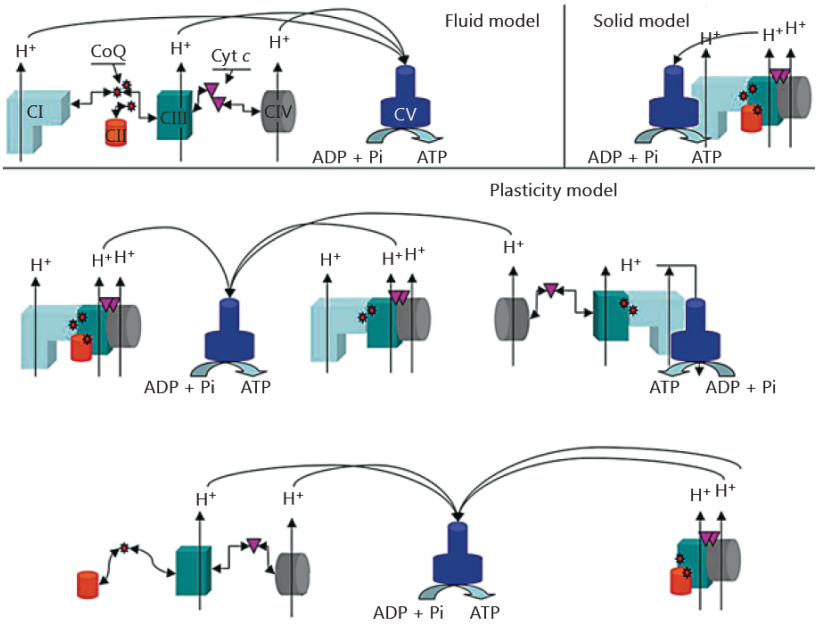

Figure 1.3 Fluid, solid and plasticity models of OXPHOS complexes organization
Representation of single complexes and electron carriers is indicated in the fluid model. Supercomplex formation in the plasticity model includes single complexes of each type, although the actual stoichiometry may vary (Enriquez and Lenaz, 2014). Illustration from (Acin-Perez et al., 2008).

The "solid model" was based on experimental co-isolation of complexes I and III, and on observations that once the electron transport system has been formed, single complexes do not dissociate upon dilution or centrifugation (Hatefi et al., 1962a; Hatefi et al., 1962b). Therefore, it was postulated that respiratory activity is based on solid supercomplex formation enabling fast intra- and inter-complex electron transfer (Green and Tzagoloff, 1966). The second, "random collision model" or "fluid model" suggests that the mitochondrial inner membrane is rather a fluid-state layer and that all membrane proteins, including the OXPHOS complexes, are freely and independently diffusing and colliding within this layer (Hackenbrock et al., 1986). Moreover, single complexes do not interact physically with electrons transported by CoQ and CytC between the complexes. Therefore, diffusion rates have controlling capacity on the electron transport kinetics. As is often the case, the truth most likely lies somewhere in between. A third, "plasticity model" suggests that two other models are extremes of the dynamic organization of complexes. In this model, single complexes form supercomplexes and so called respirasomes (supercomplexes additionally containing electron carriers CoQ or CytC, therefore able to

respire) (Acin-Perez et al., 2008). Whereas every complex can also be found freely in the IMM, mostly complex I seems to associate with other complexes in various combinations and proportions: CI+CIII, CI+CIII+CV, CI+CII+CIII+CIV or CI+CIII+CIV. However, the organization and physiological relevance of supercomplexes *in vivo* is still to be deciphered (for review see, (Enriquez and Lenaz, 2014)).

1.1.3 Mitochondrial genome

1.1.3.1 Organization

Given their common evolutionary origin, it is not surprising that mitochondria reveal similar genetic and cellular functions among eukaryotes. The most radical common development of mitochondrial genomes across taxa is that the majority of their native genes were transferred to the nucleus, where they are expressed to drive mitochondrial biogenesis or further cellular processes. Whereas the transfer event is indicated by the presence of protomitochondrial genes within the nuclear genome, it still remains unclear when, how and how often it occurred (Timmis et al., 2004). An extreme example of genome reduction can be observed in mitochondrion-related organelles, hydrogenosomes and mitosomes, which entirely lack mtDNA (for review see, (Gray, 2012)).

In all organisms that contain mitochondria, the initial mtDNA coding capacity was strongly downscaled with time. However, the outcome of this reduction shows remarkable variations in size, organization and expression among eukaryotes. In most animals it exists as a single, circular, double-stranded DNA molecule, but there are many examples of mtDNA organized as one or multiple, circular or linear chromosomes (for review, see (Gray et al., 1999; Nosek et al., 1998)). For example, mtDNA of *Polytomella piriformis*, colorless green alga, is divided into two linear chromosomes, the mitochondrial genome of *Pediculus capitis*, human head louse, is distributed on 20 circular chromosomes, each containing one to three genes (Shao et al., 2012; Smith et al., 2010). Further, the size of the chromosomes can be as small as 6 kb in protists from the *Apicomplexa* phylum, or as large as 1.6 Mb in *Cucumis sativus*, cucumber (Alverson et al., 2011; Hikosaka et al., 2010). Following variations in size, also the gene content differs between species. Freshwater protist

Reclinomonas Americana leads the field with 97 mitochondrial genes, of which 65 are protein-coding (Lang et al., 1997).

In mammals, nuclear DNA is diploid. On the contrary, the maternally inherited mitochondrial content of a single cell can vary between 1.000 and 10.000 DNA molecules, especially high in energy demanding tissues like brain and skeletal muscles (Bogenhagen and Clayton, 1974; Masuyama et al., 2005; Miller et al., 2003). This polyploidy is governed by excessive mtDNA replication, potentially resulting in two states: "heteroplasmy" and "homoplasmy". While homoplasmic state implicates only identical mtDNA molecules within one cell, heteroplasmic cells harbor at least two different mtDNA variants. In principle, mtDNA molecules can be replicated multiple times or not at all, potentially allowing clonal expansion of a mutated mtDNA, outnumbering wild type variant. However, mutated mtDNA molecules have to exceed a certain threshold, usually 60%-90%, to become pathogenic (Trifunovic et al., 2004; Tuppen et al., 2010).

There are no histones in mitochondria to pack and protect the DNA as there are in the nucleus. Instead, mtDNA forms nucleoprotein complexes (nucleoids), with, *inter alia*, mitochondrial transcription factor A (TFAM), a high-mobility group-box (HMG) containing protein. Beyond its pivotal role in transcription and replication, TFAM bends mtDNA in a U-shape manner at unspecific sequences, thus protecting the otherwise naked DNA and providing structural basis for expression activities (for review, see (Kukat and Larsson, 2013)). Superresolution experiments estimated the approximate size of a nucleoid at 100nm, prevalently containing a single copy of mtDNA (Kukat et al., 2011).

The double helix contains two differently denoted strands, "heavy" (H) and "light" (L). The nomenclature is based on the proportion of heavier G+T nucleotides leading to different sedimentation in denaturating alkaline cesium chloride gradients. Mammalian mtDNA contains 13 protein-coding genes, all subunits of OXPHOS system, 2 ribosomal rRNAs and 22 tRNAs, part of mitochondrial translation machinery (Figure 1.4). Notably, complex II subunits are exclusively encoded by nDNA. Intriguingly, whereas in the cytosol over 30 tRNAs are required to ensure amino acid specificity, in mitochondria only 22 tRNAs are used. This is due to the relaxed codon usage, when many mitochondrial tRNAs recognize any of the four nucleotides in the third anticodon position. Moreover, the "universal" code is altered in mitochondria leading to different "meanings" in 4 out of 64 codons (Alberts et al., 2002).

Dense gene packing and absence of introns characterize animal mtDNA, with few exceptions in some *Cnidaria* species (Beagley et al., 1996). Since coding sequences directly follow each other, little room is left for DNA regulatory sequences (Alberts et al., 2002). The major non-coding region is the 1kb large displacement loop (D-loop) that harbors important regulatory elements for replication and transcription. It contains promoters for light (LSP) and heavy strand (HSP) transcription, origins of heavy strand replication (O_H) and termination-associated sequences (for review, see (Falkenberg et al., 2007)).

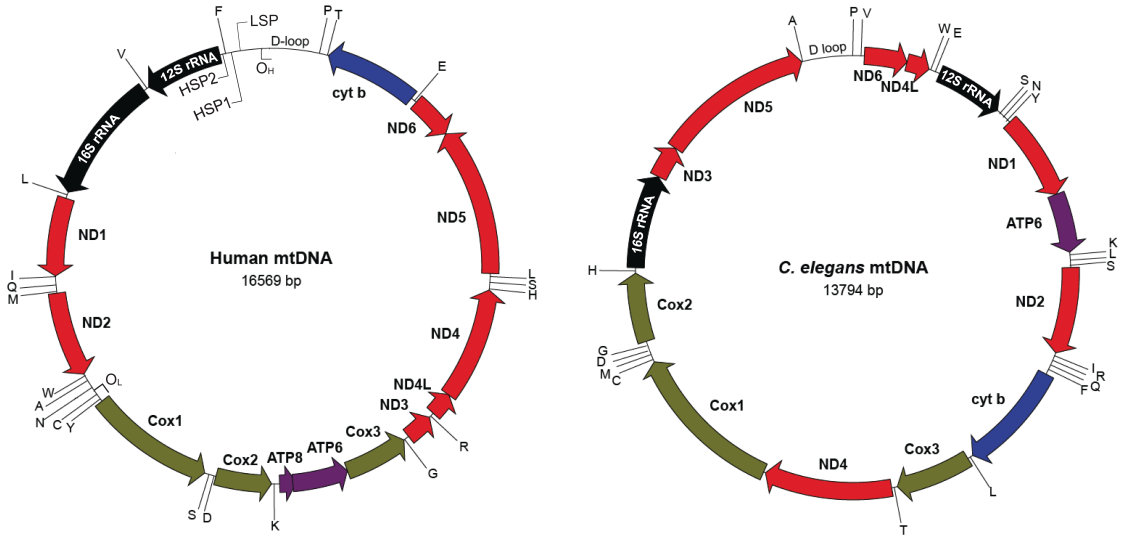

Figure 1.4 Structures of human and *C. elegans* mtDNA
Human genome contains 37 genes: 13 protein-coding genes, subunits of Complex I (ND1, ND2, ND3, ND4L, ND4, ND5, ND6), III (cyt b), IV (Cox1, Cox2, Cox3) and V (ATP6, ATP8), 2 ribosomal RNAs (12S rRNA, 16rRNA) and 22 tRNAs, indicated by single letter code for corresponding amino acid. The "D-loop" region contains replication and transcription promoter sequences. *C. elegans* mtDNA differs by the arrangement of the genes and the lack of ATP8. Images courtesy of Ivana Bratic Hench.

1.1.3.2 mtDNA transcription

In mammals there are three main transcription promoters: LSP and HSP1, residing around 150 bp from each other in the D-loop region, and HSP2 located between HSP1 and 12S rRNA gene (Figure 1.3). Transcription from LSP and HSP2 results in polycistronic precursor RNAs covering the entire coding portion of the light and heavy strand respectively. Thus, the HSP2 initiated transcript corresponds to 2 rRNA, 12 protein-coding and 14 tRNA genes, while LSP1 to 1 protein-coding and 8 tRNA genes. However,

HSP1 was found to be terminated at the end of the 16S rRNA gene, predominantly generating 12S and 16S rRNA (Montoya et al., 1983). The "tRNA punctuation" model implies that tRNA genes flank all protein and ribosomal genes, therefore their excision from polycistronic transcripts is necessary to produce mature mRNAs and rRNAs (Ojala et al., 1981).

The mammalian basic transcription machinery requires a specific set of proteins that is different from that of nuclear DNA. It includes mitochondrial DNA-directed RNA polymerase (POLRMT), TFAM and one of the two mitochondrial transcription factor B paralogs (TFB1M or TFB2M), all necessary and sufficient for transcription (Falkenberg et al., 2002; Shi et al., 2012). TFAM not only unspecifically wraps mtDNA but also specifically binds upstream of LSP and HSP1 transcription promoters (Fisher et al., 1987; Gaspari et al., 2004). The exact position of the binding sites within promoter region may imply that TFAM binding results in partial unwinding of mtDNA allowing transcription. The C-terminal tail region of TFAM interacts with the heterodimeric POLRMT:TFB2M(TFB1M) complex, thereby recruited to the initiation site. TFB1M and TFB2M additionally function as rRNA methyltransferases, whereas TFB1M is suggested to be highly active as methyltransferase and TFB2M as a transcription factor (for review, see (Falkenberg et al., 2007)).

Further mtDNA transcription is regulated by a family of mitochondrial transcription termination factor (MTERF) proteins: MTERF1-4. MTERF1 and MTERF2 are unique to vertebrates, while MTERF3 and MTERF4 are highly conserved among different phyla and can be also found in *C. elegans* (Linder et al., 2005). Initially MTERF1 was proposed to regulate mitochondrial ribosome biogenesis due to its binding downstream of rRNA genes and MTERF3 to generally repress transcription initiation (Fernandez-Silva et al., 1997; Park et al., 2007). However, a more recent study suggests that MTERF1 does not regulate heavy strand promoter activity but rather reduces light strand transcription to prevent transcriptional interference at the LSP (Terzioglu et al., 2013).

1.1.3.3 mtDNA replication

Mitochondrial DNA can be replicated independent of cell cycle and in non-dividing cells (Bogenhagen and Clayton, 1974). Similar to transcription machinery,

replication machinery is also specific to mitochondria. *In vitro* studies of minimal mammalian mtDNA replisome revealed three proteins required for sufficient replication: mitochondrial DNA polymerase γ (POLγ), mitochondrial TWINKLE helicase and mitochondrial single-stranded DNA binding protein (SSBP1 or mtSSB) (Korhonen et al., 2004). POLγ and TWINKLE are capable of using a double stranded template and generating DNA molecules of 2kb length. While TWINKLE unwinds the mtDNA duplex in an ATP dependent manner, SSBP1 coats the single strands in tetrameric fashion facilitating the opening and unwinding. Addition of SSBP1 further stimulates TWINKLE helicase activity leading to products of 16 kb of size, which corresponds to the full-length mtDNA (Korhonen et al., 2003).

Studies in mice revealed that RNA primers generated during LSP transcription are simultaneously required for replication at O_H, suggesting that the initiation process for replication and transcription is the same and that the mechanism distinguishing DNA or RNA synthesis is subject to downstream events (Brown et al., 2008; Chang et al., 1985). The initial "strand displacement" or "asynchronous" model implicates that the unidirectional replication from O_H displaces the parental heavy strand and after approximately two-thirds of the molecule exposes the origin of light strand replication (O_L) on the displaced H-strand, so that lagging strand synthesis is initiated in the opposite direction (Kasamatsu and Vinograd, 1973). The model suggests that the lagging strand synthesis is continuous, without repeated priming (Larsson, 2010). The O_L resides in a 30 bp region within five tRNAs genes and is therefore called WANCY region (Figure 1.4). The segregation of daughter molecules occurs once H-strand synthesis is finished, leaving one daughter cell intermediate with an incompletely synthesized L-strand (Robberson et al., 1972).

On the contrary, the "strand coupled" model suggests that H- and L- strands are replicated simultaneously, initiated near the O_H in the D-loop region (Holt et al., 2000; Yang et al., 2002). Discovery of multiple RNA:DNA hybrids, representing ribonucleotide incorporation on the replicating lagging strand, gave rise to the RITOLS (ribonucleotide incorporation throughout the lagging strand) replication mode, suggesting that the lagging strand is initially incorporated RNA, before subsequent conversion to DNA (Yang et al., 2002; Yasukawa et al., 2006). Data for this model was gained from employing neutral/neutral two-dimensional agarose gel electrophoresis (2D AGE), allowing for DNA

molecules to be distinguished according to size and strand configuration. Later, 2D AGE was used to reexamine the "asynchronous" model, suggesting that the RNA:DNA represent alternative replication origins for L-strand synthesis and that the "strand displacement" is the predominant mode of mtDNA replication (Brown et al., 2005). Overall it is not fully clear which is the mtDNA replication mode in mammals, possibly a combination of the two described or even more. Furthermore, little is known about the termination mechanism beyond its location or the precise relationship between replication and transcription. Eventually, discovery of new enzymes is expected to shed light onto mtDNA replication mechanics (Holt and Reyes, 2012).

However, so far investigation of mtDNA replication mechanisms was largely conducted in mammals. A recently published study in *C. elegans* suggests that in nematodes the replicating mtDNA appears as a lasso like structure with concatemeric (containing multiple DNA copies) tails, which are predicted to be eventually resolved in monomeric circles (Lewis et al., 2015). This replication mechanism is similar to the one from phages and given that POLγ and TWINKLE are believed to be derived from bacteriophages, among metazoans *C. elegans* may represent the ancestral mtDNA replication mode (for review, see (Shutt and Gray, 2006)).

1.2 Aging

"How old would you be if you didn't know how old you are?"
- Leroy Robert "Satchel" Paige, baseball player, July 9, 1948

"Aging" and "senescence" are terms universally used to describe what happens to an organism in the course of time. However, there is still no uniform scientific definition of "aging" and no consensus which components the definition should consider and which it should exclude. One of the phrasings describes aging as "deteriorative changes with time during post maturational life that underlie an increasing vulnerability to challenges, thereby decreasing the ability of the organism to survive" (Masoro, 1997). Though aging is broadly perceived as a function of time, time itself only imperfectly correlates with the physiological processes involved in aging and therefore should become an

independent parameter in biogerontology (Arking and Dudas, 1989). This seems reasonable, when for example two individuals from the same species and of the same chronological age reveal two different physiological states. Instead, three main conditions were suggested to characterize aging: they must be (i) deleterious, (ii) progressive and (iii) intrinsic (Strehler, 1982). There are only two basic ways in which aging can occur: according to a predetermined genetically driven program or due to random, accidental events (Hayflick, 2007). This duality gave rise to two major sets of aging theories: stochastic and systemic ones.

Stochastic theories describe aging in the light of thermodynamics. Condensed energy tends to disperse and to reach the state of energetic equilibrium within the system. Living organisms are constantly but randomly attacked by a variety of degenerative events and processes increasing entropy that may negatively affect functionality of biological molecules. This can be antagonized by cellular internal mechanisms and repair systems. Therefore, the aging process occurs because the energetic level of molecules shifts towards inactive or malfunctioning, and internal repair systems fail to counteract energy dispersal any more (Hayflick, 2007). Various theories suggest different explanations for aging, like increased somatic DNA mutations, telomere loss or accumulation of damaged proteins, just to name a few (for review, see (Kirkwood, 2005)).

However, systemic theories describe aging as a genetically programmed process. Whereas the theories vary in the number of specific genes and mechanisms driving aging, they all have in common the genetic basis of senescence. Nevertheless, they are not entirely deterministic as environmental impact is considered to some extent (Arking, 1998). It also becomes more obvious that organisms are programmed to survive rather than to die (Kirkwood, 2005). Still, multiple genetic interventions were shown to modulate lifespan of different organisms: inhibition of insulin/insulin-like growth factor 1 (IGF1), target of rapamycin (TOR) signaling and modest inhibition of respiration all increased lifespan in worms, flies and mice (for review, see (Kenyon, 2010)). Though these interventions prolong lifespan, they do not give an answer to the question of what causes aging. Deciphering mechanisms responsible for lifespan determination rather contribute to our understanding of longevity that is not equal to aging, as length of life describes its endpoint rather than the age-associated changes (Hayflick, 2007).

1.2.1 Mitochondrial Free Radical Theory of Aging

Almost 60 years ago Denham Harman proposed the free radical theory of aging, suggesting that aging, as well as the associated degenerative diseases, could be attributed to the deleterious effects of reactive oxygen species (ROS) on various cell compartments (Harman, 1956). As ROS are mainly produced just off the mitochondrial electron transport chain, mitochondria were later declared as a prime target for oxidative damage (Harman, 1972). Hence the free radical theory evolved into mitochondrial free radical theory of aging (MFRTA).

In the course of time, substantial evidence has emerged from various studies to lend support to this theory. It has been shown that mitochondria become larger and less numerous with age, accumulating vacuoles and cristae abnormalities (Frenzel and Feimann, 1984). In aging humans the oxidative phosphorylation capacity has been reported to decline in skeletal muscle, liver, heart and brain (Cottrell and Turnbull, 2000). Potential differences in enzymatic activities of single ETC complexes could negatively affect oxidative phosphorylation and hence promote ROS production (Kwong and Sohal, 2000). Studies in flies, worms and mammals have shown that aging is associated with increased amounts of mtDNA deletions and/or point mutations (Gadaleta et al., 1992; Lee et al., 1993; Melov et al., 1995; Piko et al., 1988; Yui et al., 2003). These mtDNA rearrangements appear to occur principally in post-mitotic tissues and their accumulation with age is a consistent feature of senescent multicellular animals (Melov et al., 1999).

More recent studies have shown that the accumulation of mitochondrial dysfunctions in aging tissues is not uniform and it is generally believed, that just a subset of cells accumulate mtDNA mutations over the threshold levels. For example the number of COX deficient cells progressively increases in skeletal and cardiac muscle and brain of elder individuals (Cottrell et al., 2001; Muller-Hocker, 1989, 1990). However, the relevance of mtDNA mutations in aging organisms is still controversial, as most of these data have been seen as a consequence rather than a driving force of aging.

1.2.2 The mtDNA mutator mouse

The first causative link between mtDNA point mutations and an

aging phenotype in mammals was provided by the mtDNA mutator mouse model, which expresses a proofreading deficient form of the nDNA-encoded mitochondrial DNA polymerase γ (Trifunovic et al., 2004). POLγ is uniquely responsible for both mtDNA replication and repair (Kaguni, 2004). Moreover, it has been shown that it is absolutely essential for mammalian embryogenesis (Hance et al., 2005). The proofreading deficiency was introduced to promote progressive, random accumulation of mtDNA mutations during the course of mitochondrial biogenesis and such homozygous knock in mice developed a mtDNA mutator phenotype with a three to fivefold increase in the levels of point mutations. The mtDNA mutator mice developed normally from birth to early adolescence but subsequently acquired some features of premature aging such as weight loss, reduced subcutaneous fat, alopecia, kyphosis, osteoporosis, anemia with progressive decrease in circulating red blood cells, reduced fertility, cardiomyopathy and sarcopenia (Trifunovic et al., 2004). In contrast to the mitochondrial theory of aging, mutator mice did not reveal increased ROS production or increased oxidative stress (Trifunovic et al., 2005). According to the "Vicious cycle" theory, mtDNA mutations accumulate exponentially during life, leading to impaired oxidative phosphorylation activity (Bandy and Davison, 1990). However mutator mice accumulated mutations proportionally and their load was already substantial very early in embryonic life, most likely due to extensive mtDNA replication (Trifunovic et al., 2005). The linear fashion of mtDNA mutation accumulation suggests no involvement of a vicious cycle as proposed by the mitochondrial theory of aging. It seems that the onset of premature ageing is not accompanied by a large *de novo* accumulation of mtDNA mutations, but is rather due to cumulative physiological damage caused by the high mutation load during adult life, and/or to segregation or clonal expansion of specific mutations. Still, it is possible that the mutation load might be an underestimate because cells with the highest levels of deleterious mutations may be lost due to cell death and/or replicative disadvantage.

1.3 Regulation of mitochondrial biogenesis in mammals

Mitochondrial biogenesis represents complex physiological process that implicates growth of pre-existing mitochondria, requiring synthesis and for the vast majority also import of mitochondrial proteins into the organelle, delivery of lipids to

mitochondrial membranes and mtDNA replication. Notably, mitochondrial mass, organization and function vary between different cell types and are dynamically adapted to environmental and intracellular stimuli. While mitochondrial biogenesis serves as long-term adaptation, transient energetic or metabolic requirements can be met by adjusted expression of a corresponding subset of nDNA-encoded mitochondrial genes (Hock and Kralli, 2009). As the nuclear genome harbors most of the genes with regard to mitochondrial function, nuclear factors are designated to orchestrate their expression. In 1989, analysis of mammalian cytochrome *c* promoter revealed specific nuclear factor binding sites. The corresponding nuclear respiratory factor 1 (NRF-1) was proposed to control expression of nDNA-encoded mitochondrial genes in addition to housekeeping transcription factors (Evans and Scarpulla, 1989). Ever since further genes and factors were identified to be involved in regulation of mitochondrial biogenesis, some of which are presented below (for review, see (Kelly and Scarpulla, 2004; Scarpulla, 2008)).

1.3.1 Nuclear transcription factors

NRF-1/2: Palindromic binding sites of NRF-1 are most frequently found in proximal promoters of ubiquitously expressed genes, however presence of regulatory elements does not necessarily means biological relevance (FitzGerald et al., 2004). Human NRF-1 was found to occupy promoter elements of 691 genes, many of which are mitochondria-related, representing OXPHOS enzymes, mitochondrial translation, transcription and import machineries (Cam et al., 2004). More recent analysis employing Chromatin ImmunoPrecipitation followed by deep sequencing (ChIP-seq) identified 2.470 NRF-1 target genes in human neuroblastoma cells, underlying its central role in regulation of mitochondrial biogenesis, but also extra-mitochondrial processes such as cell cycle progression, DNA damage repair or RNA metabolism (Satoh et al., 2013). NRF-1 exerts its positive regulatory activity on transcription as a homodimer, with multiple phosphorylatable serine residues, which enhance its binding and *trans*-activation functions (Gugneja and Scarpulla, 1997). Phosphorylation of NRF-1 is induced in quiescent fibroblasts upon serum exposure and in hepatoma cells upon exposure to exogenous oxidants (Herzig et al., 2000; Piantadosi and Suliman, 2006).

Human NRF-2 (homolog of murine GA-binding protein (GABP)) acts as heterodimer consisting of two α and two β subunits. Initially it was shown to bind essential elements in the gene encoding the cytochrome oxidase subunit IV (COXIV). Later NRF-2, along with NRF-1, was proposed to regulate all 10 nDNA-encoded COX subunits (Ongwijitwat and Wong-Riley, 2005). Neuronal stimulation by depolarization is suggested to induce NRF-2 translocation from the cytoplasm to the nucleus, thereby facilitating its transcriptional activity (Yang et al., 2004; Zhang and Wong-Riley, 2000). Apart from regulation of *COX* genes, NRF-2 binding elements were discovered in *cis*-regulatory elements of other nDNA-encoded mitochondrial genes, e.g. complex II subunits and mtDNA transcription machinery. Whereas both, NRF-1 and NRF-2 binding sites, can frequently be found in the proximal promoters of mitochondrial genes, some genes reveal either NRF-1 or NRF-2, which can also differ between rodents and humans (for review, see (Kelly and Scarpulla, 2004)). Together NRF-1 and NRF-2 directly control expression of nDNA-encoded OXPHOS subunits and genes involved in mitochondrial transcription, offering a mechanism for bigenomic transcriptional control (Figure 1.5).

PPARα/β (or δ)/γ: Peroxisome-proliferator activated receptor α (PPARα) was the first nuclear receptor, shown to regulate mitochondrial metabolism. Originally implicated in peroxisomal fatty acid oxidation (FAO), now PPARs are known to transcriptionally control mitochondrial FAO enzymes (for review, see (Madrazo and Kelly, 2008)). FAO enzymes act within the mitochondrial matrix, oxidizing fatty acids to Acetyl Coenzyme A (Acetyl-CoA), which is fed into the TCA cycle, generating energy-rich intermediates, which provide electrons for the ETC. PPARα is predominantly enriched in tissues with high FAO capacity such as heart, liver or brown adipose tissue. The PPAR family is completed by ubiquitously expressed PPARβ (also known as δ) also participating in FAO and adipose-enriched PPARγ that directs programs involved in adipocyte differentiation and fat storage (Figure 1.5). PPARs reveal different but overlapping spatial and temporal expression patterns. Upon binding their ligand, PPARs form heterodimers with retinoid X receptor (RXR) and occupy cognate DNA response elements. Next to ligand-mediated activation, PPARs are activated by endogenously produced ligands regulated by transcriptional coactivators and corepressors.

ERRα/β/γ: The second family of nuclear receptors consists of estrogen related receptors (ERR). Structural characteristics have determined their

nomenclature, however classic estrogens do not activate them. Similar to PPARs, high levels of ERRα were initially shown to activate expression of FAO enzymes in oxidative tissues like heart, kidney and brown fat (Sladek et al., 1997; Vega and Kelly, 1997). Later, studies on heart and skeletal muscle revealed that ERRs regulate the expression of genes involved in effectively all mitochondrial energy producing pathways (Huss et al., 2004) (Figure 1.5). As nonobligatory heterodimers ERRα and ERRγ occupy promoters of genes involved in TCA, FAO and OXPHOS, which additionally reveal NRF-1 and cAMP response element-binding protein (CREB) binding sites (Dufour et al., 2007).

CREB, SP1 and YY1: In addition to the NRF-1 binding site, cytochrome *c* gene revealed recognition sites for common transcriptional activators: specificity protein 1 (SP1) and CREB (Evans and Scarpulla, 1989). Phosphorylation of CREB determines its activity and is critical for its interaction with PRC and NRF-1 (Vercauteren et al., 2006). It appears that CREB function is required for rapid cytochrome *c* response to cyclic adenosine monophosphate (cAMP) and to serum-stimulated cell growth (Gopalakrishnan and Scarpulla, 1994; Herzig et al., 2000). However CREB binding sites do not represent common promoter elements of nDNA-encoded mitochondrial genes (Scarpulla, 2008) (Figure 1.5).

Next to cytochrome *c*, SP1 is involved in transcriptional regulation of cytochrome c_1 (subunit of complex III) and adenine nucleotide translocase 1 (ANT1), both lacking NRF binding sites (Li et al., 1996a; Li et al., 1996b). The number and organization of GC box binding sites for SP1 determine its contribution to transcriptional activity, which can also be repressing (Zaid et al., 1999). More recent studies in mice show Sp1 family members mediated transcriptional regulation of all thirteen genes encoding cytochrome *c* oxidase subunits (Dhar et al., 2013; Johar et al., 2013). Similar to NRFs, transcriptional regulation of the three mtDNA-encoded *COX* genes is indirect via expression of control genes involved in mtDNA transcription.

The ubiquitous Ying Yang 1 (YY1) transcription factor was initially reported to be involved in regulation of mammalian COX gene expression (Figure 1.5). The promoter region of murine *Cox5b* contains three YY1 binding elements and appears to be suppressed by the presence of YY1 (Basu et al., 1997). However, the minimal promoter of bovine *COX7B* contains two YY1 binding elements, which are essential for promoter activity (Seelan and Grossman, 1997). Yet it remains to be deciphered, which mechanisms underlie

the dual function of YY1. Nevertheless, more recent studies not only show that YY1 binding motifs are enriched in a variety of mitochondrial genes regulated by rapamycin and PGC-1α, but also that YY1 function is required for mammalian TOR-mediated control of mitochondrial function (Cunningham et al., 2007). Moreover skeletal-muscle-specific YY1 knockout mice reveal decreased levels of nDNA-encoded mitochondrial gene transcripts resulting in bioenergetics deficiencies, accompanied by reduced OXPHOS protein levels and impaired respiratory activity (Blattler et al., 2012).

1.3.2 Nuclear coactivators

Over the years accumulating data suggests that a manageable number of transcription factors, including the aforementioned ones, directly regulate expression of nDNA-encoded mitochondrial genes. Moreover, they appear to indirectly regulate mtDNA expression and maintenance by activating transcription of nuclear genes composing respective machineries. Consequently the question arises, how activities of various transcription factors are coordinated to enable mitochondrial biogenesis regulation. The discovery of PGC-1 family of coactivators has offered one possible mechanism integrating physiological stimuli and orchestration of transcription activity in vertebrates.

Initially, the name giving interaction of PPARγ coactivator-1 α (PGC-1α) with PPARγ was observed in murine brown adipose tissue, pointing toward cold-induced PGC-1α activity as a requirement for adaptive thermogenesis (Puigserver et al., 1998). In this study PGC-1α was shown to induce expression of uncoupling protein-1 (*Ucp-1*), indicating promoted uncoupled respiration to generate heat. Soon after, PGC-1α was reported to coactivate NRF-1, NRF-2 and PPARα (Vega et al., 2000; Wu et al., 1999) (Figure 1.5). Based on the structural similarity to PGC-1α, identification of PGC-1β and PGC-related coactivator (PRC) has completed the PGC-1 family (Andersson and Scarpulla, 2001; Kressler et al., 2002; Lin et al., 2002). Generally, PGC-1 family members bind to the respective transcription factor, and are thereby recruited to the transcription site of the target gene. At the same time PGC-1 recruits histone acetyltransferases and the Mediator complex to enhance transcription initiation (Hock and Kralli, 2009). The critical feature of these coactivators is their high versatility in interacting with distinct transcription factors, including NRF-1/2, PPARs, ERRs and YY1 (Figure 1.4). This allows them to activate

different biological programs in a tissue specific manner (for review, see (Lin et al., 2005)). In mitochondria-rich tissues like heart, brown fat and muscles, expression of PGC-1α is strongly induced upon cold exposure and short-term exercise, resulting in increased expression of mitochondrial genes (Baar et al., 2002; Lehman et al., 2000; Wu et al., 1999). Importantly, mice lacking only PGC-1α or only PGC-1β are viable, showing modest decrease in mitochondrial gene expression (Lelliott et al., 2006; Lin et al., 2004). However PGC-1αβ double knockout animals die shortly after birth, with severe cardiac dysfunction and abnormalities in brown adipose tissue, suggesting at least partial functional redundancy between PGC-1α and PGC-1β (Lai et al., 2008).

This level of mitochondrial-nuclear communication integrates distinct physiological stimuli like cold exposure, caloric intake or exercise, which stimulate mitochondrial biogenesis through PGC-1 activity. Transcriptional and posttranscriptional control mechanisms of PGC-1α add further plasticity to the biogenic response (for review, see (Ryan and Hoogenraad, 2007)). External temperature decrease can be sensed by β-adrenergic receptors, which transduce the signal via cAMP pathway, eventually leading to transcriptional activation of PGC-1α (Puigserver et al., 1998). Another case of mitochondrial biogenesis upregulation can be observed in long-term exercising mice. Muscular energy deprivation is sensed by AMP activated kinase (AMPK) which activates calcium/calmodulin-dependent protein kinase IV (CaMK IV), eventually enhancing *Pgc-1α* expression (Schaeffer et al., 2004; Zong et al., 2002). On the posttranscriptional level, for example, mitogen-activated protein kinases (MAPK) transmit extracellular signals, such as chemical and physical stress, into intracellular responses (for review, see (Cargnello and Roux, 2011)). One member of MAPK family, p38 MAPK, has been shown to stabilize PGC-1α by phosphorylation, thereby extending its half-life from ~2 to ~6 hours (Knutti et al., 2001; Puigserver et al., 2001). In response to cytokines, p38 MAPK activated PGC-1α targeted nDNA-encoded mitochondrial gene expression in muscle cells (Puigserver et al., 2001). Another example is the lysine deacetylase sirtuin 1 (SIRT-1) that is induced in response to fasting signals and consequently acetylates PGC-1α (Rodgers et al., 2005). Notably, in contrast to p30 MAPK, SIRT-1 regulates PGC-1α activity targeting genes with gluconeogenic but not mitochondrial function. The mentioned examples by far do not cover all facets of how PGC-1 family members are regulated on genetic and protein levels.

However, variety and tissue specificity of transcription factors and cofactors emphasize highly versatile modes of mitochondrial biogenesis regulation in mammals.

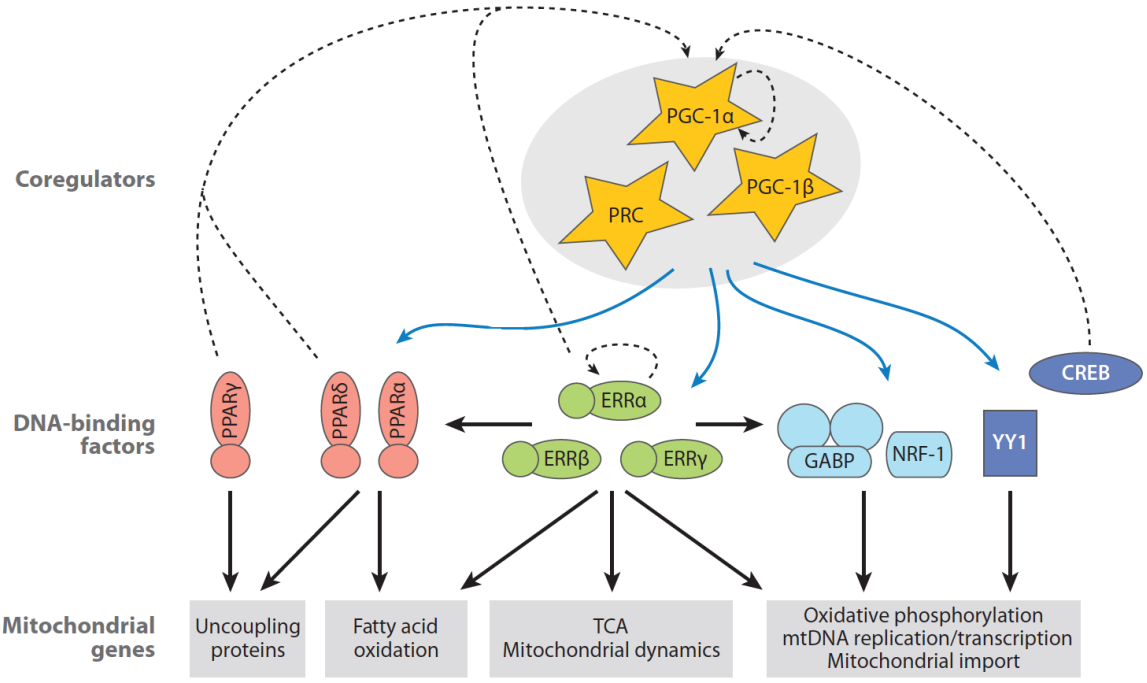

Figure 1.5 Mitochondrial biogenesis regulatory framework in mammals
Nuclear DNA-binding factors regulate transcription of distinct but overlapping sets of mitochondrial genes (thick black lines). Their activity is orchestrated and fine-tuned by coregulators (blue lines). Additionally, there is interregulation among the transcription factors (horizontal black lines). Feed-forward and feedback loops (dashed lines) further activate the system. Specificity is achieved by tissue and trigger signal specificity (not indicated). PGC-1, peroxisome proliferator-activated receptor coactivator-1; PRC, PGC-1 related coactivator; PPAR, peroxisome proliferator-activated receptor; ERR, estrogen related receptor; GABP (NRF-2), GA-binding protein; NRF-1, nuclear respiratory factor; YY1, Ying Yang 1; CREB, cAMP response element-binding protein. Illustration adapted from (Hock and Kralli, 2009).

1.4 Retrograde response

Another mechanism of mitochondrial-nuclear communication suggests adaptations in nuclear transcription in response to changes in mitochondrial functional state. Such changes in mitochondria can be caused by the loss of mtDNA, oxidative stress or protein aggregation. The mtDNA mutator mouse model has demonstrated that accumulation of mtDNA mutations can lead to a premature aging phenotype with reduced respiratory capacity. While proof-reading-deficient polymerase predominantly introduced a plethora of random point mutations into mtDNA, there is a number of

mitochondrial diseases characterized by defects in one single mitochondrial protein-coding gene (for review, see (Schapira, 2012)). But also "naturally" aged tissues reveal increased load of mtDNA mutations and decline in the mitochondrial respiratory enzyme activities (Cortopassi et al., 1992; Lezza et al., 1994). Initially mitochondrial diseases were classified as disorders caused by a dysfunction of the mitochondrial respiratory chain originating from sequence changes in either mitochondrial or nuclear DNA. However, malfunction of other mitochondrial proteins not directly involved in oxidative phosphorylation are associated with further human pathologies. Mitochondrial metabolic and structural anomalies are observed in Alzheimer's and Parkinson's diseases, although it is not clear whether mitochondrial defect is causative (for review, see (Perier and Vila, 2012; Santos et al., 2010)). But also less drastic interventions like food deprivation or temperature shift affect mitochondrial functionality (Mollica et al., 2005; Schulz et al., 2007). While some of the mentioned examples could transiently enhance or reduce mitochondrial activity, others potentially deteriorate mitochondrial function causing sustainable and eventually malicious proteostatic and metabolic changes.

However caused, antagonizing mitochondrial dysfunction is vital for cellular and consequently organismal survival. As the vast majority of mitochondrial proteins are encoded in nuclear DNA, it is consequential to assume that there are mechanisms for communication between mitochondria and the nucleus in order to adapt expression of nuclear genes in response to changes in mitochondrial functional state but also to ensure similar synthesis rate of respiratory chain subunits. The directed signaling from mitochondria to the nucleus is termed "mitochondrial retrograde signaling" or more broadly mitochondrial stress responses (MSR), and it modulates cellular activities under both normal and pathophysiological conditions (Butow and Avadhani, 2004). While such retrograde signaling is conserved from yeast to mammals, the molecular mechanisms governing the communication do differ between species, most likely reflecting their specific metabolic and environmental peculiarities (Liu and Butow, 2006).

1.4.1 … in yeast

First studies suggesting existence of mitochondrial-nuclear communication reach back to 1987, when defects inducing the expression of nuclear genes

were observed in mtDNA (Parikh et al., 1987). Since then, retrograde signaling in the budding yeast, *Saccharomyces cerevisiae*, was intensively studied and core regulatory elements were identified.

The major physiological role of the RTG pathway is to assure glutamate availability. Glutamate represents 85% of total cellular nitrogen content and is the essential source for amino nitrogen used in biosynthetic reactions like amino acid synthesis (Magasanik and Kaiser, 2002). As mentioned earlier, succinate dehydrogenase fulfills dual function, feeding electrons into the electron transport chain and converting succinate to fumarate within the scope of the TCA cycle (Figure 1.2). Thus in respiratory-deficient cells that are devoid of mtDNA (ρ^0), the TCA cycle fails to operate in full, thereby reducing the production of α-ketoglutarate (α-KG), which when transaminated gives rise to glutamate. Two groups of *RTG*-target genes play a role in the maintenance of glutamate supply in cells with compromised mitochondrial function (Figure 1.6 A). The most prominent and best-studied member of the first group is the peroxisomal isoform of citrate synthase (*CIT2*). Displaying increased expression in ρ^0 cells, *CIT2* generates citrate within the glyoxylate cycle, which can be fed into the mitochondrial TCA cycle (Liu and Butow, 2006). The second group contains genes with unchanged expression, encoding the first three reactions of the TCA cycle, namely mitochondrial citrate synthase (*CIT1*), aconitase (*ACO1*) and isocitrate dehydrogenases (*IDH1/2*). In cells with stable mitochondrial function expression of TCA cycle genes is under the control of Hap transcription factors, while in respiratory deficient cells the control is dominated by *RTG*-genes (Liu and Butow, 1999). With the control of these two gene groups, the RTG pathway carries out metabolic adaptation to assure TCA cycle dependent α-KG supply, required for glutamate synthesis.

Next to its essential role for the TCA cycle functionality, *ACO1* expression is reported to be essential for mtDNA maintenance, as a packaging substitute of Abf2p (homolog of mammalian TFAM) (Chen et al., 2005). Thereby, ACO1 links retrograde signaling to mitochondrial biogenesis, independently of its TCA cycle function, as other mutations of Krebs cycle enzymes did not induce mtDNA loss to the same extent as *aco1Δ* (McCammon et al., 2003).

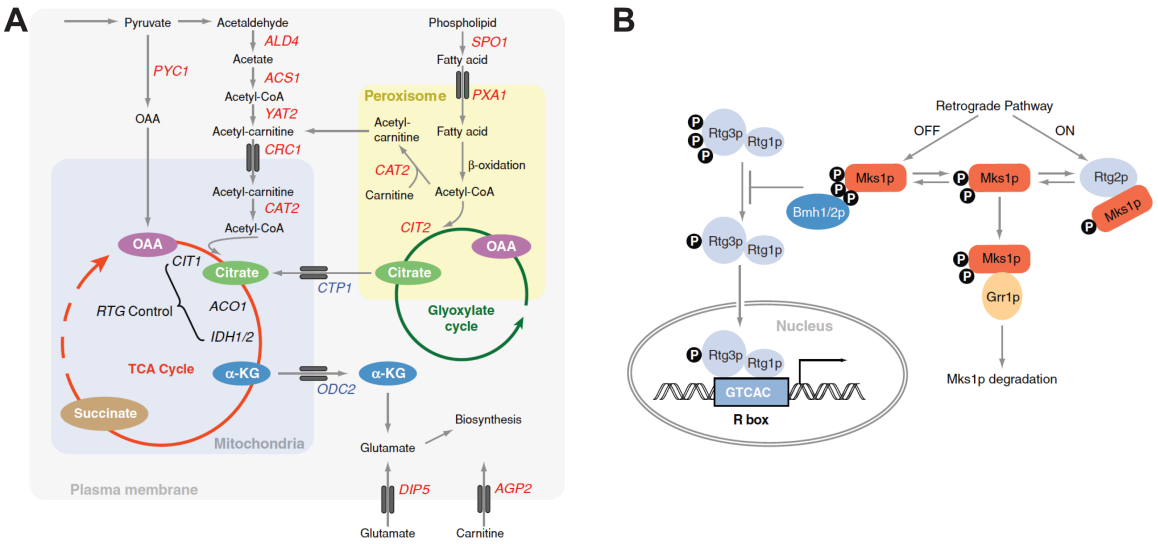

Figure 1.6 Metabolic events modulating retrograde response and the RTG pathway
(A) Respiratory deficiency as in ρ^0 cells disrupts TCA cycle, represented by the dashed line. Drop in α-KG levels induces expression of genes involved in anaplerotic pathways, indicated in red. These genes are involved in anaplerotic pathways, replenishing TCA cycle with metabolic intermediates. Blue genes indicate potential RTG pathway targets. **(B)** Key players of the RTG pathway. When active, Rtg2p binds Mks1p, reducing its inhibitory effect on the Rtg1/3p complex. Dephosphorylated Rtg3p enables the complex translocation to the nucleus, where it initiates transcription of genes with R-box containing promoters. Grr1p mediates free Mks1p ubiquitination. Bmh1/2p protects Mks1p from degradation when retrograde pathway is inactive. Illustrations from (Liu and Butow, 2006).

The RTG pathway consists of four positive, Rtg1p, Rtg2p, Rtg3p, Grr1p and four negative regulators, Mks1p, Lst8p, Bmh1p and Bmh2p (for review, see (Liu and Butow, 2006)). *RTG1* and *RTG3* encode transcription factors that form heterodimers to bind to the promoter region of target genes at the so-called R box (Figure 1.6 B). Out of these two, only Rtg3p is a phosphoprotein that can be phosphorylated at multiple sites. The inactive cytosolic Rtg1/3p complex is activated upon dephosphorylation of Rtg3p and consequently joint nuclear translocation (Sekito et al., 2000). The cytosolic Mks1p inhibits the RTG pathway by promoting Rtg3p phosphorylation. In turn, Rtg2p is indirectly responsible for Rtg3p dephosphorylation by reversibly binding Mks1p, thus diminishing its activity. The inhibitory effect of Mks1p can also be abrogated by Grr1p, which mediates ubiquitination of the former, leading to its degradation. Lst8p is an integral component of both TOR complexes. Data from genetic experiments suggest that it acts as a negative regulator of the RTG pathway at two sites, upstream of Rtg2p and between Rtg2p and Rtg1/3p (Liu et al., 2001). Lastly, two functionally redundant Bmh1p and Bmh2p proteins, which are highly conserved among eukaryotes, were suggested to negatively regulate the

RTG pathway by protecting Mks1p from ubiquitination or by binding Rtg3p, thereby keeping it in the cytosol.

Much insight was gained into relations between positive and negative regulators, and obviously Rtg2p and Mks1p interaction is central for RTG pathway activity. However, it is still not clear what signal determines their interaction. The designated task of the RTG system is to maintain α-KG supply for glutamate synthesis, which in turn potently suppresses the RTG pathway (Liu and Butow, 1999). On the other hand, reduced α-KG levels in damaged mitochondria result in elevated ammonium ion (NH_4^+) levels, suggesting a positive effect of accumulating ammonia on *RTG*-dependent gene expression (Tate and Cooper, 2003). Rtg2p also contains an N-terminal ATP binding domain that is necessary for Rtg2p-Mks1p interaction, whereas the interaction most likely happens via the C-terminal region (Liu and Butow, 2006; Liu et al., 2003). This raises the possibility of ATP dependent regulation of Rtg2p activity (Liu and Butow, 2006).

1.4.2 … in mammals

The central players in the mediation of the RTG pathway, Rtg2p and Mks1p, were found in multiple fungal species. By contrast, in mammals, RTG related genes have not been identified thus far. However, several studies suggest calcium and ROS as mediators of mitochondrial stress (for review, see (Whelan and Zuckerbraun, 2013)).

Depletion of mtDNA in murine C2C12 skeletal myocytes causes loss of mitochondrial membrane potential and elevated concentration of free calcium in the cytoplasm (Biswas et al., 1999). Partial mtDNA depletion in human lung cancer cells revealed 3-fold increase in steady state cytosolic calcium, accompanied by induction of antiapoptotic genes, tumor-specific markers and calcium pathways (Amuthan et al., 2002; Amuthan et al., 2001). Rising calcium levels are sensed by CaMK IV, which was shown to activate CREB, involved in cytochrome *c* expression, and induce *Pgc-1α* expression (Gopalakrishnan and Scarpulla, 1994; Wu et al., 2002). Yet, it remains to be determined whether observed calcium concentrations and proposed pathways are physiologically relevant.

Next to disturbed calcium homeostasis, mitochondrial stress can lead to excessive ROS production. This in turn can be sensed by nuclear factor erythroid

2-like 2 (NFE2L2, previously known as NRF2, hence frequently confused with NRF-2). In non-stressed cells, ubiquitous NFE2L2 is constantly degraded in the cytoplasm by Keap1. In the presence of ROS, Keap1 undergoes conformational changes, thereby releasing NFE2L2, which subsequently translocates to the nucleus (Itoh et al., 1999). There, NFE2L2 binds antioxidant response elements (ARE), thus inducing expression of cytoprotective genes involved in the antioxidant response, such as heme oxygenase 1 and glutathione *S*-transferases (Hayes et al., 2000) (Itoh et al., 1999). Notably, ARE have also been found in the promoter region of NRF-1, indicating a connection between antioxidant response and mitochondrial biogenesis (for review, see (Vomhof-Dekrey and Picklo, 2012)).

A specific branch of retrograde signaling is the mitochondrial unfolded protein response (UPR^{mt}). Mitochondria are equipped with a certain range of intramitochondrial proteases and molecular chaperones to facilitate degradation of *e.g.* damaged proteins, or folding of nascent peptides respectively (for review, see (Rugarli and Langer, 2012)). Accumulation of un-/misfolded proteins within the organelle leads to disturbed proteostasis and requires assistance from nDNA-encoded auxiliary proteins. Surveying and signaling such changes ideally leads to full restoration of mitochondrial function. The mammalian UPR^{mt} is poorly understood, however treatment with ethidium bromide, which depletes mtDNA, leads to increased transcript and protein levels of heat shock proteins 60/10 (Hsp60/10), two chaperonins involved in protein folding in the mitochondrial matrix along with heat shock 70kDa protein 9 (HSPA9 also known as mtHSP70) (Martinus et al., 1996; Ryan et al., 1997). Analysis of *Hsp60/10* promoters revealed the presence of CAAT/enhancer-binding protein (C/EBP) and C/EBP homologous protein (CHOP) binding sites, required for efficient transcription (Zhao et al., 2002a). A possible mechanism for integration of mitochondrial status is that accumulation of protein aggregates leads to elevated CHOP levels (Zhao et al., 2002a). However, in the human genome 3.899 nuclear genes contain CHOP-C/EBP binding elements, suggesting additional factors involved in UPR^{mt} activation and it is still enigmatic how the signal is transduced from mitochondria to the nucleus (Ryan and Hoogenraad, 2007).

1.4.3 … in *C. elegans*

Not much is known about nuclear factors modulating mitochondrial biogenesis in worms. The search for homologs of NRF-1/2, PGC-1 and *RTG*-genes in lower eukaryotes including nematodes has been unsuccessful so far (Knutti et al., 2000; Lin et al., 2005). Functional homologs of mammalian PPARα/β/γ and ERRα/β/γ have not been identified yet but might exist in worms, as 5 times more nuclear hormone receptors are present in nematodes than in humans (for review, see (Antebi, 2006)). However, it appears only natural that there are mechanisms regulating mitochondrial biogenesis, for the simple fact alone that a single *C. elegans* hermaphrodite produces around 300 eggs, all equipped with functional mitochondria. Moreover, the dynamic nature of this regulation is underlined by observations that mtDNA content of somatic cells increases in the course of development, but exposure to heat shock reduces the content of mitochondrial proteins (Bratic et al., 2009; Liang et al., 2014).

Though initial observations that mitochondrial stress specifically activates expression of mitochondria-directed chaperones were made in mammals (described in previous paragraph), more insight into the mechanism of the UPR^{mt} was gained from studies on *C. elegans* (Figure 1.7 A). Expression of two mitochondrial chaperones HSP-6 and HSP-60, mammalian mtHSP70 and HSP60 respectively, is activated in response to mitochondria-specific stress, which is not inducing endoplasmatic reticulum or cytosolic stress responses (Yoneda et al., 2004). This kind of stress can be generated by ethidium bromide treatment or disturbing protein folding environment via inhibition of enzymes involved in mitochondrial protein homeostasis, such as metalloprotease SPG-7 (homolog of human paraplegin, part of m-AAA protease). The foundation stone to the understanding of UPR^{mt} regulation was laid, when RNAi screens for factors mediating UPR^{mt} identified ubiquitin-like protein UBL-5 and homeobox transcription protein DVE-1, shown to bind the *hsp-60* promoter (Benedetti et al., 2006; Haynes et al., 2007). Proteolytic activity of the mitochondrial protease CLPP-1 was designated as an important step in the stress signal generation, which leads to DVE-1 and UBL-5 activation (Haynes et al., 2007). Major progress in deciphering signal transduction from mitochondria to the nucleus was made by the identification of the bZip activating transcription factor associated with stress-1 (ATFS-1) (Haynes et al., 2010). It possesses a nuclear localization signal (NLS) and

N-terminal mitochondrial targeting sequence (MTS) (Nargund et al., 2012). In unstressed worms, ATFS-1 is constantly imported into mitochondria, where it is degraded most likely by the Lon mitochondrial protease (encoded by *C34B2.6*). Under mitochondrial stress ATFS-1 import into mitochondria is attenuated and it partially translocates to the nucleus, where it activates expression of 391 genes, binding directly to 70 of the respective gene promoters, including mitochondrial chaperones and transporters but also genes involved in ROS detoxification and glycolysis (Nargund et al., 2015; Nargund et al., 2012). Import of ATFS-1 into the mitochondria is attenuated by the activity of the mitochondrial inner-membrane localized ABC transporter, HAF-1, which is proposed to contribute to the export of unfolded proteins, previously cleaved by CLPP-1 protease (Haynes et al., 2010). However, HAF-1 most likely has modulating effects on the UPR^{mt}, as some mitochondrial stresses like *spg-7* knockdown do not require HAF-1 activity for response induction (Nargund et al., 2012). Notably, a truncated version of ATFS-1, lacking MTS, robustly and constitutively induces *hsp-60* expression (Nargund et al., 2012). A recent study suggests that during mitochondrial stress ATFS-1 not only induces transcription of aforementioned genes, but also coordinates bigenomic expression of OXPHOS genes (Nargund et al., 2015). ChIP-seq analysis revealed that ATFS-1 binds not only promoters of nuclear genes but also the non-coding region of mtDNA. While ATFS-1 enhanced transcription of mitochondrial protective genes and genes involved in OXPHOS assembly, expression of mtDNA- and nDNA-encoded OXPHOS genes was reduced in an ATFS-1 dependent manner (Nargund et al., 2015). The mechanism by which ATFS-1 reduces mtDNA transcripts is unclear, however identification of a second, shorter isoform of ATFS-1 was shown to be upregulated during mitochondrial stress and proposed to be responsible for reduced OXPHOS transcripts (Nargund et al., 2015). Hence, ATFS-1 is suggested to simultaneously promote mitochondrial proteostasis and to match OXPHOS complexes generation to the current mitochondrial functional state (Nargund et al., 2015) (Figure 1.7 B).

Additionally, in a pathway complementary to ATFS-1 mediated UPR^{mt}, mitochondrial stress attenuates cytosolic translation (Figure 1.7 A). ROS production, presumably resulting from impaired respiration, is sensed by the cytosolic general control non-derepressible-2 kinase (GCN-2), which phosphorylates eukaryotic translation initiation factor 2 α, eIF2α, thereby diminishing cytosolic translation initiation (Baker et al., 2012).

Thereby, attenuation of cytosolic translation may be seen as auxiliary mechanism to protect stressed mitochondria from the additional burden of imported proteins.

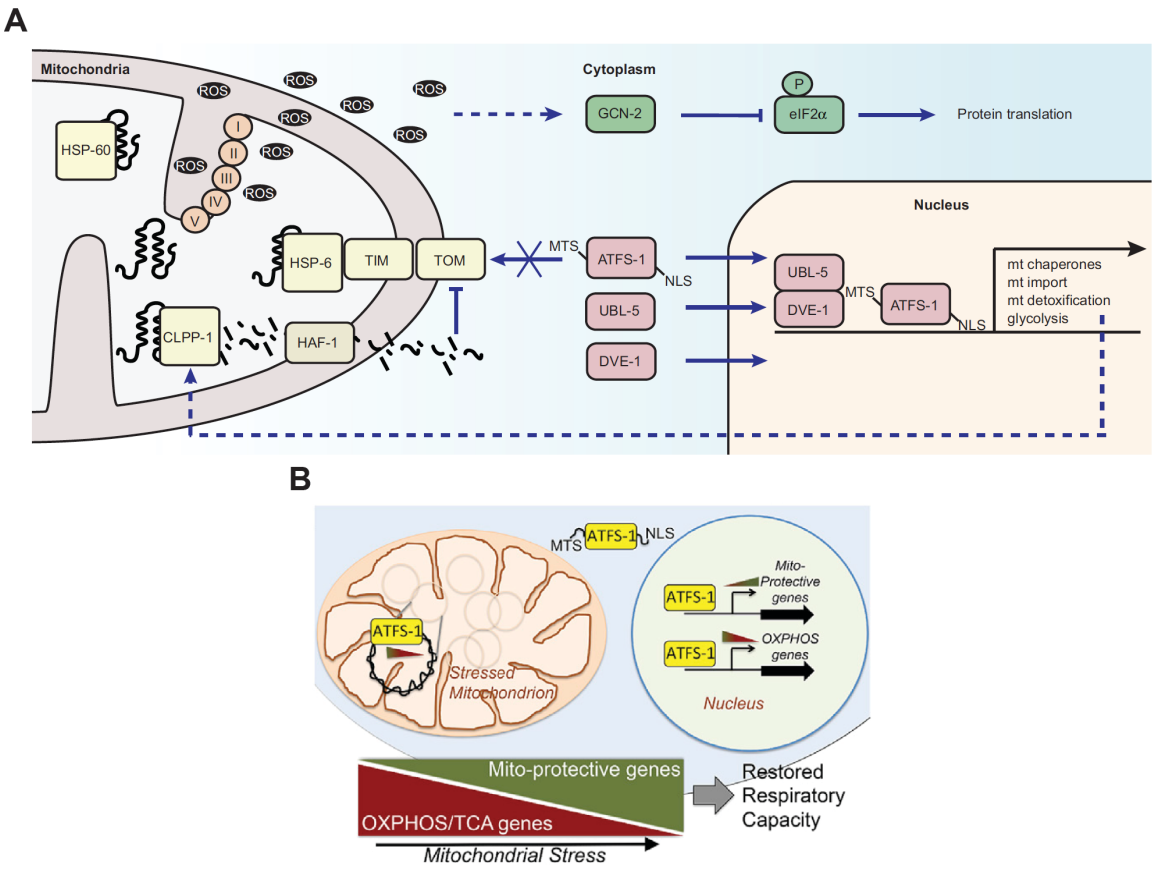

Figure 1.7 Mitochondrial stress signaling in *C. elegans*
(A) Molecular chaperones HSP-6 and HSP-60 ensure protein folding within mitochondria. Accumulating un-/misfolded proteins are cleaved by CLPP-1 protease and exported into the cytoplasm by HAF-1 transporter, where they attenuate mitochondrial import of ATFS-1. Thus ATFS-1, which possesses MTS and NLS, translocates to the nucleus where it, most likely in a complex with ubiquitin-like protein UBL-5 and homeobox transcription protein DVE-1, initiates transcription of mitochondrial protective genes, which restore mitochondrial environment and function. In a complementary pathway increased ROS production induces GCN-2 mediated eIF2α phosphorylation, thereby inhibiting cytosolic translation, consequently reducing protein-folding burden in mitochondria. Illustration from (Jovaisaite et al., 2014). **(B)** During stress cytosolic ATFS-1 translocates to the nucleus where it induces transcription of OXPHOS assembly and mitochondrial protective genes. At the same time ATFS-1 has inhibitory effect on the expression of nuclear- and mtDNA-encoded OXPHOS genes. In this way ATFS-1 adapts OXPHOS complexes biogenesis to the disturbed mitochondrial environment until full organelle recovery. HSP, heat shock protein; ATFS-1, activating transcription factor associated with stress-1; MTS, mitochondrial targeting sequence; NLS, nuclear localization signal; GCN-2, general control non-derepressible-2 kinase; TIM, translocase of inner membrane; TOM, translocase of outer membrane; ROS, reactive oxygen species; eIF2α, eukaryotic translation initiation factor 2 α. Illustration from (Nargund et al., 2015)

1.5 Objectives

Mitochondrial biogenesis is a complex process, which implicates coordinated expression of different subsets of genes defining mitochondrial functionality. Given that genes with mitochondrial function are distributed between two genomes, orchestration mechanisms are required to ensure efficient expression and assembly of OXPHOS complexes. Further, the rate of mitochondria-related gene expression integrates internal and external, chemical and physical stimuli, allowing cellular adaptation to current nutrient availability and energy requirements, increasing organismal chances of survival. Eventually, mitochondrial dysfunction becomes prominent during aging and is also associated with various types of diseases (for review, see (Nunnari and Suomalainen, 2012; Schapira, 2012; Terman and Brunk, 2004)).

Various mammalian nuclear transcription factors and coactivators were identified as pivotal regulators of mitochondria-related gene expression. By contrast, to date, no homologs of these factors were found in *C. elegans* (Knutti et al., 2001; Lin et al., 2005). This raises the possibility of an alternative or as of yet neglected meshwork of factors determining mitochondrial biogenesis in worms. Deciphering regulatory mechanisms in *C. elegans* may contribute to further understanding of mitochondrial biology in mammals. Thus, we designed a reporter strain to monitor mitochondrial replication that is associated with the dynamics of mitochondrial biogenesis (Schultz et al., 1998). Using this model, we conducted large-scale RNA interference (RNAi) screens allowing us to identify factors potentially involved in mitochondrial biogenesis and characterized selected candidates with regard to their role in various aspects of mitochondrial functions.

2 Materials and Methods

2.1 Chemicals and biological materials

Size markers for agarose gel electrophoresis (Gene Ruler DNA Ladder Mix) and for SDS-PAGE (Page Ruler Prestained Protein Ladder Mix) were obtained from. Chemicals and material used in this work are listed in (Table 2.1). Solutions were prepared with double distilled water. StrataPrep Plasmid Miniprep Kit (Agilent Technologies, USA) was used for plasmid isolation from bacteria and NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, Germany) was used for DNA extraction from agarose gel.

Chemical/Enzyme	Supplier
2-methyl-2-butanol	Sigma Aldrich, USA
agar	VWR, USA
agarose ultra pure	Thermo Fisher
ammoniumpersulfat (APS)	Sigma Aldrich, USA
ampicillin	Sigma Aldrich, USA
bovine serum albumin (BSA)	Sigma Aldrich, USA
bromophenol blue	Merck, Germany
calcium chloride dihydrate	VWR, USA
chloroform	Merck, Germany
cholesterol	Sigma Aldrich, USA
deoxynucleotides (dNTPs)	Sigma Aldrich, USA
dimethylsulfoxide (DMSO)	Merck, Germany
disodium phosphate	Merck, Germany
dithiothreitol (DTT)	Sigma Aldrich, USA
enhanced chemiluminescence (ECL)	PanReac AppliChem, Germany
ethanol, absolute	PanReac AppliChem, Germany
ethidium bromide	Sigma Aldrich, USA
ethylendiamine tetraacetate (EDTA)	PanReac AppliChem, Germany
glycerol	Sigma Aldrich, USA
glycine	PanReac AppliChem, Germany
glycogen	Roche, Switzerland
hydrochloric acid (37%)	VWR, USA
IPTG	PanReac AppliChem, Germany
isopropanol (2-propanol)	PanReac AppliChem, Germany
magnesium chloride	Merck, Germany
magnesium chloride hexahydrate	Sigma Aldrich, USA

Chemical/Enzyme	Supplier
magnesium sulfate	Merck, Germany
methanol	PanReac AppliChem, Germany
nitrogen (liquid)	Linde, Germany
nonfat dried milk powder	PanReac AppliChem, Germany
nonidet P40 (NP-40)	Sigma Aldrich, USA
nystatin suspension	Sigma Aldrich, USA
phosphate buffered saline (PBS)	PanReac AppliChem, Germany
potassium chloride	PanReac AppliChem, Germany
potassium phosphate dibasic	VWR, USA
potassium phosphate monobasic	Sigma Aldrich, USA
protease Inhibitor Cocktail Tablets	Roche, Switzerland
proteinase K	PanReac AppliChem, Germany
Rotiphorese Gel 40 (37.5:1)	Roth, Germany
sodium azide	Sigma Aldrich, USA
sodium chloride	PanReac AppliChem, Germany
sodium dodecyl sulfate (SDS)	PanReac AppliChem, Germany
sodium hydroxide	PanReac AppliChem, Germany
sodium hypochlorite 14%	VWR, USA
tetracyclin hydrochloride	PanReac AppliChem, Germany
tetramethylethylenediamine (TEMED)	Sigma Aldrich, USA
trishydroxymethylaminomethane(Tris)	PanReac AppliChem, Germany
Tween 20	PanReac AppliChem, Germany
β-mercaptoethanol (BME)	PanReac AppliChem, Germany

Table 2.1 Chemicals and biological materials used in this study

2.2 Worm experiments

2.2.1 Maintenance of *C. elegans*

Standard techniques were used for growing and maintaining of *C. elegans* populations (Stiernagle, 2006). In brief, animals were kept on nematode growth medium (NGM) agar plates (0,25% bacto-peptone, 0,3 % NaCl, 1,7 % agar, 1mM $CaCl_2$, 1mM $MgSO_4$, 25mM KPI buffer pH 6, 5 µg/ml cholesterol, nystatin 25 units/ml) at 20°C in air permeable boxes and fed *Escherichia Coli* OP50 strain bacteria, unless otherwise indicated. In all experiments worms were used as synchronized populations.

2.2.2 Nematode strains

Strain	Genotype
N2, Bristol	*wild type*
ATR0011	*mtss-1$_{pr}$ ::gfp III*
ATR0031	*Ex[W04D2.4$_{pr}$ ::gfp]*
ATR0028	*Ex[myo-3$_{pr}$ ::W04D2.4::mcherry]*
ATR0025	*Ex[aco-2$_{pr}$ ::gfp]*
ATR0026	*Ex[cts-1$_{pr}$ ::gfp]*
ATR0027	*Ex[hmg-5$_{pr}$ ::gfp]*
ATR0018	*glp-4(bn2) I;mtss-1$_{pr}$ ::gfp III*
ATR0024	*gcn-2(ok871) II;mtss-1$_{pr}$ ::gfp III*
ATR0022	*ife-2(ok306) X;mtss-1$_{pr}$ ::gfp III*
MQ989	*isp-1(qm150)IV;ctb-1(qm189)*
SJ4005	*zcIs4 [hsp-4$_{pr}$ ::gfp V]*
SJ4100	*zcIs13 [hsp-6$_{pr}$ ::gfp V]*
SJ4058	*zcIs9 [hsp-60$_{pr}$ ::gfp V]*
SJ4143	*zcIs17 [ges-1$_{pr}$ ::gfpmt]*
SJ4103	*zcIs14 [myo-3$_{pr}$ ::gfpmt]*
ATR1010	*isp-1(qm150)IV;ctb-1(qm189);hsp-6$_{pr}$ ::gfp*
ATR1040	*isp-1(qm150)IV;ctb-1(qm189);gst-4$_{pr}$ ::gfp*
TB2601	*polg-1(ok1548)/+ II; +/mln [dpy-10(e128)mls14] II*

Table 2.2 List of strains with respective genotypes used in this study

2.2.3 Generating synchronized worm population

Gravid animals were washed off the plates and collected in 3.5 ml M9 buffer (20mM KH_2PO_4, 40 mM Na_2HPO_4, 80 mM NaCl, 1mM $MgSO_4$). A mix containing 0.5 ml of 5M NaOH and 0.356 ml Sodium Hypochlorite 14% (VWR, USA) was added to the worms. Shaking every 2 min, animals were incubated until dissolution of somatic tissues after ~10 min. Unharmed eggs were centrifuged for 30 sec at 1.200x*g* and washed with fresh M9. After repeating the centrifugation and washing cycles 4 times, eggs were either directly used for subsequent experiments or incubated on a shaker at 20°C over night in M9. Next day synchronized population of L1 staged animals were transferred to fresh NGM plates.

2.2.4 Biosorter

Measurement of GFP signal intensity in large populations of worms was performed using BioSorter®INTRUMENT and FlowPilot™ software (Version 1.5.5.4) (Union Biometrica). Synchronized worms were collected at day 1 of adulthood with M9 buffer and washed multiple times to get rid of bacteria. At least 100 worms per condition were used for analysis, determining

2.2.5 Crossing of *C. elegans* strains

In order to outcross a mutation or cross two different genotypes, crossings were set up by placing 2 L4 staged hermaphrodites of one of the strains together with 10 males of the other strain on the same NGM plate. If mating was successful (~50% occurrence of male progeny), L4 hermaphrodite progeny (F1 generation) were singled on new NGM plates and allowed to lay eggs. Then F1 generation animals were analyzed for desired genotype by single worm PCR and respective progeny was allowed to develop. Eventually F2 animals were singled again in order to lay eggs. After analyzing F2 animals, F3 worms of desired genotype were kept as working strain.

2.2.6 Lifespan assays

The RNAi effect was followed during entire lifespan. Synchronized populations were used for lifespan measurements. At least 100 animals were used per condition and scored every other day. Worms that died due to internally hatched eggs, vulva protrusion, desiccation or due to crawling out of the plate were censored. All lifespan assays were conducted at 20°C, unless otherwise indicated.

2.2.7 Oxygen consumption

Oxygen consumption rates were measured using the Oxygraph-2k high-resolution respirometry system (Oroboros Instruments GmbH, Austria). 300 animals of desired age were manually picked and transferred to non-seeded NGM plates from where they were

immediately recollected with M9 buffer. The worms were resuspended in 50µl M9 buffer and introduced into the Oxygraph chamber containing 2ml of M9 buffer, maintained at 20°/25°C. Oxygen consumption was measured for a minimum of 15 min. The slopes of the linear portions of the plots were used to calculate oxygen consumption rates. All measurements were performed alternating the chambers and at least 6 times. Data were analyzed using DatLab4 software (Version 4.3).

2.3 Molecular biology and biochemistry

2.3.1 RNAi

To inhibit specific gene function we used a standard feeding RNAi protocol (Kamath et al., 2001). In brief, either eggs (all screening experiments) or L1 stage larvae were placed on NGM-RNAi plates (NGM plates additionally containing 100 µg/ml ampicillin, 25 µg/ml tetracycline, 1mM IPTG) seeded with E. coli bacteria expressing double-stranded RNA of a specific gene. All RNAi clones were retrieved from the Ahringer RNAi library and checked before their use by sequencing. If the sequence was wrong or bacteria did not grow, respective clone was taken from the Vidal RNAi library. For RNAi screens 384-pin replicator was used to transfer bacteria from glycerol stock library into fresh LB-medium containing deep-well plates. Worms feeding on bacteria carrying the empty vector (L4440) were used as control. When performing double RNAi experiments *e.g.* A+B, for control condition single RNAi bacteria were diluted with L4440 vector carrying bacteria *e.g.* A+L4440 to ensure equal availability of dsRNA. After reaching day 1 of adulthood worms were scored, collected for further biochemical experiments or imaged.

2.3.2 Cloning

The *mtss-1$_{pr}$ ::gfp* reporter was constructed by amplifying ~1kb genomic region containing the putative promoter of *C. elegans mtss-1* using the primers Pmtss.SphI and Pmtss.AgeI and ligating the fragment into the *Sph*I–*Age*I sites of pPD95.75 plasmid to create Pmtss1.pPD95.75, subsequently injected into N2 animals to create *mtss-1$_{pr}$ ::gfp III* (ATR0011) strain. Construct was integrated using UV-irradiation as described (Evans,

2006). After successful integration the strain was outcrossed 4 times with N2, following GFP expressing animals. The *W04D2.4$_{pr}$ ::gfp* reporter was constructed by amplifying ~1kb genomic region containing the putative promoter of *C. elegans W04D2.4* using the primers PW04.HindIII and PW04.XbaI and ligating the fragment into the *Hind*III–*Xba*I sites of pPD95.75 plasmid to create PW04.pPD95.75, subsequently injected into N2 animals to create *Ex[W04D2.4$_{pr}$ ::gfp]* (ATR0031) strain. The *myo-3$_{pr}$ ::W04D2.4::mcherry* reporter was constructed by amplifying the *C. elegans W04D2.4* coding sequence using the primers W04.XbaI and W04.XbaI and ligating the fragment into the *Xba*I site of pCFJ104 plasmid to create W04.pCFJ104, subsequently injected into N2 animals to create *Ex[myo-3$_{pr}$ ::W04D2.4::mcherry]* (ATR0028) strain. The *hmg-5$_{pr}$ ::gfp* reporter was constructed by amplifying 300bp genomic region containing the putative promoter of *C. elegans hmg-5* using the primers Phmg5.SphI and Phmg5.AgeI and ligating the fragment into the *Sph*I–*Age*I sites of pPD95.75 plasmid to create Phmg.pPD95.75, subsequently injected into N2 animals to create *Ex[hmg-5$_{pr}$ ::gfp]* (ATR0027) strain. For all constructs *rol-6(su1006dm)*pRF4 was used as co-injection marker. Injection mix containing reporter construct and co-injection marker did not exceed 100ng/µl DNA concentration. ATR0025 and ATR0026 were generated as described (Yoneda et al., 2004). All DNA fragments were amplified using Pfu DNA polymerase (Promega, USA) using respective primers (Table 2.3) and sequenced by GATC Biotech, Germany. Digestion reactions were performed using restriction enzymes (New England Biolabs, USA) and ligations were performed using T4 DNA Ligase (Thermo Fisher, USA) following manufacturer's instructions. One Shot TOP10 chemically competent *E. coli* cells (Thermo Fisher, USA) were used for transformation. All other strains were purchased from Caenorhabditis Genetics Center (CGC) (Table 2.2).

Name	Genotype	Sequence 5'-3'	Application
mtss.SphI	*mtss-1*	GCGGCATGCTTTACAATAAGAAAGT	cloning
mtss.AgeI		AATACCGGTTTTCTATAATTTTGGTTTATCGA	
Phmg5.SphI	*hmg-5*	TGCGCATGCAGAAGAAGAAATATC	cloning
Phmg5.AgeI		AGTACCGGTCTTCGAAAATAAGAGTTTTAAG	
PW04.HindIII.fw	*W04D2.4*	TATAAAGCTTCGCTCGTAGAGTTCCTTCGA	cloning
PW04.XbaI.rev		ATCTGGCTCTAGATCGATATCATCAGTCAT	
W04.XbaI.fw	*W04D2.4*	TGACTTCTAGAATGACTGATGATATCGATGA	cloning
W04.XbaI.rev		TGACTTCTAGATAGCTCCAAATTCCAGTATT	

Table 2.3 Oligonucleotides used for cloning
The restriction site is indicated in the primer name.

2.3.3 Single worm lysis

For genotyping experiments single animals were collected in a PCR stripe, one worm per tube, in 2 µl of single worm lysis buffer (30 mM Tris pH 8, 8 mM EDTA, 100 mM NaCl, 0.7% NP40, 0.7% Tween 20, proteinase K 100 g/ml). Next, samples were put at -80°C and subsequently lysis program (65°C for 1h, 90°C for 15 min) was run in the Veriti® 96-well Thermal Cycler (Thermo Fisher, USA). Eventually 6 µl of double distilled water were added to each sample (PanReac AppliChem, Germany). GoTaq DNA polymerase (Promega, USA) and respective primers (Table 2.4) were used for genotyping experiments in 20 µl reaction volume (1 µl lysis product as template, 0.5 µM forward primer, 0.5 µM reverse primer, 0.2 mM dNTP mix, 1X GoTaq buffer, 0.05 U GoTaq polymerase, water to 20µl)

Name	Genotype	Sequence 5'-3'	Application
ok871.int.fw	*gcn-2(ok871)*	GTTTTCGGGATATTCGCAGA	genotyping
ok871.ext.rev		TGTGGACCCGAAACAGTACA	
ok306.ife2.ext.fw	*ife-2(ok306)*	AAACATTCGTTCATTTCCGC	genotyping
ok306.ife2.int.rev		CGTTTTGCCAATCGAATTTT	

Table 2.4 Oligonucleotides used for genotyping

2.3.4 Determination of mtDNA copy number

Worms at day 1 of adulthood were singled and lyzed by standard protocol (Bratic et al., 2009). The mtDNA copy number was measured by quantitative PCR as previously

described (Bratic et al., 2009). In brief, absolute mtDNA content was determined in single animal by amplifying mtDNA-encoded endogenous *nd1*. Standard curve was generated using as template serial dilutions of pCR2.1 plasmid containing *nd1*. Quantitative PCR was performed for at least 6 independent samples. Real-time PCR conditions as in *measurement of transcript levels*.

2.3.5 Quantification of nucleic acids

DNA and RNA concentrations were quantified by measuring the sample absorption at 260 nm and 280 nm with a NanoDrop 8000 spectrophotometer (Thermo Fisher, USA). A ratio greater than 2 of absorptions at 260 nm (DNA/RNA) divided by the absorption at 280 nm (protein) was used as an index of purity of DNA/RNA.

2.3.6 Analysis of gene expression

Transcript levels were analyzed in hermaphrodite animals at day1 of adulthood by quantitative real-time PCR using coding sequence specific primer pairs (Table 2.5). Total RNA was isolated with TRIzol (Thermo Fisher, USA) and contaminating DNA was removed using TURBO DNA-free™ kit (Thermo Fisher, USA). Then 0.8 µg of total mRNA was reversely transcribed using High Capacity cDNA Reverse Transcription Kit (Thermo Fisher, USA). Samples representing one condition were obtained from 6 biological replicates. Real time PCR was performed using QuantStudio 12K Flex Real Time PCR System (Thermo Fisher, USA) with the following PCR conditions: 3 min at 95°C, followed by 40 cycles of 5 sec at 95°C and 16 sec at 60°C. Amplified products were detected with SYBR Green (Brilliant III Ultra Fast SYBR Green qPCR Master Mix, Agilent Technologies, USA). Relative quantification was performed against *Y45F10D.4* (Hoogewijs et al., 2008). Relative gene expression was determined using $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). At least 6 biological replicates were used during isolation.

Name	Genotype	Sequence 5'-3'	Application
Y45F10D.4.fw	*Y45F10D.4*	GTCGCTTCAAATCAGTTCAGC	qPCR
Y45F10D.4.rev		GTTCTTGTCAAGTGATCCGACA	
hmg-5.fw	*hmg-5*	AAAGAAGTACACAGACGAAGC	qPCR
hmg-5.rev		TTTCTGGAGGACGACATGG	
mtss-1.fw	*mtss-1*	CGATCTCCAAGTCTACCGTC	qPCR
mtss-1.rev		GTCATCAACCTCTTGCTTGC	
ndufs-3.fw	*nuo-2*	TTCGTGTTCGTACATACACTG	qPCR
ndufs-3.rev		CTTCACGCTCAAACCAGTC	
MTCE.26.fw	*ctc-1*	GGTGAACAGTCTACCCACC	qPCR
MTCE.26. rev		GCTAAATCTACTCTACTTCCAGG	
sptf-3.fw	*sptf-3*	CAACAACACCTGATGGATCAC	qPCR
sptf-3.rev		GGAATGAATTGCACCTGTCTC	
tomm-20.fw	*tomm-20*	ACAACTCCTGTCTATCTTCCA	qPCR
tomm-20.rev		GAACATTTCCGCAAGACGT	
gfp.fw	*gfp*	CATGGCAGACAAACAAAAGAATG	qPCR
gfp.rev		CTGCTAGTTGAACGCTTCCATC	
vdac.fw	*vdac-1*	GATCCCACAATACGGAATCACTTT	qPCR
vdac.rev		CTTGAGTCCACGTCCGAATTG	
hmg-5.fw	*hmg-5*	AGATAAGTACACAGAACTCTCG	qPCR
hmg-5.rev		CCTCAGTAGATAGTTTCATGAAGTC	
nd-1.fw	*nduo-1*	AGCGTCATTTATTGGGAAGAAGAC	qPCR
nd-1.rev		AAGCTTGTGCTAATCCCATAAATGT	

Table 2.5 Oligonucleotides used for qPCR-based gene expression analysis

2.3.7 RNA-seq

The quality of isolated RNA samples was determined using Experion system (BioRad, USA) and biological triplicates of required quality (RIN>8, OD260/280=1.8-2.1 and OD260/230>1.5) were send to Cologne Center for Genomics (CGC) for RNA sequencings. In brief, libraries were prepared using the Illumina® TruSeq® RNA sample preparation Kit. After poly-A selection, mRNA was purified, fragmented and underwent reverse transcription using random primers, followed by second strand cDNA synthesis with DNA Polymerase I and RNase H. After end repair and A-tailing, indexing adapters were ligated. The products were then purified and amplified (14 PCR cycles) to create the final cDNA libraries. After library validation and quantification (Agilent Technologies 2200 TapeStation), equimolar amounts of library were pooled. The pool was quantified by using the Peqlab KAPA Library Quantification Kit and the Applied Biosystems 7900HT Sequence Detection System. The pool was sequenced by using an Illumina TruSeq PE Cluster Kit v3 and an Illumina TruSeq

SBS Kit v3-HS on an Illumina HiSeq 2000 sequencer (Illumina, USA) with a paired-end (101x7x101 cycles) protocol. NGS data were analyzed by CECAD Bioinformatics facility as described (Wagle et al., 2015).

2.3.8 Protein isolation

Worms were collected and pelleted in 1.5 ml Eppendorf tubes, then resuspended in 150 µl of lysis buffer (25 mM Tris, 0.15 M NaCl, 1 mM EDTA, 1% NP-40, freshly added: 5 mM DTT and 1X protease inhibitor cocktail (Sigma-Aldrich, USA), pH 7.4). After 5 consecutive freezing (liquid nitrogen) thawing (37°C) worms were sonicated for 3 min at 4°C using Bandelin Sonorex RK 102 P (Sigma-Aldrich, USA). Subsequently, samples were centrifuged at 4°C, 14.000x*g*. The supernatant was transferred to a new tube and the protein concentrations were measured by using Bradford reagent (Sigma-Aldrich, USA) according to the manufacturer's instructions. Eventually, proteins were stored at -80 °C until used. At least 4 biological replicates were used during isolation.

2.3.9 Western Blot analysis

For Western blots (Tris-Glycine gel and buffer system) protein samples were boiled for 10 min in SDS-PAGE sample loading buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 1% b-mercaptoethanol, 12.5 mM EDTA. 0.02% bromphenol blue). Proteins were separated by SDS-PAGE, 30 µg of protein per lane, and blotted onto nitrocellulose membranes (GE Healthcare, United Kingdom). Next, membranes were blocked at room temperature for 1h in 5%-milk, PBST (PBS containing 0.05% Tween 20), further referred to as 5% MPBST, solution. Subsequently, primary antibodies, diluted in 5% MPBST were applied for overnight incubation at 4 °C. Next day, membranes were washed 3 x 10 min with PBST. Respective secondary antibody was diluted in 5% MPBST and applied for 1 h at room temperature. All antibodies and working dilutions listed in (Table 2.6). The membrane was again washed 3 x 10 minutes with PBST and signal was visualized using ECL solution according to manufacturers manual (GE Healthcare, United Kingdom) and eventually exposing to FUJIFILM Super RX (Fujifilm, Japan). After development using CURIX 60 tabletop processor (Agfa, Belgium) films were scanned using HP LaserJet 500

color MFP M575 (Hewlett-Packard, USA).

Antibody (Cat. #)	Distributor	Dilution
primary		
anti-Tubulin (T6074)	Sigma-Aldrich	1:5.000
anti-GFP (A-11122)	Thermo Fisher	1:2.000
anti-HSC70 (sc-7298)	Santa Cruz	1:10.000
anti-COXIV-1 (459600)	Thermo Fisher	1:1.000
anti-NDUFS3 (ab14711)	Abcam	1:1.000
anti-SSBP1 (HPA002866)	Sigma-Aldrich	1:500
anti-Phospho-eIF2α [Ser51] (#3597)	Cell Signaling	1:1000
anti-SPTF-3 (M82) (Hirose and Horvitz, 2013)	kind gift from Horvitz lab, MIT	1:2.000
secondary		
anti-mouse IgG peroxidase (A4416)	Sigma-Aldrich	1:2.000
anti-rabbit IgG peroxidase (A6154)	Sigma-Aldrich	1:2.000

Table 2.6 Antibodies used in this study

2.4 Computer analyses and microscopy

2.4.1 Photomicrographs

Photomicrographs were taken using Hamamatsu camera (OrcaR^2) and AxioVision software 4.8 on the epifluorescence microscope (AxioImager Z.1) with Colibri illumination system. Worms were mounted on glass slides with 5% agarose pads in the middle. Animals were immobilized using 50mM sodium azide. Whenever possible, worms to be compared were placed on the same slide.

2.4.2 Statistical analyses and graphical representation

A two-tailed unpaired Student's t-test was used to determine statistical significance. Error bars represent standard error of the mean (SEM). All p values below 0.05 were considered significant: $p^{*}<0.05$; $p^{**}<0.01$; $p^{***}<0.001$; $p^{****}<0.0001$. All statistical analyses and generation of graphs were performed in GraphPad Prism5.0d. (GraphPad Software, USA). All photomicrographs and Western blot scans were arranged using Adobe Illustrator CS6 16.0.4 (Adobe Systems, USA). Quantification of protein amount was based on calculated intensity per mm^2 using ImageJ 1.47 software (National Institutes of Health, USA). Hardware and protocols for Western blotting from BioRad, USA.

3 Results

3.1 Transcription factors RNAi screen using *mtss-1$_{pr}$ ::gfp*

In order to identify genes affecting mitochondrial biogenesis we designed reporters to monitor changes in mtDNA replication state. In mammals, the minimal mtDNA replisome consists of three proteins: POLγ, TWINKLE and SSBP1 (also known as mtSSB) (Korhonen et al., 2004). We generated respective reporters coupling green fluorescent protein (GFP) expression to the transcriptional activity of the *polg-1, F46G11.1* and *mtss-1* putative promoter regions. Only injection of *mtss-1$_{pr}$ ::gfp* led to successful expression of the extrachromosomal array, which was subsequently integrated by ultraviolet (UV) irradiation into the genome at chromosome III.

When kept at standard laboratory maintenance temperature of 20°C, GFP was expressed predominantly in the pharynx and the posterior part of the worm. Grown at 25°C, *mtss-1$_{pr}$ ::gfp* expression was robustly upregulated in the intestine (Figure 3.1 A). The upregulation of the fluorescent signal was confirmed by analysis using BioSorter®INSTRUMENT (Union Biometrica) (Figure 3.1 B). Importantly, at no point was GFP detected in cells of the reproductive system. In this way we were able to observe changes in *mtss-1* expression independent of the germ line based mtDNA proliferation. The screen was carried out at 25°C, with eggs from bleached worms exposed to bacteria expressing double stranded RNA throughout development. After reaching day 1 of adulthood we looked for animals expressing GFP at a lower level than control worms fed empty vector (Figure 3.1 C).

For the screen we used two different RNAi libraries: one transcription factor library from Source BioScience which is publicly available on the company's website; the second library was generated by the Cole M. Haynes laboratory from Memorial Sloan Kettering Cancer Center and is based on a genome-wide *in silico* screen for genes with putative DNA interacting domains. In total, these libraries were comprised of 388 and 341 targeted genes respectively. We identified five genes whose RNAi mediated knockdown decreased *mtss-1$_{pr}$ ::gfp* expression (Table 3.1). *ceh-20* and *lin-40* knockdown

induced the least fluorescence decrease. Therefore, we decided to focus on *sptf-3, gei-17 and W04D2.4*.

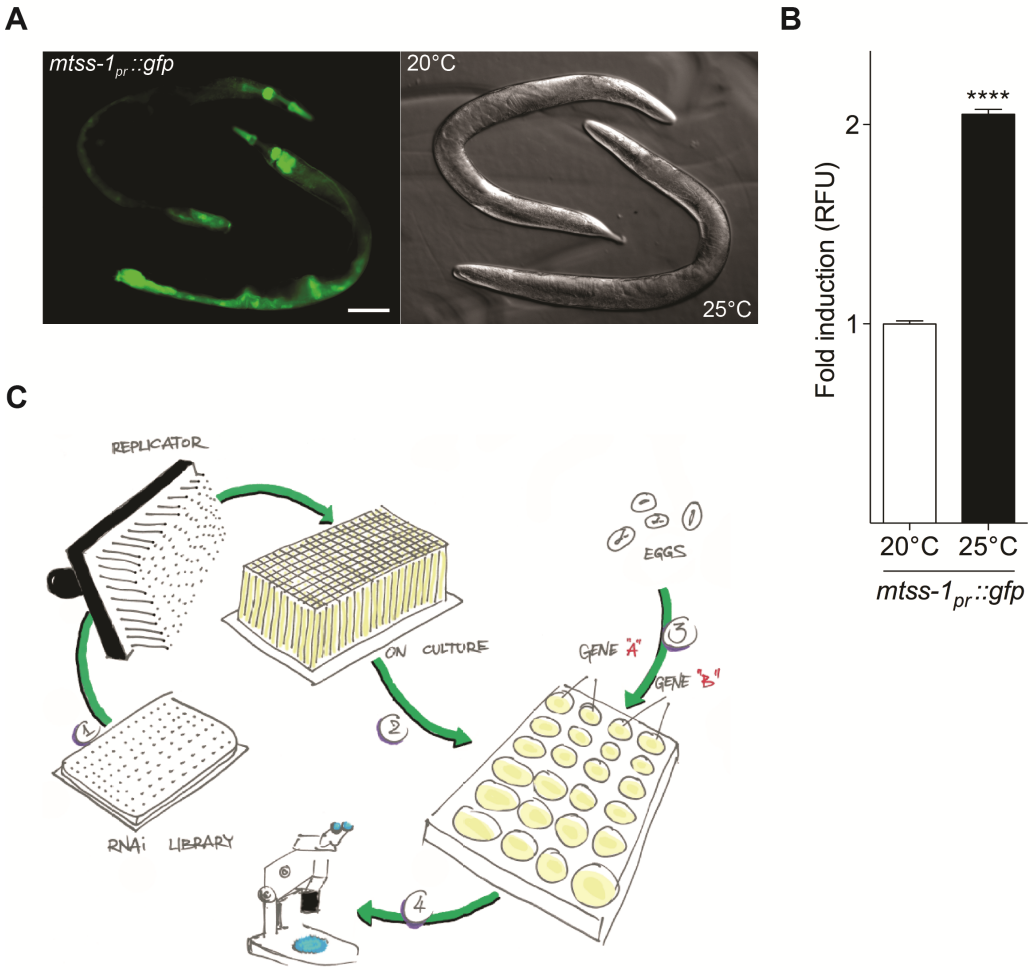

Figure 3.1 Experimental outline to detect genes involved in mitochondrial biogenesis
(A) Representative photomicrographs of *mtss-1$_{pr}$::gfp* transgenic worms raised on control (20°C) or increased (25°C) temperature. Scale bar, 0.1 mm. **(B)** GFP expression levels in *mtss-1$_{pr}$::gfp* transgenic worms measured with BioSorter®INSTRUMENT (Bars represent mean ± SEM, [Student's t test], p****<0.0001, n=400). RFU, relative fluorescence units. **(C)** RNAi screen workflow.

Gene	Brief description	DNA binding domain	GFP signal
Transcription factor library (www.sourcebioscience.com)			
ceh-20	homeodomain protein	HD - TALE	↘
lin-40	part of NURD complex	ZF - GATA, MYB	↘
In silico library (Cole M. Haynes)			
sptf-3	member of Sp1 family of transcription factors	ZF - C2H2 - 3 fingers	↓
gei-17	SUMO E3 protein ligase	ZF - MIZ	↓
W04D2.4	n/a	ZF - C2H2 - 1 finger	↓↓
control	L4440 empty vector		↑

Table 3.1 Candidates from RNAi screen conducted at 25°C

3.2 Knockdowns of *sptf-3, gei-17* and *W04D2.4* decrease lifespan

As perturbation of many genes with mitochondrial function affects *C. elegans* lifespan, we next investigated whether knockdown of *sptf-3, gei-17* and *W04D2.4* results in a similar phenotype. As we isolated the candidates performing the screen at 25°C, initially we performed lifespan experiments at the same temperature. Both *gei-17* and *W04D2.4* knockdown decreased the lifespan of wild type worms in a similar manner by ~ 25%, compared to control (Figure 3.2 A) (Table 3.2).

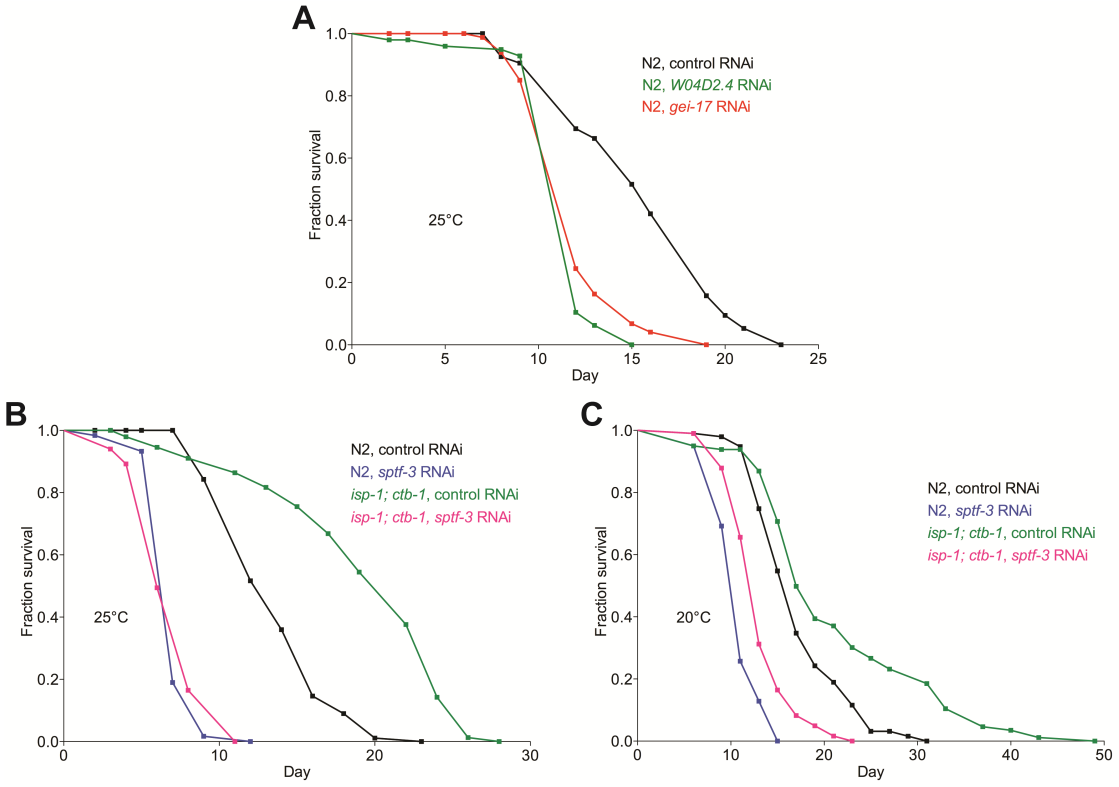

Figure 3.2 Lifespan assays upon inhibition of candidate genes
(A) Lifespan of N2 (wild type) worms subjected to RNAi mediated knockdown of *gei-17* and *W04D2.4* at 25°C. **(B)** Lifespan of N2 and *isp-1(qm150);ctb-1(qm189)* worms subjected to RNAi mediated knockdown of *sptf-3* at 25°C or **(C)** at 20°C.

Furthermore, we tested the effect of *sptf-3* knockdown on long lived, mitochondrial *isp-1(qm150);ctb-1(qm189)* double mutants. Both single amino acid substitutions target mitochondrial respiratory chain complex III subunits: mutations are located in the head domain of ISP-1, "Rieske" iron sulfur protein, and mtDNA-encoded cytochrome b, respectively. While *isp-1(qm150)* leads to slowed development and extended lifespan, no phenotype was found associated with *ctb-1(qm189)* (Feng et al., 2001).

Nevertheless, *ctb-1(qm189)* suppresses developmental delay but has no effect on lifespan of *isp-1(qm150)* mutant. Knockdown of *sptf-3* function in *isp-1(qm150);ctb-1(qm189)* background reduced mean lifespan by up to 72% at 25°C (Figure 3.2 B) (Table 3.2). Remarkably, the lifespan extension phenotype of *isp-1(qm150);ctb-1(qm189)* was completely abolished. At 20°C, the lifespan of wild type worms and *isp-1(qm150);ctb-1(qm189)* double mutants was reduced by *sptf-3* knockdown in a similar manner as at 25°C, though to a lesser extent (Figure 3.2 C) (Table 3.2). Together these results indicate vital functions of candidate genes specifically pronounced at higher temperature.

Strain	°C	RNAi	Median Adult Lifespan	Total Number of Animals (Censored)	% Lifespan Change *vs.* Control	*p*-Value *vs.* Control
wild type	25	L4440	16	100 (5)	-	-
wild type	25	*gei-17*	12	100 (23)	-25	<0.0001
wild type	25	*W04D2.*	12	100 (4)	-25	<0.0001
wild type	25	L4440	14	100 (11)	-	-
wild type	25	*sptf-3*	7	61 (3)	-50	<0.0001
isp-1(qm150); ctb-1(qm189)	25	L4440	22	100 (20)	-	-
isp-1(qm150); ctb-1(qm189)	25	*sptf-3*	6	100 (25)	-72	<0.0001
wild type	20	L4440	17	100 (6)	-	-
wild type	20	*sptf-3*	11	100 (48)	-35	<0.0001
isp-1(qm150); ctb-1(qm189)	20	L4440	17	100 (13)	-	-
isp-1(qm150); ctb-1(qm189)	20	*sptf-3*	13	100 (30)	-23	<0.0001

Table 3.2 Lifespan summary

3.3 Endogenous *mtss-1* transcripts are reduced upon *sptf-3* and *W04D2.4* knockdown

To confirm that changes in GFP expression coincide with changes in the endogenous *mtss-1* expression, we determined the respective transcript levels in worms raised at 25°C (Figure 3.3 A). *gei-17* knockdown appeared to have no effect on transcript levels of *mtss-1* or other genes with mitochondrial function, so we decided to exclude it from future experiments. *sptf-3* and *W04D2.4* knockdown decreased the expression of *gfp* and *mtss-1* transcripts. *sptf-3* knockdown also led to a decrease in *hmg-5* (ortholog of mammalian TFAM) and *tomm-20* (ortholog of human translocase of outer mitochondrial membrane (yeast)-like, TOMM20L) transcript levels, whereas *vdac-1* (ortholog of human voltage dependent anion channel, VDAC1) transcripts seemed not to be affected by knockdown of these two transcription factors. Additionally, the visually observed decrease in GFP expression was confirmed at the protein level and the efficacy of *sptf-3* knockdown was

examined using *C. elegans* specific anti-SPTF-3 antibody (Figure 3.3 B). Knockdown of *W04D2.4* and *sptf-3* caused the strongest reduction of *mtss-1* transcript levels out of the various transcription factors, which were tested. Therefore, we decided to further investigate the role of these two candidates in mitochondrial biogenesis.

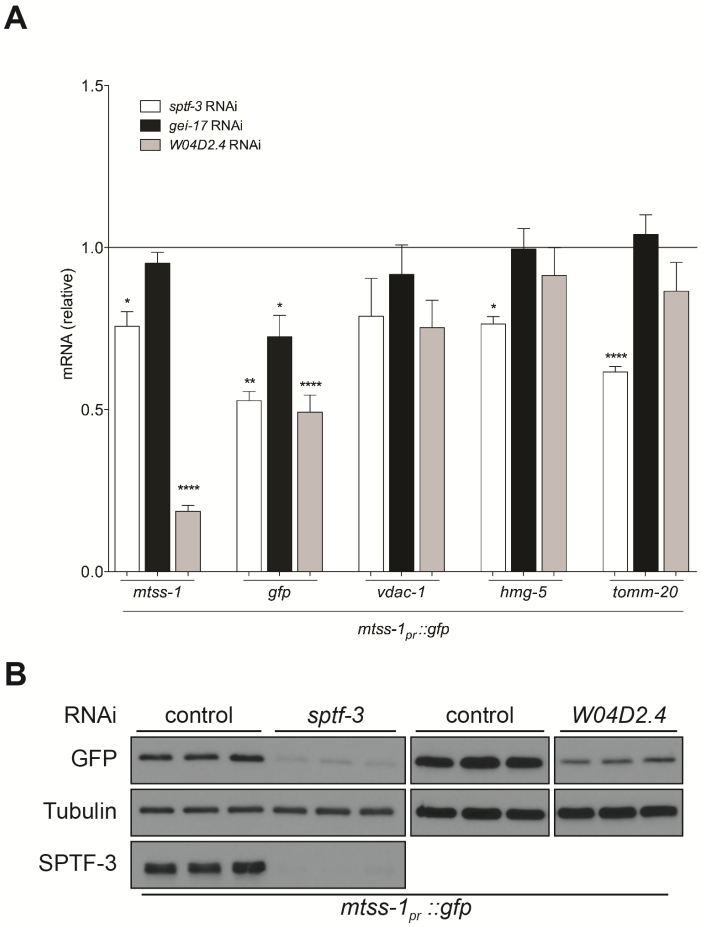

Figure 3.3 Transcript and protein levels of genes with mitochondrial function upon *sptf-3* knockdown

(A) Relative transcript levels of *mtss-1*, *gfp*, *vdac-1*, *hmg-5* and *tomm-20* in worms raised at 25°C and exposed to RNAi. Control indicated by grid line, bars represent mean ± SEM, [Student's t test], p*<0.05, p**<0.01, p****<0.0001, n=6. **(B)** Western blot analysis of GFP and SPTF-3 levels in total protein extracts from worms raised on control, *W04D2.4* or *sptf-3* RNAi at 25°C. Tubulin used as loading control. Irrelevant samples between control and *W04D2.4* knockdown were removed.

3.4 W04D2.4 localizes to the nucleus at 25°C

To shed light on the nature of W04D2.4, we generated a transcriptional reporter strain with *gfp* driven by the 5` upstream promoter region of *W04D2.4*. The highest level of GFP expression was observed in the pharynx, tail and nervous system. To obtain information about subcellular localization of W04D2.4, we

generated a translational reporter. Instead of the internal W04D2.4 promoter region, we used the muscle specific *myo-3* promoter, driving the expression of the *W04D2.4::mcherry* construct. Expression of fusion proteins in muscle cells is convenient in terms of cell size, number and location, which makes localization of the fluorescent signal relatively easy. In contrast, cells that have shown *W04D2.4* promoter activity are unsuitable for protein localization studies. Contrary, one disadvantage of using the *myo-3* promoter is that in body wall muscle cells it drives the expression in a declining manner, from strong to weak towards adulthood (Chen et al., 2010; Nargund et al., 2012). Therefore in adult worms we did not observe strong and clear mCherry signal (data not shown), thus our observations were restricted to larval stages. At 20°C mCherry signal was diffused without being directed to any cell compartment specifically, whereas at 25°C the signal was detected predominantly in the nuclei (Figure 3.5). These results indicate that W04D2.4 is an inducible transcription factor, translocating to the nucleus upon temperature increase.

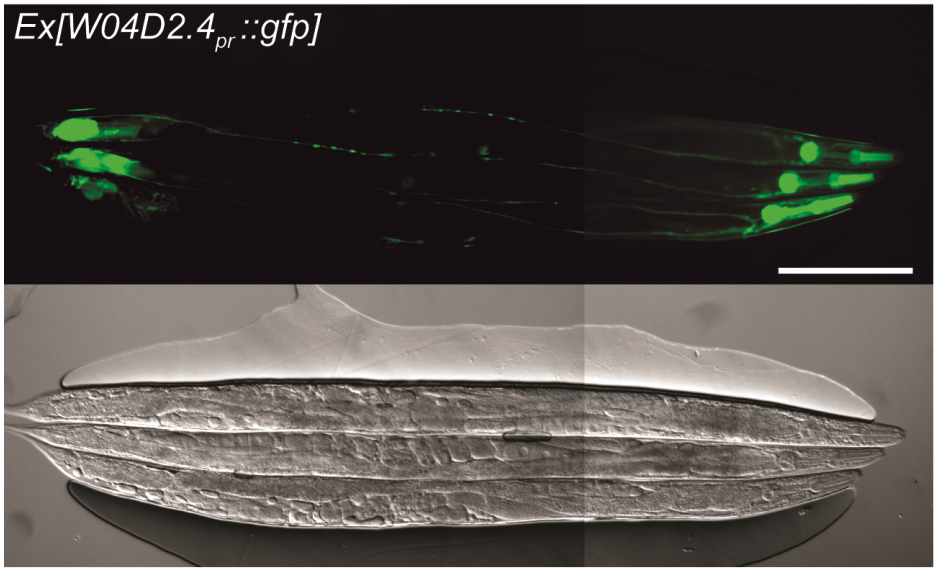

Figure 3.4 *W04D2.4* transcriptional reporter
Representative photomicrographs of *Ex[W04D2.4$_{pr}$::gfp]* at 20°C. Scale bar, 0.1 mm.

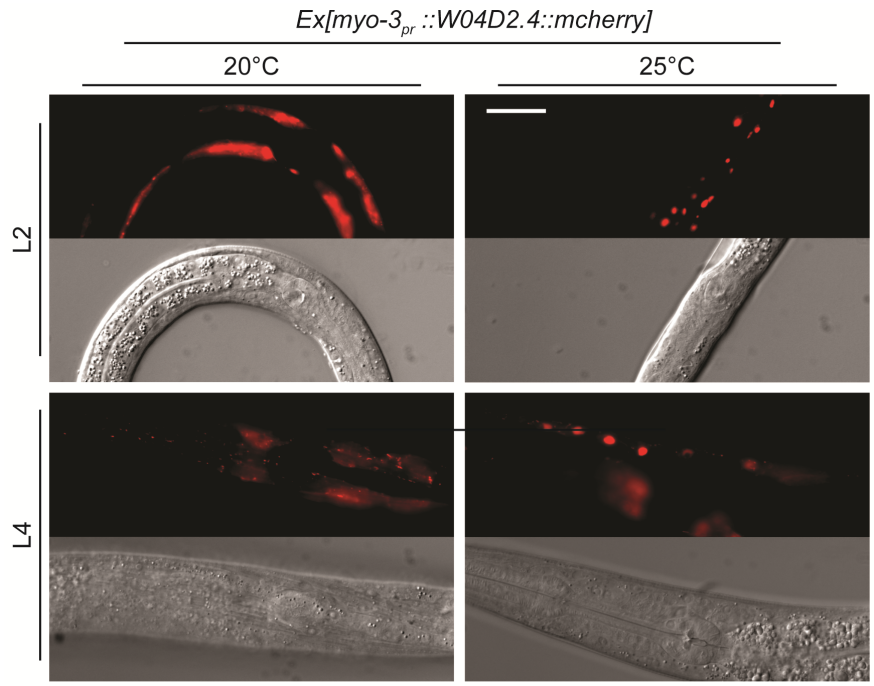

Figure 3.5 Localization of W04D2.4-mCherry
Representative photomicrographs of *Ex[myo-3$_{pr}$ ::W04D2.4::mcherry]* at L2 and L4 larval stages at 20°C/25°C. Scale bar, 20 µm.

3.5 W04D2.4 is involved in the regulation of genes with mitochondrial function

Our localization studies suggest that W04D2.4 is a transcription factor, activated by stress, as it strongly translocates to the nucleus upon temperature increase. We next performed RNA sequencing (RNA-seq) analysis to compare transcriptomes of worms exposed to *W04D2.4* RNAi at 25°C and worms fed empty vector at 20°C or 25°C. As we were interested in deciphering the regulation of mitochondrial biogenesis, we looked closely at all annotated genes with mitochondrial function that were up- or downregulated in animals exposed to *W04D2.4* (25°C) *vs.* control (25°C) RNAi, with p-value <0.01. In total we identified 222 transcripts that met our criteria. Further, we analyzed transcript levels of those genes in three possible combinations: (1) *W04D2.4* (25°C) *vs.* control (25°C); (2) *W04D2.4* (25°C) *vs.* control (20°C) and (3) control (25°C) *vs.* control (20°C). Transcript levels of 94 genes were changed in all three conditions whereas 88 were changed exclusively due to inhibited function of *W04D2.4* (Figure 3.6 A). The vast majority of the 222 transcripts were downregulated in worms upon *W04D2.4* knockdown (*W04D2.4*

(25°C) *vs.* control (25°C)) with only 22 transcripts being upregulated (Figure 3.6 B, Appendix Table 8.1).

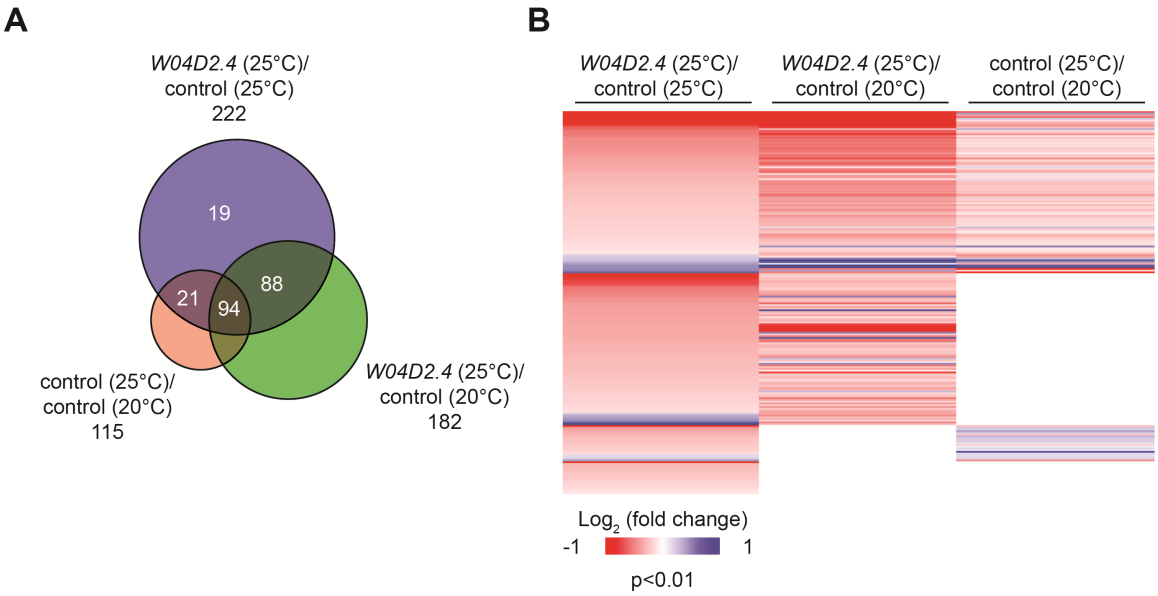

Figure 3.6 RNA-seq transcriptome analysis after *W04D2.4* knockdown.
(A) Venn diagram illustrating transcripts of genes with mitochondrial function altered due to *W04D2.4* knockdown and/or temperature shift and their overlap (only transcripts changed at 25°C in *W04D2.4* (RNAi) *vs.* control (RNAi) were taken into consideration). **(B)** Heat map comparing expression patterns of genes with mitochondrial function in worms raised on *W04D2.4* (RNAi) or control (RNAi) at 20°C/25°C. Color code indicates Log_2 (fold changes).

Strikingly, at 25°C transcripts of eleven mtDNA-encoded genes were downregulated between worms exposed to *W04D2.4* and control RNAi (Table 3.3). Seven of those transcripts were downregulated exclusively due to *W04D2.4* knockdown, while four genes showed also reduced transcript abundance already due to temperature increase. Further, 31 nDNA-encoded RC (Respiratory Chain) subunit genes were downregulated at 25°C upon *W04D2.4* knockdown. Moreover, out of the 65 annotated mitochondrial ribosomal protein genes (RPG) in *C. elegans*, downregulation of *W04D2.4* reduced levels of 28 respective transcripts. Additionally, two key chaperones of mitochondrial unfolded protein response (*hsp-6* and *hsp-60*) showed reduced transcript levels upon *W04D2.4* knockdown. Importantly, *mtss-1* levels were highly reduced by diminished *W04D2.4* function and slightly upregulated when the worms were exposed to higher temperature only. This not only supports the role of W04D2.4 in *mtss-1* regulation but also validates the correlation between *gfp* and *mtss-1* expression in the *mtss-1*$_{pr}$ *::gfp* reporter. Taken together, the RNA-seq data indicate that W04D2.4 primarily affects expression of the

mitochondrial genome. Under the chosen temperature of 25°C, knockdown of *W04D2.4* reduces transcript levels of mtDNA genes. W04D2.4 likely indirectly affects replication and translation processes within mitochondria, by modifying transcript levels of a broad range of mitochondrial RPGs and/or parts of the replication machinery (*polg-1*, *mtss-1)*. Additionally, W04D2.4 might further directly affect mitochondrial respiratory chain complexes by altering expression of RC subunits encoded by the nuclear genome. Though W04D2.4 showed intriguing features like nuclear translocation upon temperature increase and high probability of being involved in the expression of mtDNA and RC subunits, it seems to be nematode specific, as there are no distinct *W04D2.4* homologs in any other organisms. At this point we decided to focus exclusively on the second candidate from the screen, namely *sptf-3*.

Gene	*W04D2.4* 25°C/ control 25°C	*W04D2.4* 25°C/ control 20°C	control 25°C/ control 20°C
RC Subunits (mtDNA)			
ND5	-1.19	-1.52	-0.36
ND4	-0.76	-1.13	-0.4
CYTB	-0.22	-0.58	-0.39
ND1	-0.97	-0.15	-
ND2	-0.88	-0.2	-
ND3	-0.82	-0.22	-
ND6	-1.2	-0.21	-
COX2	-0.46	-0.4	-
ATP6	-0.75	-0.28	-
COX3	-0.39	-	0.22
ND4L	-1.01	-	-
RC Subunits (nDNA)			
C18E9.4	-0.33	-0.61	-0.32
nuo-1	-0.2	-0.31	-0.14
nuo-5	-0.22	-0.4	-0.21
nuo-2	-0.17	-0.26	-0.12
C33A12.1	-0.19	-0.3	-0.14
T20H4.5	-0.2	-0.29	-0.12
sdha-1	-0.22	-0.42	-0.23
T02H6.11	-0.13	-0.22	-0.12
ucr-1	-0.32	-0.45	-0.16
cyc-1	-0.16	-0.22	-0.09
isp-1	-0.19	-0.24	-0.09
cyc-2.1	-0.11	-0.16	-0.08
Y71H2AM.5	-0.12	-0.23	-0.14
atp-2	-0.95	-1.19	-0.28
atp-4	-0.21	-0.34	-0.16
atp-5	-0.43	-0.55	-0.15
Y82E9BR.3	-0.18	-0.36	-0.2
R53.4	-0.24	-0.4	-0.19
atp-3	-0.09	-0.18	-0.12
H28O16.1	-0.43	-0.53	-0.13
asb-1	-0.39	-0.43	-0.07
nuaf-3	-0.48	-0.3	-
F45H10.3	-0.2	-0.29	-
F57B10.14	-0.53	-0.19	-
ZK1128.1	-0.23	-0.08	-
cco-2	-0.16	-0.16	-
F58F12.1	-0.26	-0.33	-
R04F11.2	-0.16	-0.29	-
nduf-6	-0.17	-	0.09
Y94H6A.8	0.15	-	0.14
nuaf-1	-0.25	-	-
Y71H2AM.4	-0.2	-	-
Replication/Transcription/ Translation			
mtss-1	-2.5	-2.1	0.38
polg-1	-0.31	-0.2	-
mrps-23	-2.97	-3.6	-0.66
mrpl-23	-0.21	-0.43	-0.25
mrps-15	-0.19	-0.41	-0.25
mrps-25	-0.45	-0.65	-0.23
W03F8.3	-0.61	-0.27	0.31
mrps-31	-0.51	-0.19	-
mrpl-49	-0.46	0.48	-
mrpl-24	-0.37	-0.16	-
mrps-10	-0.35	-0.34	-
mrpl-19	-0.34	-0.35	-
mrpl-55	-0.31	0.47	-
mrps-16	-0.3	-0.5	-
mrps-9	-0.28	-0.29	-
mrpl-35	-0.27	-0.21	-
mrps-5	-0.27	-0.23	-
mrps-26	-0.24	-0.09	-
mrps-17	-0.22	-0.23	-
mrpl-51	-0.19	-0.29	-
mrpl-32	-0.18	0.26	-
mrpl-50	-0.14	-0.38	-
mrps-18C	-0.14	-0.16	-
mrpl-11	-0.13	-0.4	-
mrpl-18	-0.31	-	0.36
mrpl-45	-0.24	-	0.27
mrps-24	-0.23	-	0.2
mrpl-41	-0.22	-	-
mrps-7	-0.22	-	-
tsfm-1	-0.15	-	-
CD4.3	-0.16	-	-
UPR^{mt}			
hsp-60	-1.09	-1.16	-0.11
clpp-1	-0.72	-0.22	-
hsp-6	-0.32	-1.7	-

Table 3.3 RNA-seq results for genes involved in RC, mtDNA expression and UPR^{mt}
Values indicate Log_2 (fold change). No value means unchanged expression, under set criteria.

3.6 *sptf-3* is the only sptf gene affecting *mtss-1$_{pr}$::gfp* expression

In mammals 9 SP and 16 KLF transcription factors belong to the Specificity Protein/ Kruppel-like factor (SP/KLF). In *C. elegans* the Sp family includes *sptf-1*, *sptf-2*, *sptf-3* and *tlp-1* genes (Ulm et al., 2011; Zhao et al., 2002b). In order to test whether, in addition to *sptf-3,* also other Sp family members affect *mtss-1* expression, we downregulated the three *sptf* family members, namely *sptf-1-3,* and compared *mtss-1$_{pr}$ ::gfp* induction levels (*tlp-1* still to be tested). Only *sptf-3* knockdown was able to reduce *mtss-1$_{pr}$::gfp* expression, which was most obvious in worms raised at 25°C (Figure 3.7 A). Neither *sptf-1*, nor *sptf-2* had any effect on *gfp* expression, independent of temperature conditions. In order to quantitatively confirm the effect of *sptf-3* knockdown, we determined the fluorescence signal using BioSorter®INSTRUMENT (Figure 3.7 B). At 25°C downregulation of *sptf-3* clearly induced a reduction in GFP signal intensity compared to untreated control worms. These data suggest that from the *sptf* gene family members only *sptf-3* is required for the upregulated *mtss-1* expression.

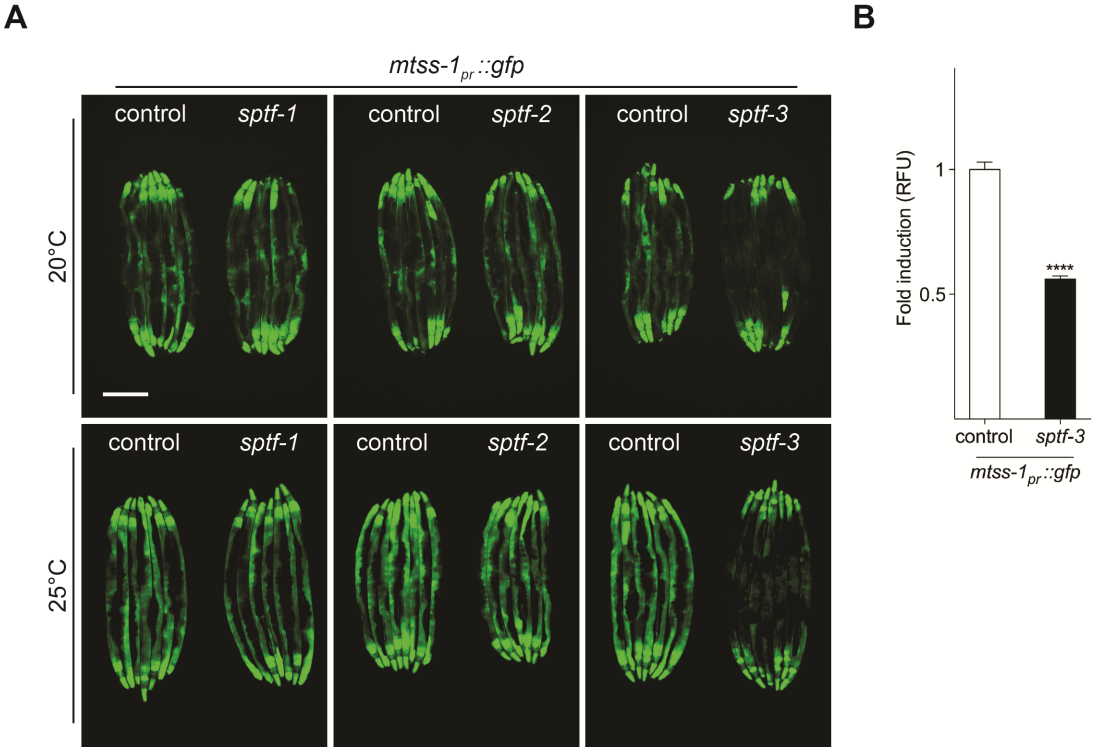

Figure 3.7 Only *sptf-3* knockdown prevents *mtss-1$_{pr}$::gfp* induction
(A) Representative photomicrographs of *mtss-1$_{pr}$::gfp* transgenic worms raised on control or *sptf* RNAi at 20°C/25°C. Scale bar, 0.2 mm. **(B)** GFP expression levels in *mtss-1$_{pr}$::gfp* transgenic worms measured with BioSorter®INSTRUMENT (Bars represent mean ± SEM, [Student's t test], p****<0.0001, n=100). RFU, relative fluorescence units.

3.7 *sptf-3* knockdown affects induction of reporter constructs representing TCA cycle and mtDNA transcription

Next, we aimed to investigate whether loss of *sptf-3* affects expression of other genes involved in mitochondrial functions. To monitor mtDNA transcription we generated the *hmg-5*$_{pr}$ *::gfp* reporter, with *hmg-5* as functional *C. elegans* ortholog of mammalian TFAM (Sumitani et al., 2011). TFAM function is involved in mtDNA packaging, maintenance, initiation of transcription and replication (Kukat and Larsson, 2013). Additionally, we generated previously described reporters representing two enzymes of the TCA cycle: aconitase and citrate synthase (Yoneda et al., 2004). The enzymatic activity of citrate synthase is often used in mammalian systems as a marker for mitochondrial mass. Populations of worms carrying extrachromosomal arrays were then exposed to *sptf-3* RNAi at 20°C and 25°C (Figure 3.8). On control plates all three reporters showed an increase in GFP fluorescence simply by keeping the worms at higher temperatures. However knockdown of *sptf-3* at 25°C reduced *Ex[cts-1*$_{pr}$ *::gfp]* and *Ex[hmg-5*$_{pr}$ *::gfp]* expression to the level of animals kept at 20°C. Moreover, *sptf-3* knockdown moderately decreased GFP signal in *Ex[aco-2*$_{pr}$ *::gfp]* reporter at 20°C and 25°C (Figure 3.8 A). At 20°C the expression of *Ex[cts-1*$_{pr}$ *::gfp]* (Figure 3.8 B) and *Ex[hmg-5*$_{pr}$ *::gfp]* (Figure 3.8 C) reporters was not altered by *sptf-3* knockdown. Additionally, results from *Ex[hmg-5*$_{pr}$ *::gfp]* experiments are supported by our previously observed reduction in *hmg-5* transcript levels upon *sptf-3* knockdown (Figure 3.3 A). Taken together, these experiments indicate that *sptf-3* mediates regulation of *cts-1* and *hmg-5* expression, genes involved in the TCA cycle and mtDNA transcription/maintenance, respectively. These data suggest that the role of *sptf-3* in mitochondrial function is not restricted to the transcriptional regulation of *mtss-1*.

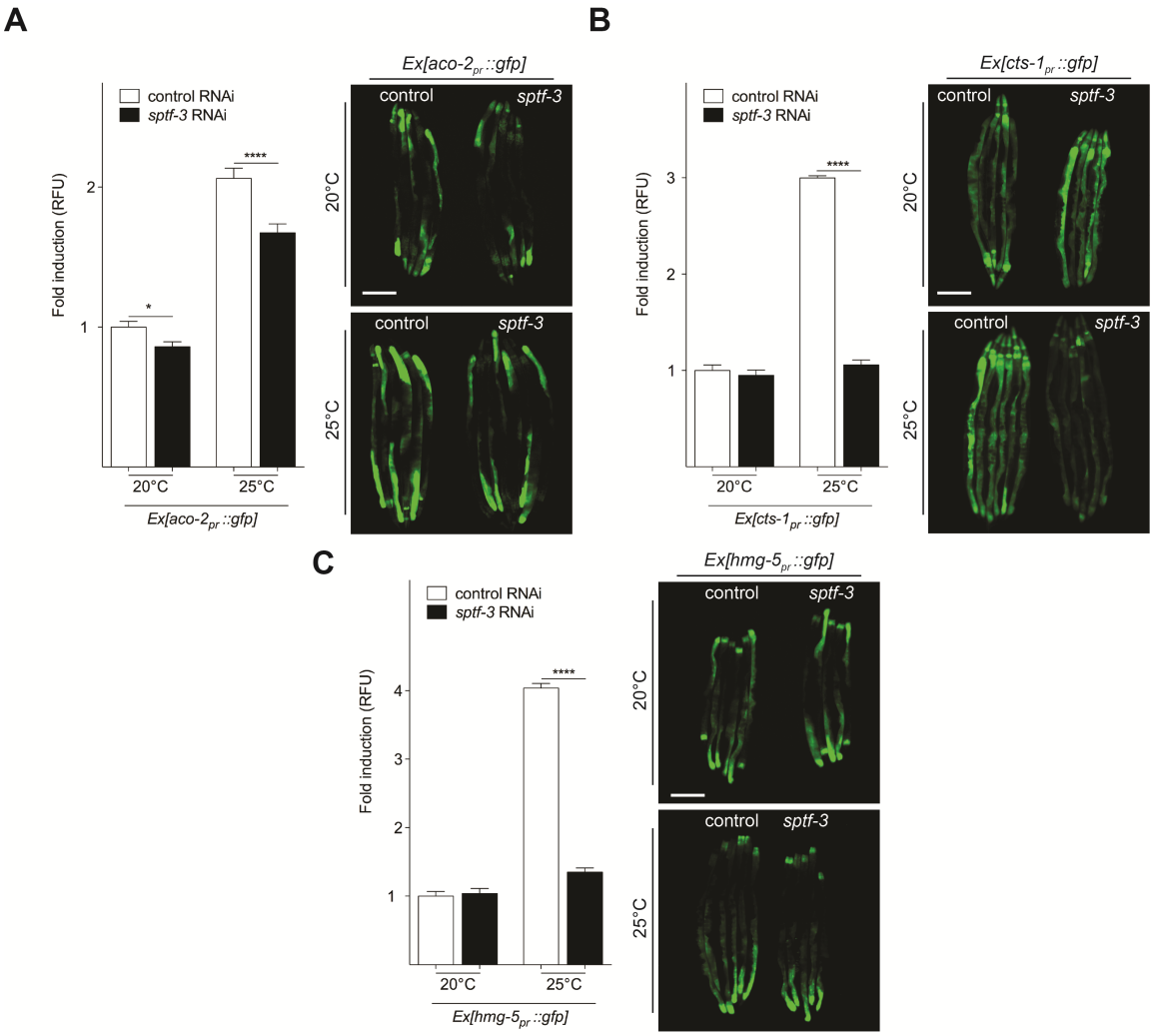

Figure 3.8 *sptf-3* knockdown affects mitochondrial reporters
Representative photomicrographs of **(A)** *Ex[aco-2$_{pr}$::gfp]*, **(B)** *Ex[cts-1$_{pr}$::gfp]* and **(C)** *Ex[hmg-5$_{pr}$::gfp]* transgenic worms with respective quantification using BioSorter®INSTRUMENT raised on control or *sptf-3* RNAi at 20°C/25°C. Scale bar, 0.2 mm. Bars represent mean ± SEM, [Student's t test], p*<0.05, p****<0.0001, n≥100). RFU, relative fluorescence units. Scale bar, 0.2 mm.

3.8 *sptf-3* knockdown promotes mitochondrial hyperfusion without significantly affecting mitochondrial mass

Given the fact that *sptf-3* knockdown affected expression of transcriptional reporters representing respective mitochondrial functions, we wanted to know whether *sptf-3* knockdown affects mitochondrial morphology. For this purpose we used another reporter strain *zcls14* (*myo-3$_{pr}$::gfpmt*), in which the muscle specific *myo-3* promoter drives expression of GFP targeted to mitochondria. Once worms reached day 1 of adulthood, mitochondrial morphology was investigated in the 19th ventral muscle pair where we

observed three different states: linear, hyperfused and fragmented (Figure 3.9 A). Whereas in control worms at 20°C the three states seem to be almost equally presented within the investigated number of individuals, *sptf-3* knockdown appears to drive the network towards the hyperfused state (Figure 3.9 B). The temperature increase promotes fragmentation of mitochondrial networks in control and *sptf-3* RNAi conditions, whereby the number of cells with hyperfused networks is lower in control condition. These results indicate that *sptf-3* knockdown facilitates formation of hyperfused mitochondrial networks. Mitochondrial fission and fusion processes are governed by distinct sets of proteins, which are highly conserved in yeast, flies, worms and mammals (for review, see (Westermann, 2010)). Reduction of SPTF-3 levels possibly affects expression of genes directly involved in fusion/fission events. On the other hand, inhibition of *sptf-3* function may lead to changes in the mitochondrial functional state resulting in alteration of fusion/fission ratio.

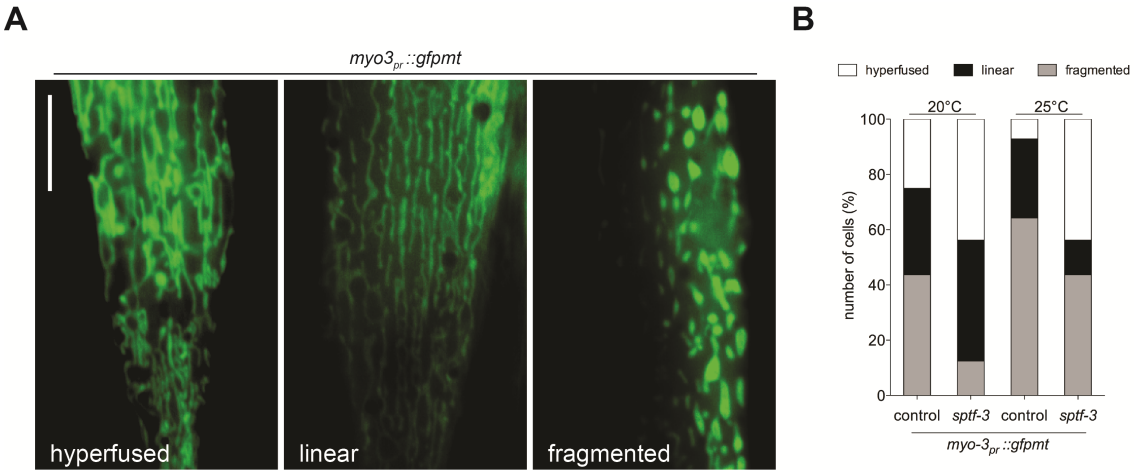

Figure 3.9 Mitochondrial network morphology upon *sptf-3* knockdown
(A) Representative photomicrographs of mitochondrial networks in *myo-3$_{pr}$::gfpmt* transgenic worms raised on control or *sptf-3* RNAi at 20°C/25°C. Scale bar, 10µm. **(B)** Classification of observed mitochondrial morphology in the 19th ventral muscle pair (n=14).

Next we tested whether *sptf-3* knockdown influences general mitochondrial mass. One way to access this information is to quantify the amount of GFP targeted to mitochondria. To this end, we used *myo-3$_{pr}$::gfpmt* and *ges-1$_{pr}$::gfpmt* (*ges-1* encodes a gut-specific type B carboxylesterase) reporters. Knockdown of *sptf-3* slightly reduced the GFP signal: in muscle specific reporter by 2% (Figure 3.10 A) and in gut specific reporter by 10% (Figure 3.10 B), yet statistically significant in both cases. However, *sptf-3* knockdown might affect the levels of GFP at any point: from transcription initiation to mitochondrial import. Previously we observed reduction in *tomm-20* transcript levels

upon *sptf-3* inhibition (Figure 3.3 A). This reduction may lead to changes in the mitochondrial import efficiency, which would possibly lead to misinterpretations of mitochondrial mass based on the amount of the imported GFP. To compare mitochondrial mass between two conditions via fluorescent signal, at least equal amounts of marker should be present in the cell and it should penetrate mitochondria independent of mechanisms possibly affected by chosen conditions. Therefore mitochondrial mass should be determined differently, *e.g.* using mitotracker.

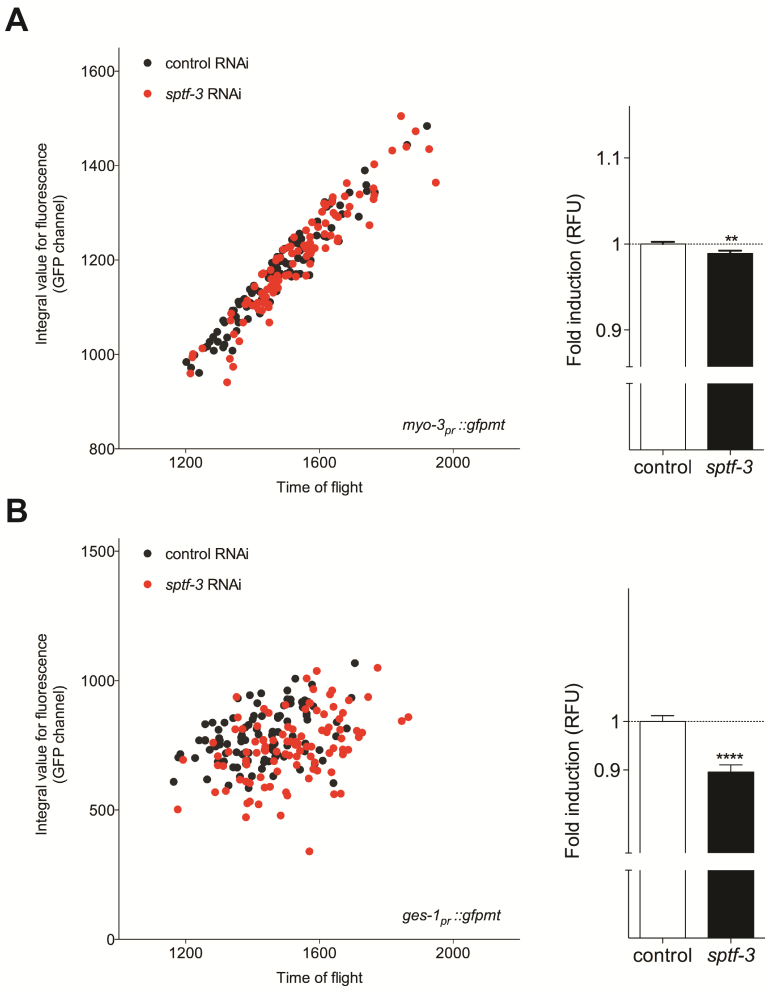

Figure 3.10 Mitochondrial mass upon *sptf-3* knockdown
BioSorter®INSTRUMENT analysis of mitochondrial mass reflected by GFP signal in **(A)** *myo-3$_{pr}$ ::gfpmt* and **(B)** *ges-1$_{pr}$ ::gfpmt* raised on control or *sptf-3* RNAi at 25°C. Bars represent mean ± SEM, [Student's t test], p**<0.01, p****<0.0001, n=100). RFU, relative fluorescence units.

3.9 *sptf-3* knockdown reduces mtDNA copy number but does not affect respiration

We next investigated whether SPTF-3 affects mtDNA copy number, using a *polg-1(ok1548)* mutant as the ultimate control for impaired mtDNA replication (Bratic et al., 2009). We found that *sptf-3* knockdown reduced the absolute number of mtDNA molecules by ~22% at 20°C (Figure 3.11 A). However the decrease was not as prominent as ~86% observed in *polg-1(ok1548)* mutants. Maintaining worms at elevated temperature increased mtDNA content by 19% in control animals and 29% upon *sptf-3* knockdown (Figure 3.11 A). To further investigate mild reduction in the mtDNA content upon *sptf-3* knockdown, we determined mRNA levels of *polg-1*, encoded by nDNA, and of *nduo-1*, encoded by mtDNA (Figure 3.11 B). Reduction in *polg-1* transcript levels via *sptf-3* knockdown was mild and apparently not strong enough to mimic total loss of *polg-1*. Most likely the residual POLG-1 activity is sufficient to maintain mtDNA at almost normal levels. *nduo-1* levels were not significantly reduced, however declining tendency might result from reduced *hmg-5* transcript levels upon *sptf-3* knockdown (Figure 3.3 A). Taken together, these data indicate that *sptf-3* regulates transcripts of genes involved in mtDNA replication and transcription. Regardless, residual activity of either SPTF-3 or the target genes is sufficient for mtDNA expression.

As the integrity of mitochondrial genome and the sufficient production of RC subunits might correlate with mitochondrial mass and their functional state, we decided to investigate the respiration of worms upon *sptf-3* knockdown. The respiration rate was increased with developmental state but also with higher temperature (Figure 3.12 A). Nevertheless, *sptf-3* knockdown did not affect oxygen consumption compared to control animals. These results suggest that mild decrease in mtDNA levels orchestrated by the loss of *sptf-3* does not have an effect on expression of RC subunits and therefore does not affect respiration. It also indicates that RC capacity is not directly connected to the level of mtDNA, as we observed minor increase in mtDNA content of wild type worms at 25°C compared to 20°C (Figure 3.11 A), while respiration rates where increased several fold (Figure 3.12 A). As previously reported, reduced *sptf-3* activity leads to reduced brood size underlining its role in embryonic development (Ulm et al., 2011). Also in our hands, RNAi mediated knockdown of *sptf-3* resulted in decreased number of progeny,

however mtDNA experiments suggest that this developmental function of *sptf-3* is largely independent of mtDNA content (Figure 3.12 A, B).

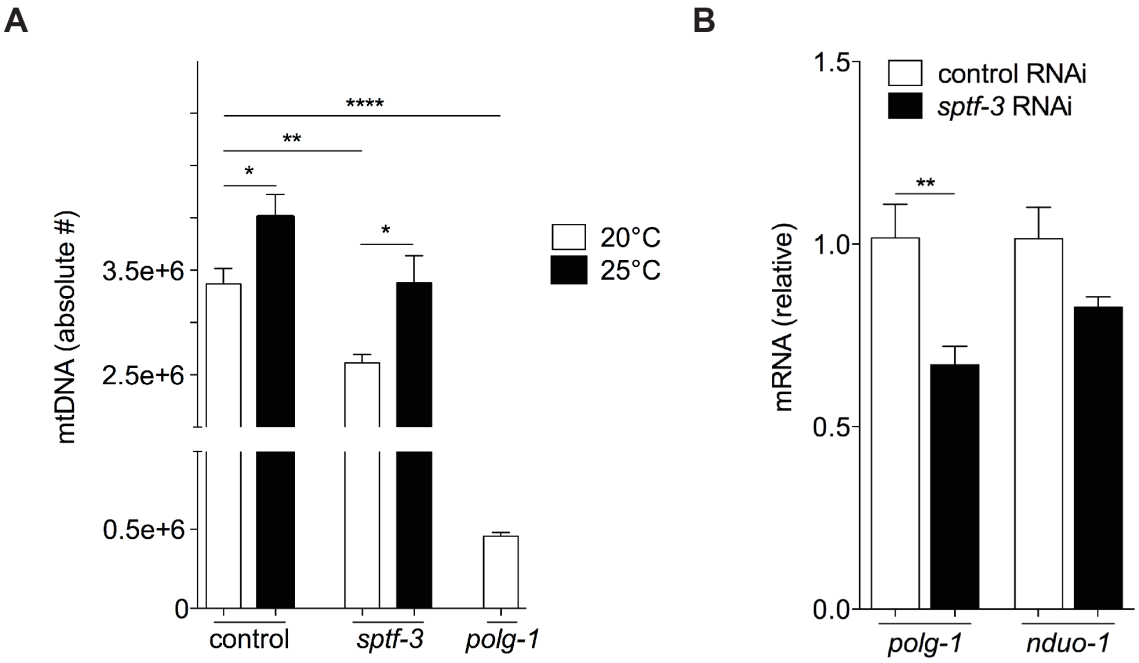

Figure 3.11 mtDNA copy number upon *sptf-3* knockdown
(A) Absolute mtDNA copy number determined by quantitative real-time (PCR) in wild type worms raised at 25°C and exposed to control, *sptf-3* RNAi or and in *polg-1(ok1548)* mutants. Bars represent mean ± SEM, [Student's t test], p*<0.05, p**<0.01, p****<0.0001, n=6. **(B)** Relative transcript levels of *polg-1* and *nduo-1* in worms raised at 25°C and exposed to control or *sptf-3* RNAi. Bars represent mean ± SEM, [Student's t test], p**<0.01, n=6.

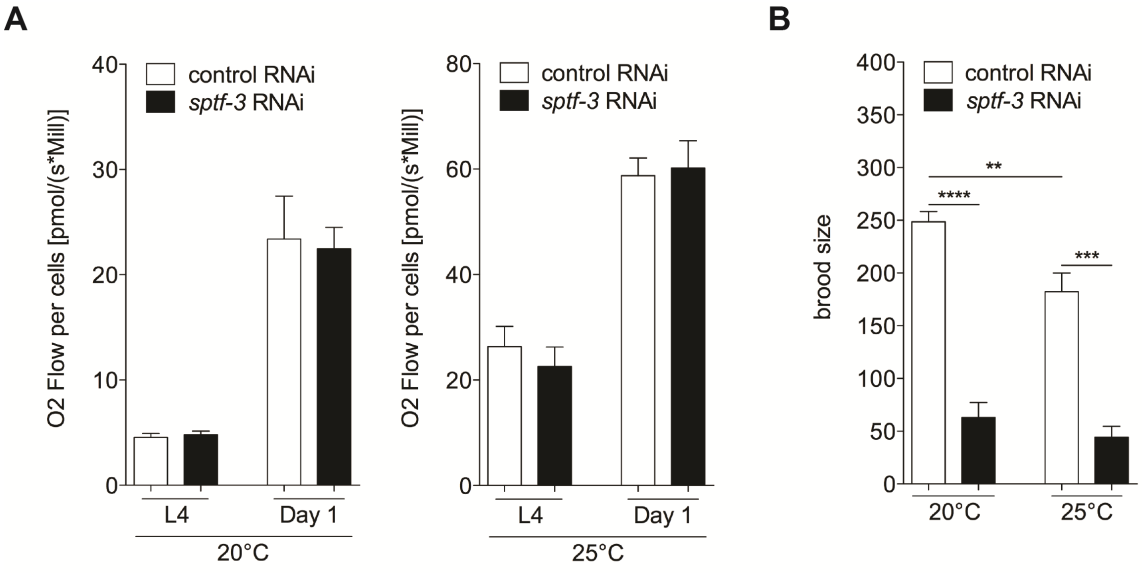

Figure 3.12 Respiration capacity and brood size upon *sptf-3* knockdown
(A) Oxygen consumption measured in animals exposed to control or *sptf-3* RNAi, at 20°C/25°C after reaching L4/day 1 of adulthood (n=6). **(B)** Laid eggs were counted per single animal raised on control or *sptf-3* RNAi at 20°C/25°C. Bars represent mean ± SEM, [Student's t test], p**<0.01, p***<0.001, p****<0.0001, n=6.

3.10 SPTF-3 is a broad-spectrum transcription factor

Recent ChIP-seq experiments revealed that SPTF-3 binds 2,459 genomic regions, indicating its direct transcriptional targets (Hirose and Horvitz, 2013). In that study, two different *C elegans* specific SPTF-3 antibodies, namely N81 and M82, were used for immunoprecipitation and the respective list was generated, containing genes with SPTF-3 binding sites in the respective proximal promoter regions (Hirose and Horvitz, 2013). In that list we searched for genes encoding mitochondrial proteins (Table 3.4). Supporting our previous findings SPTF-3 binding motif was found in the promoter region of *mtss-1* and *cts-1*. No SPTF-3 binding site was found in the promoter region of *aco-2*, suggesting that we observed an indirect effect of *sptf-3* knockdown on *aco-2* expression (Figure 3.8 A). Interestingly *polg-1* and *hmg-5* promoter regions do not appear to contain SPTF-3 binding motifs. However both genes are predicted to belong to distinct operons, together with *pqn-87* and *nrde-4* respectively, which in turn possess SPTF-3 binding elements in proximal promoters. Additionally, SPTF-3 bound regions were detected upstream of a number of RC subunits and mitochondrial ribosomal protein genes. These ChIP-seq data suggests that SPTF-3 is involved in a variety of biological processes, including mitochondrial respiration, translation and mtDNA expression.

Gene	Brief description	M82	N81
Respiratory chain subunits (Human orthologs)			
F31D4.9	NDUFA1	+	+
Y53G8AL.2	NDUFA9	+	+
C34B2.8	NDUF15	+	+
Y18D10A.3	NDUF15	+	+
C50B8.3	NDUFAF1	-	+
Y116A8C.30	NDUFAF2	+	+
Y51H1A.3	NDUFB8	+	+
nuo-5	NDUFS1	+	+
nuo-2	NDUFS3	+	+
nduf-5	NDUFS5	+	-
W10D5.2	NDUFS7	+	+
nuo-1	NDUFV1	+	+
C03G5.1	SDHA	+	+
cyc-1	CYC1	+	+
cyc-2.1	CYCS	+	+
ucr-2.2	UQCRC2	+	+
ucr-2.1	UQCRC2	+	+
ucr-1	UQCRC1	+	+
W09C5.8	COX4I1	+	+
cco-2	COX5A	+	+

Gene	Brief description	M82	N81
Respiratory chain subunits (Human orthologs)			
cco-1	COX5B	+	+
tag-174	COX6A1	-	+
H28O16.1	ATP5A1	+	+
atp-2	ATP5B	+	+
asb-2	ATP5F1	+	+
Y82E9BR.3	ATP5G1	+	+
Y82E9BR.3	ATP5G2	+	+
Y82E9BR.3	ATP5G3	+	+
atp-3	ATP5O	+	+
R04F11.2	ATP5I	+	+
T05H4.12	ATP5J	+	+
asg-2	ATP5L	+	+
Mitochondrial ribosomes (Human orthologs)			
mrps-7	MRPS7	+	+
mrps-12	MRPS12	-	+
mrps-17	MRPS17	+	+
mrps-23	MRPS23	+	+
mrps-34	MRPS34	+	+
mrpl-9	MRPL9	-	+
mrpl-15	MRPL15	+	+
mrpl-17	MRPL17	+	+
mrpl-20	MRPL20	+	+
mrpl-28	MRPL28	+	+
mrpl-32	MRPL32	+	+
mrpl-47	MRPL47	-	+
mrpl-49	MRPL49	+	+
mrpl-51	MRPL51	+	+
mrpl-53	MRPL53	-	+
mrpl-55	MRPL55	+	+
Other mitochondrial proteins			
clpp-1	ClpP	+	+
ZC376.7	ATFS-1	+	+
mtss-1	ortholog of human SSBP1	+	+
cts-1	Citrate Synthase	+	+
Y67H2A.4	MICU 1	+	+
tag-61	ANT-1.1	+	+
pdp-1	Pyruvate Dehydrogenase Phosphatase homolog	+	+
T25G3.4	a putative mitochondrial glycerol-3-phosphate dehydrogenase	+	+
nmat-2	Nicotinamide Mononucleotide AdenylylTransferase homolog	+	-
H25P06.1	hexokinases	+	-
timm-23	tim23	+	+
rpom-1	RNA POlymerase, Mitochondrial	+	-
sco-1	SCO1 cytochrome c oxidase assembly protein	+	+
mtch-1	Mitochondrial carrier homolog 1	+	+
atad-3	ATAD3, mitochondrial membrane bound ATPase	+	+
cchl-1	Cytochrome C Heme-Lyase	+	+
mecr-1	Mitochondrial trans-2- Enoyl-CoA Reductase	+	+
tufm-1	TU elongation Factor (EF- Tu), Mitochondrial	+	+
idhg-1	(Isocitrate DeHydrogenase Gamma)	+	+
akap-1	A-Kinase Anchor Protein	+	+
mdh-2	encodes a homolog of malate dehydrogenase	+	+

Gene	Brief description	M82	N81
	Other mitochondrial proteins		
Y66A7A.2	subunit shared by the endoribonucleases RNase MRP and RNAse P	+	-
wah-1	Worm AIF (apoptosis inducing factor) Homolog	+	-
mai-2	Mitochondrial ATPase Inhibitor family	+	+
srs-1	predicted mitochondrial seryl-tRNA synthetase (SerRS)	+	-
ogdh-1	OxoGlutarate DeHydrogenase	+	+
acl-3	ACyLtransferase-like protein with role in triacylglycerol metabolism	+	+
ech-2	enoyl-coenzyme A hydratase involved in mitochondrial beta-oxidation of fatty acids	+	+
tgt-1	tRNA-guanine transglycosylase	+	+
nkcc-1	Na-K-Cl Cotransporter homolog	+	-
coq-3	involved in COenzyme Q (ubiquinone) biosynthesis	+	+
dhs-13	short-chain dehydrogenase	+	+
tomm-20	Translocase of Outer Mitochondrial Membrane	+	+
letm-1	(Leucine zipper, EF-hand, TransMembrane mitochondrial protein	+	+
pus-1	putative tRNA pseudouridine synthase	+	+
immt-1	Inner Membrane of MiTochondria protein homolog	+	+
mai-1	Mitochondrial ATPase Inhibitor family	+	+
got-2.2	Glutamate Oxaloacetate Transaminase	+	+
dct-1	DAF-16/FOXO Controlled, germline Tumor affecting	+	+
dhb-1	homolog of the beta subunit of an NAD+-dependent mitochondrial isocitrate dehydrogenase	+	+

Table 3.4 Putative transcriptional targets of SPTF-3
(+)/(-) Indicates the precipitation status of the genomic region with the respective antibody. List adapted from (Hirose and Horvitz, 2013).

We next wanted to know whether *sptf-3* knockdown affects transcript and consequently protein levels of genes with mitochondrial function. Inhibiting *sptf-3* activity was shown to affect oocyte and vulval formation, sometimes leading to animals retaining the eggs (Ulm et al., 2011). Trapped embryos might skew results from transcript and protein level analysis of parental postmitotic tissues. Therefore we decided to use animals, which lack progeny. Thus we crossed the *mtss-1$_{pr}$ ::gfp* reporter strain to the temperature sensitive mutant *glp-4(bn2)*, which gets sterile if shifted to 25°C. We determined transcript and protein levels of *nuo-2*, ortholog of human *NDUFS3* and mtDNA-encoded *ctc-1*, ortholog of human *COXIV-1* (Figure 3.13 A, B, C). Though we detected decreased transcript levels of *ctc-1* upon *sptf-3* knockdown, we did not observe any changes on protein level, neither for NDUFS-3 nor for COXIV-1. Decreased levels of *mtss-1* and *hmg-5* upon *sptf-3* knockdown reproduced our results from Figure 3.3 A, once again supporting SPTF-3 involvement in the expression of these genes (Figure 3.13 A). At 25°C, impaired *sptf-3* function led to GFP decrease on mRNA and protein levels (Figure 3.13 B, C). Due to the lack of working antibodies in *C. elegans* we were not able to investigate protein quantities of further candidates from the list. Collectively, these data indicate that

under chosen conditions, *sptf-3* knockdown reduces transcript but not protein levels of tested RC subunits, which is supported by unchanged respiration rates (Figure 3.12 A).

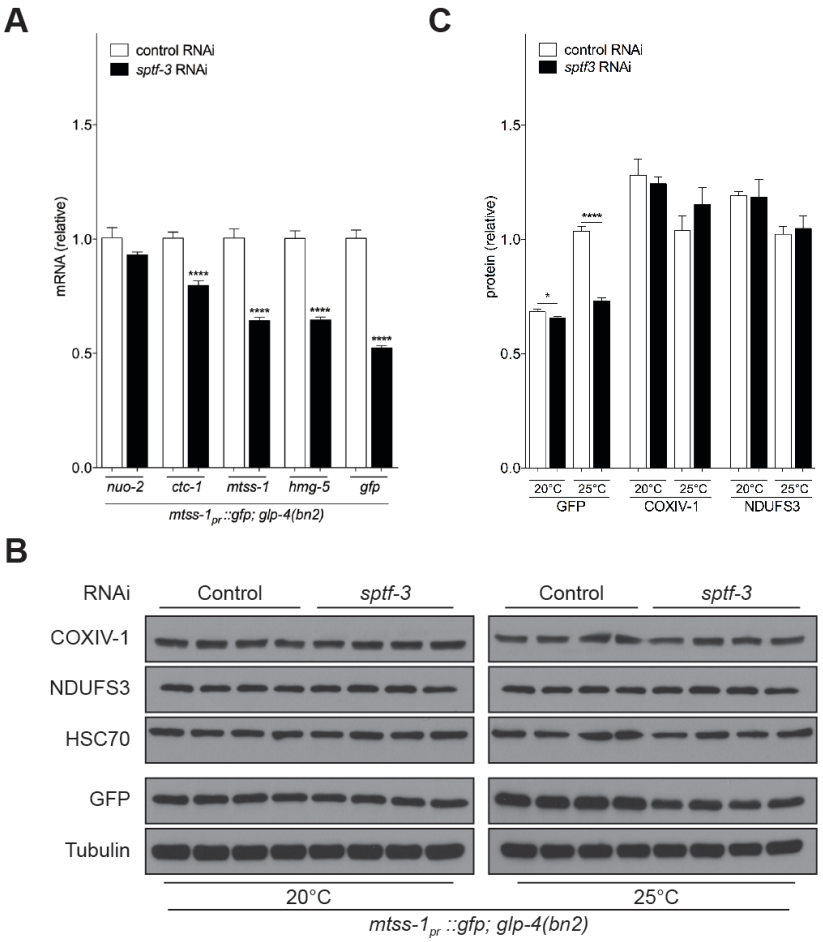

Figure 3.13 Transcript and protein levels of genes with mitochondrial function upon *sptf-3* knockdown
(A) Relative transcript levels of *nuo-2, ctc-1, mtss-1*, *hmg-5* and *gfp* in *mtss-1$_{pr}$::gfp; glp-4(bn2)* worms raised at 25°C and exposed to RNAi. Bars represent mean ± SEM, [Student's t test], p****<0.0001, n=6. **(B)** Western blot analysis of COXIV-1, NDUFS3, and GFP levels in total protein extracts from worms raised on control or *sptf-3* RNAi at 20°C/25°C. HSC70 and Tubulin used as loading control. **(C)** Relative quantification of COXIV-1 and NDUFS3 amounts normalized to HSC70; GFP normalized to Tubulin. Eventually relative amounts were normalized to their respective control RNAi, 25°C sample. Bars represent mean ± SEM, [Student's t test], p*<0.05, p****<0.0001, n=4.

3.11 *mtss-1$_{pr}$::gfp* suppressor screen

Disclaimer: Paragraphs 3.11, 3.12 and 3.13 contain experiments partly conducted by Estela Cepeda Cores and Brianne Nesbitt, unpublished data.

Another screening approach was set up to identify candidate genes potentially suppressing mitochondrial biogenesis. The experimental outline was the same as in Figure 3.1 C, except this time we were interested in finding knockdown conditions, leading to increased mitochondrial biogenesis, represented by induced *mtss-1$_{pr}$ ::gfp* expression. Hence, the worms were raised at standard temperature of 20°C, at which the expression level of GFP is low (Figure 3.1 A). When exposing animals to RNAi, we were looking for genes whose knockdown resulted in enhanced GFP signal. Importantly, candidates from the suppressor screen do not necessarily actively suppress reporter expression. There is also the possibility that only their inhibition leads to *mtss-1$_{pr}$ ::gfp* induction.

Screening through the *C. elegans* chromosome III, testing ~2.100 RNAi clones, we found 31 candidates that increased GFP expression compared to control treatment (Table 3.5). Notably, 7 of the candidates represent genes encoding distinct cytosolic ribosomal proteins. Among 31 candidates, knockdown of RPGs caused the strongest GFP induction. Moreover, diminished RPG function resulted in delayed development, with a large part of the animals not being able to pass the larval stages at all (Figure 3.14 A). They were smaller in size and the ones that reached adulthood had massively reduced brood size. To overcome the developmental arrest we exposed worms to RNAi from the L3 stage on and still could observe strong induction of GFP expression (Figure 3.14 B). Over 80% of worms exposed to RNAi mediated RPG knockdown exhibited strong GFP expression, based on visual observations (Figure 3.14 C) and Western blot analysis (Figure 3.14 D). Remarkably, the effect of RPG knockdown was so robust that the level of GFP expression was higher than in control worms even in animals kept at 25°C (Figure 3.14 A, B). Together these data indicate that *mtss-1* expression is induced upon knockdown of cytoplasmic ribosomal protein genes, independently of temperature and developmental stage. At first sight, it seems irrelevant, whether small or large ribosomal subunit is affected.

Gene	Brief description	GFP signal
control	L4440 empty vector	→
rps-0	Small ribosomal subunit SA	↑↑
rps-12	Small ribosomal subunit S12	↑↑
rps-1	Small ribosomal subunit S3A	↑↑
rps-22	Small ribosomal subunit S15a protein	↑↑
rpl-9	Ribosomal protein L9	↑↑
rpl-35	Ribosomal protein L35	↑↑
rpl-3	Ribosomal protein L3	↑↑
C34C12.8	Adenine nucleotide exchange factor of DnaK (Hsp70)-type ATPases	↗
T24C4.5	DNA primase, catalytic (small) subunit	↗
W06E11.1	DNA-directed RNA polymerase III subunit RPC5	↗
hum-5	Myosin heavy chain	↗
atp-2	F1 subunit of the ATP synthase subunit	↑
cdtl-7	*cdc-2* related protein kinase	↗
rpc-2	RNA polymerase III, second largest subunit	↑
polq-1	Most closely related to vertebrate DNA polymerase theta (POLQ)	↗
him-10	Structurally related to the Nuf2 kinetochore proteins	↗
F01F1.11	Canopy FGF signalling regulator 1	↗
mig-21	Transmembrane protein	↗
pars-1	Cytoplasmic prolyl-tRNA synthetase	↗
ubq-1	Ortholog of human ubiquitin C	↗
hmg-4	High mobility group protein SSRP1	↗
hpl-2	Negatively regulates RNA-mediated interference (RNAi)	↗
Y47D3A.29	Catalytic subunit of DNA polymerase alpha	↗
rabx-5	Rabex-5 Rab5 guanine-nucleotide exchange factor	↗
ubq-2	ubiquitin peptide and large ribosomal subunit protein L40 peptide domains	↗
rpt-6	Triple A ATPase, subunit of the 26S proteasome 19S RP base subcomplex	↗
ccf-1	Homolog of subunit 7 of CCR4-NOT transcription complex from *S. cerevisiae*	↗
mrg-1	Chromodomain-containing protein orthologous to mammalian MRG15	↗
T27E9.2	Ubiquinol-cytochrome c reductase hinge protein	↗
ubl-1	Protein similar to Drosophila ubiquitin/ ribosomal protein S27a	↗
ral-1	Ras-related GTPase homolog	↗

Table 3.5 Candidates from the suppressor screen conducted at 20°C

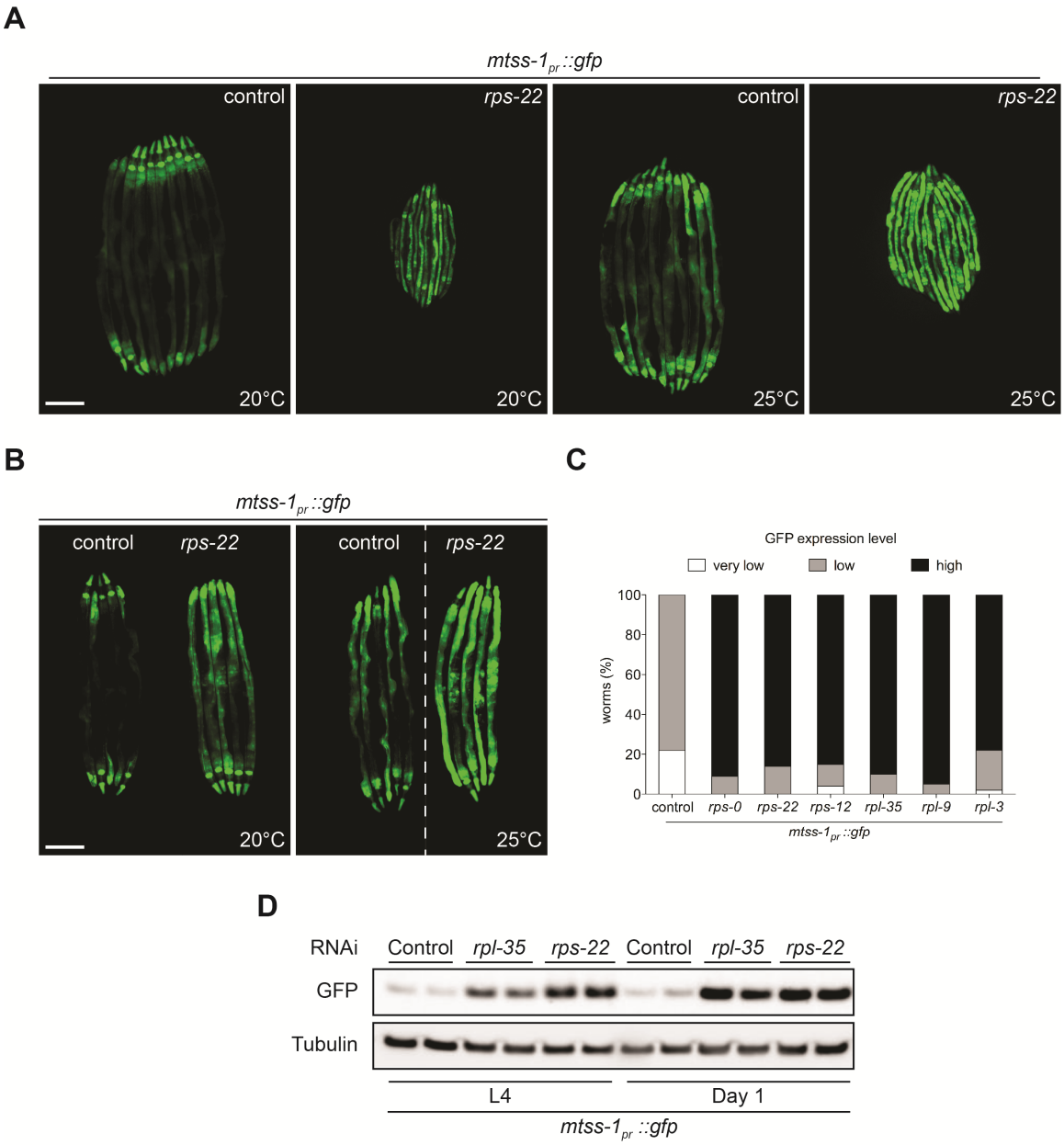

Figure 3.14 Reducing cytoplasmic ribosomes activates *mtss-1$_{pr}$::gfp* reporter
Representative photomicrographs of *mtss-1$_{pr}$::gfp* transgenic worms raised on control or *rps-22* RNAi at 20°C/25°C **(A)** from egg or **(B)** from L3 stage. Dashed line indicates two independent snap shots, yet identical exposure time and color adjustments. Scale bar, 0.2 mm. **(C)** GFP expression levels were compared between singe *mtss-1$_{pr}$::gfp* transgenic worms raised on control or *rps-0, rps-22, rps-12, rpl-35, rpl-9 or rpl-3* RNAi at 20°C and classified as very low, low and high (n=100). **(D)** Western blot analysis of GFP levels in total protein extracts from worms raised on control, *rpl-35* or *rps-22* RNAi at 20°C and collected at L4 stage or day 1 of adulthood. Tubulin was used as a loading control.

3.12 From factors involved in cytoplasmic translation only knockdown of ribosomal protein genes induces *mtss-1$_{pr}$::gfp* expression

Given the fact that ribosomes are not solely forming translation machinery we next tested the hypothesis that impairing other genes involved in translation would likely induce GFP expression. Additionally, we tested whether knockdown of the remaining RPGs results in the previously observed phenotype. We subjected *mtss-1$_{pr}$::gfp* worms to all available RNAi clones covering the entire spectrum of annotated cytosolic ribosomal proteins, translation initiation/elongation/termination factors and factors known to influence translation rates (Table 3.6). Strikingly, 85% of all silenced RPGs induced *mtss-1$_{pr}$::gfp* expression. Again, it did not matter whether subunits of small or large ribosomes were affected. By contrast, knockdown of all the other selected factors, involved in translation associated processes or in modulating translation rates, did not alter *mtss-1$_{pr}$::gfp* expression.

TOR kinase is a potent regulator of cellular growth and metabolism and can be activated in response to nutrients, growth factors and energy status of the cell. TOR acts in two distinct complexes, TORC1 and TORC2, and interestingly ribosomes are required for TORC2 signaling independently of protein synthesis (Zinzalla et al., 2011). This prompted us to take a closer look into *C. elegans* TOR (*Ce*TOR) signaling, as only 2 out of 5 known components of TOR complexes were among initially tested factors, namely *let-363* and *daf-15* (Table 3.6). In *C. elegans*, TORC1 is comprised of *daf-15*, *let-363* and *C10H11.8*, while TORC2 is composed of *let-363*, *C10H11.8*, *rict-1* and *sinh-1*. For practical reasons, we chose one RPG, namely *rps-22,* and systematically silenced it in double RNAi experiments with genes encoding components of *Ce*TOR complexes (Figure 3.15 A). Additionally we tested the effect of reduced *Ce*TORC subunits only. Eventually we analyzed the effect of all RNAi combinations on *mtss-1$_{pr}$::gfp* expression using BioSorter®INSTRUMENT (Figure 3.15 B). Knockdown of single genes encoding subunits of *Ce*TOR complexes either mildly decreased (in case of *daf-15*, *sinh-1*) or increased (in case of *let-363*, *C10H11.8*, *rict-1*) GFP levels. Notably, conditions that induced *mtss-1$_{pr}$::gfp* expression did not reach induction level of *rps-22* knockdown. Further, single *Ce*TORC gene knockdown, combined with *rps-22* knockdown, did not alter GFP levels, compared to worms exposed to *rps-22* RNAi only. Only silencing of *C10H11.8* in combination with

rps-22 showed a slight decrease in GFP levels (Figure 3.15 B). Overall, the effect of *Ce*TORC related gene knockdown on *mtss-1$_{pr}$::gfp* expression was inconsistent and the amplitude of reporter expression changes was much smaller compared to the *rps-22* RNAi condition. Collectively these data demonstrate that from all translation related factors only knockdown of ribosomal protein genes has the capacity to strongly activate the *mtss-1$_{pr}$::gfp* reporter and this effect is largely independent of *Ce*TOR signaling. Moreover, these results suggest that not the global translation rate but altered stoichiometric balance among ribosomal proteins and the associated consequences are responsible for reporter induction.

Sequence	Name	GFP
control	L4440	➔
Cytosolic ribosomes		
Y71F9AL.13	*rpl-1*	↑↑
F10B5.1	*rpl-10*	↑↑
T22F3.4	*rpl-11.1*	↑↑
F07D10.1	*rpl-11.2*	↑↑
JC8.3	*rpl-12*	↑↑
C32E8.2	*rpl-13*	↑↑
C04F12.4	*rpl-14*	↑↑
M01F1.2	*rpl-16*	↑↑
Y48G8AL.8	*rpl-17*	↑↑
Y45F10D.12	*rpl-18*	↑↑
C09D4.5	*rpl-19*	↑↑
B0250.1	*rpl-2*	↑↑
E04A4.8	*rpl-20*	↑↑
C14B9.7	*rpl-21*	↑↑
B0336.10	*rpl-23*	↑↑
D1007.12	*rpl-24.1*	↑↑
F28C6.7	*rpl-26*	↑↑
C53H9.1	*rpl-27*	↑↑
F13B10.2	*rpl-3*	↑↑
Y106G6H.3	*rpl-30*	↑↑
T24B8.1	*rpl-32*	↑↑
F10E7.7	*rpl-33*	↑↑
C42C1.14	*rpl-34*	↑↑
ZK652.4	*rpl-35*	↑↑
F37C12.4	*rpl-36*	↑↑
C54C6.1	*rpl-37*	↑↑
C06B8.8	*rpl-38*	↑↑
C09H10.2	*rpl-41*	↑↑
F54C9.5	*rpl-5*	↑↑
R151.3	*rpl-6*	↑↑
F53G12.10	*rpl-7*	↑↑
Y24D9A.4	*rpl-7A*	↑↑
R13A5.8	*rpl-9*	↑↑
B0393.1	*rps-0*	↑↑
F56F3.5	*rps-1*	↑↑
D1007.6	*rps-10*	↑↑
F40F11.1	*rps-11*	↑↑
F54E7.2	*rps-12*	↑↑
C16A3.9	*rps-13*	↑↑
F37C12.9	*rps-14*	↑↑
T01C3.6	*rps-16*	↑↑
T08B2.10	*rps-17*	↑↑
Y57G11C.16	*rps-18*	↑↑
C49H3.11	*rps-2*	↑↑
Y105E8A.16	*rps-20*	↑↑
F37C12.11	*rps-21*	↑↑
F53A3.3	*rps-22*	↑↑
F28D1.7	*rps-23*	↑↑
T07A9.11	*rps-24*	↑↑
F39B2.6	*rps-26*	↑↑
F56E10.4	*rps-27*	↑↑
Y41D4B.5	*rps-28*	↑↑
B0412.4	*rps-29*	↑↑
C26F1.4	*rps-30*	↑↑
Y43B11AR.4	*rps-4*	↑↑
T05E11.1	*rps-5*	↑↑
ZC434.2	*rps-7*	↑↑
F42C5.8	*rps-8*	↑↑
F40F8.10	*rps-9*	↑↑
T05F1.3	*rps-19*	↑↑
K11H12.2	*rpl-15*	➔
C03D6.8	*rpl-24.2*	➔
F52B5.6	*rpl-25.2*	➔
R11D1.8	*rpl-28*	➔
B0513.3	*rpl-29*	➔
B0041.4	*rpl-4*	➔
C09H10.1	*rpl-42*	➔
F36A2.6	*rps-15*	➔
K02B2.5	*rps-25*	➔
C23G10.3	*rps-3*	➔
Translation initiation factors		
T27F7.3	*eif-1*	➔
H06H21.3	*eif-1.A*	➔
E04D5.1	(eIF2A)	➔
Y37E3.10	(eIF2S1)	➔
K04G2.1	*iftb-1*	➔
C01G10.9	(MRI1)	➔
ZK1098.4	(eIF2B1)	➔
Y47H9C.7	(eIF2B2)	➔
D2085.3	(eIF2BE)	➔
C27D11.1	*egl-45* (eIF3A)	➔
F55H2.6	*clu-1*	➔
Y54E2A.11	*eif-3.B*	➔
T23D8.4	*eif-3.C*	➔
R08D7.3	*eif-3.D*	➔
B0511.10	*eif-3.E*	➔
D2013.7	*eif-3.F*	➔
F22B5.2	*eif-3.G*	➔
C41D11.2	*eif-3.H*	➔
Y40B1B.5	*eif-3.J*	➔
T16G1.11	*eif-3.K*	➔
F57B9.6	*inf-1*(EIF4A)	➔
C07H6.5	*cgh-1*	➔
C26D10.2	*hel-1*	➔
F53A2.6	*ife-1* (eIF4E)	➔
R04A9.4	*ife-2* (eIF4E)	➔
B0348.6	*ife-3* (eIF4E)	➔
C05D9.5	*ife-4* (eIF4E)	➔
M110.4	*ifg-1* (eIF4G)	➔
C37C3.2	(eIF5)	➔
F54C9.1	*iff-2* (eIF5A2)	➔
T05G5.10	*iff-1* (eIF5A)	➔
Y54F10BM.2	*iffb-1* (eIF5B)	➔
Translation elongation factors		
F31E3.5	*eef-1A.1*	➔
R03G5.1	*eef-1A.2*	➔
Y41E3.10	*eef-1B.2*	➔
F54H12.6	*eef-1B.1*	➔
F25H5.4	*eef-2*	➔
ZK328.2	*eftu-2*	➔
Translation termination factors		
T05H4.6	*erfa-1*	➔
H19N07.1	*erfa-3*	➔
Translational control		
Y71H2B.3	*ppfr-4*	➔
Y75B8A.30	*pph-4.1*	➔
Y49E10.3a	*pph-4.2*	➔
C12D8.10	*akt-1*	➔
F46C3.1	*pek-1*	➔
K08A8.1	*mek-1*	➔
F42G10.2	*mkk-4*	➔
C04G6.1	*mpk-2*	➔
F09C12.2	(MAPK)	➔
C41C4.4	*ire-1*	➔
B0261.2	*let-363*	➔
T06E4.3	*atl-1*	➔
ZC8.6	(PI4K2A/B)	➔
C56A3.8	(PI4K2A/B)	➔
C10C5.6	*daf-15*	➔
Y48G9A.3	*gcn-1*	➔
Y81G3A.3	*gcn-2*	➔

Table 3.6 List of factors related to translation and their knockdown effect on *mtss-1$_{pr}$ ::gfp* expression

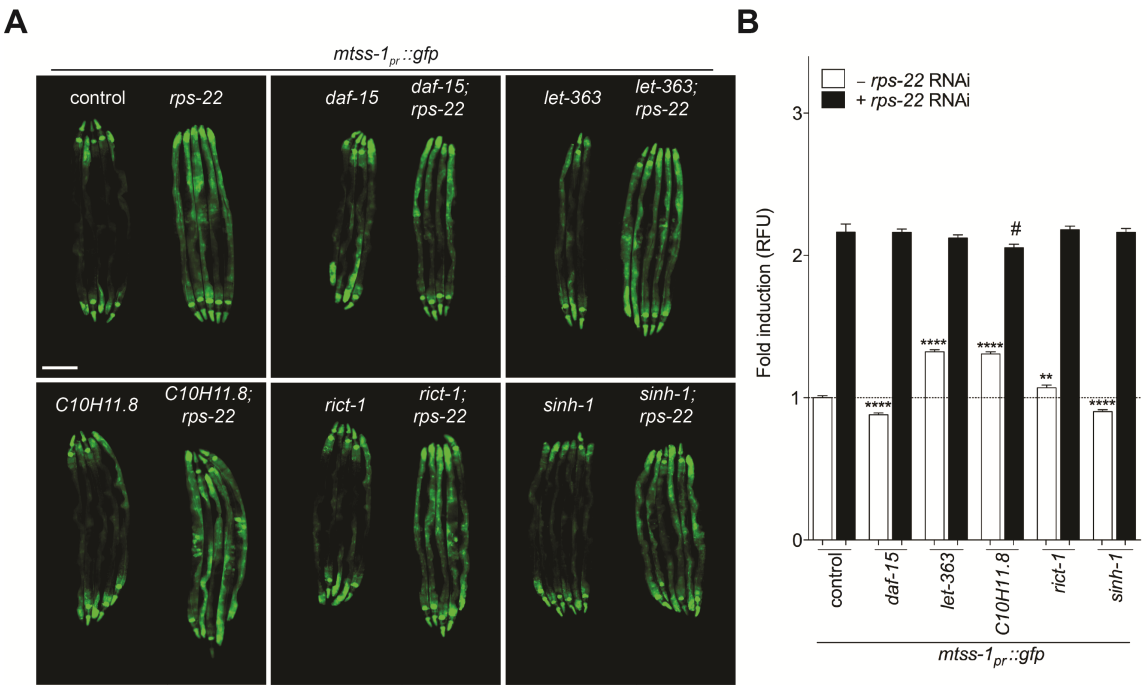

Figure 3.15 *Ce*TOR signaling and *rps-22* knockdown
(A) Representative photomicrographs and respective **(B)** BioSorter®INSTRUMENT analysis of *mtss-1$_{pr}$::gfp* transgenic worms raised on control or *rps-22, daf-15, let-363, C10H11.8, rict-1; sinh*-1 alone and in combination with *rps-22* RNAi at 20°C. Scale bar, 0.2 mm. Bars represent mean ± SEM, [Student's t test], p#<0.05, p**<0.01, p****<0.0001, n≥230. * represents analysis of fluorescent signal intensity between worms exposed to single RNAi and empty vector, white bars. # represents analysis of fluorescent signal intensity between worms exposed to double RNAi and *rps-22* RNAi, black bars. RFU, relative fluorescence units. RNAi from L3 stage.

3.13 SPTF-3 is the only transcription factor able to alter *rps-22* knockdown mediated *mtss-1$_{pr}$::gfp* induction

Next we wanted to gain more insight into the relation between ribosomal subunits and *mtss-1* expression. Silencing of RPGs increases GFP protein levels. We assumed that it also increases *gfp* transcripts, which most likely is mediated by a transcription factor. Therefore, in double RNAi experiments we screened for transcription factors, whose knockdown would abolish GFP induction achieved by *rps-22* knockdown. From 729 transcription factors tested, only knockdown of *sptf-3* abolished the effect of downregulated *rps-22* (Figure 3.16 A). Moreover, the loss of SPTF-3 prevented temperature dependent GFP induction, consistent with previous observations. Not only were GFP levels reduced but also endogenous MTSS-1 protein levels, which were determined using polyclonal mouse antibody raised against human SSBP1 (Figure 3.16 B). Further, we wanted

to know whether RPG knockdown affects *sptf-3* expression. Strikingly, *rps-22* knockdown strongly increased SPTF-3 protein levels (Figure 3.16 C). This effect was so robust, that upon *sptf-3*; *rps-22* double knockdown, SPTF-3 was still detectable at the level of control samples. Importantly, this induction was not a direct consequence of induced *sptf-3* transcription, as *sptf-3* mRNA levels were unchanged upon *rps-22* knockdown (Figure 3.16 D).

Collectively, these data suggest that *sptf-3* function is required for enhanced *mtss-1$_{pr}$::gfp* expression, induced by *rps-22* knockdown. Further, reduction of ribosomal subunits increases MTSS-1 and SPTF-3 protein levels. Moreover, increased SPTF-3 protein but unchanged *sptf-3* mRNA levels indicate that *rps-22* knockdown leads to enhanced *sptf-3* translation.

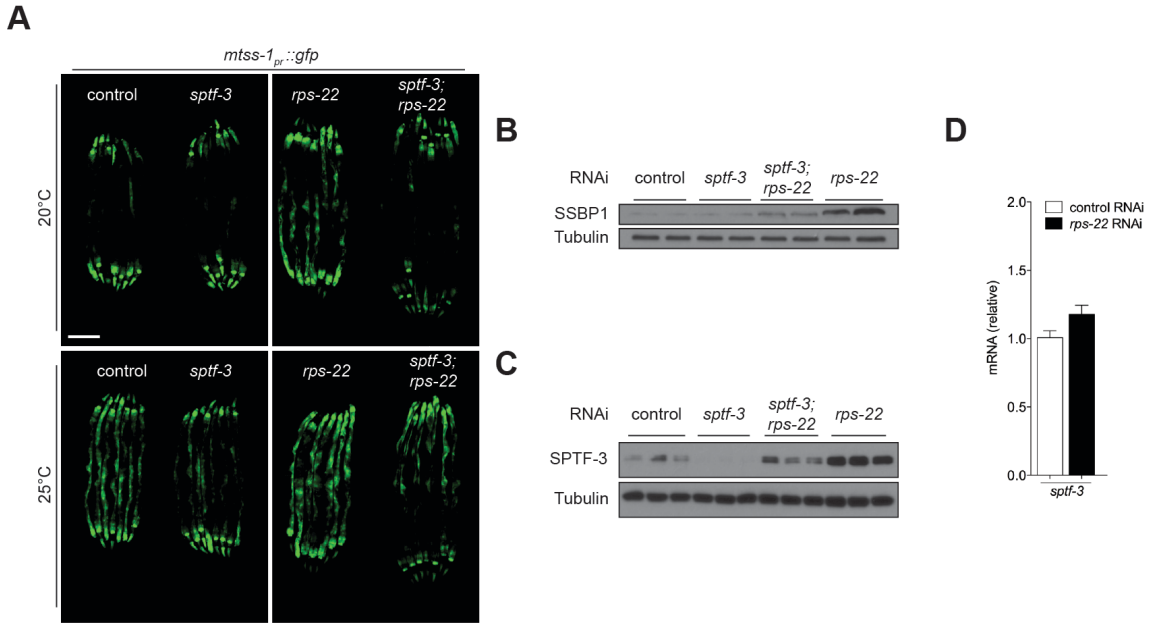

Figure 3.16 SPTF-3 is required for *mtss-1$_{pr}$::gfp* expression induced by *rps-22* knockdown
(A) Representative photomicrographs of *mtss-1$_{pr}$ ::gfp* transgenic worms raised on control, *sptf-3*, *rps-22* or *sptf-3*; *rps22* double RNAi at 20°C/25°C. RNAi from L3 stage. Scale bar, 0.2 mm. **(B)** Western blot analysis of SSBP1 and **(C)** SPTF-3 levels in total protein extracts from worms raised on control, *sptf-3*, *rps-22* or *sptf-3*; *rps22* double RNAi at 20°C and collected at day 1 of adulthood. Tubulin used as loading control. **(D)** Relative transcript levels of *sptf-3* in *mtss-1$_{pr}$ ::gfp* worms, exposed to control and *rps22* RNAi at 20°C. Bars represent mean ± SEM, [Student's t test], n=6.

3.14 *rps-22* knockdown effect is not mediated via GCN-2 kinase

Accumulation of misfolded proteins in the endoplasmic reticulum (ER) activates a specific, multilane stress response. One of the response components is the reduction of cytosolic translation rate by attenuating translation initiation. For that, ER stress activates PEK-1 kinase (homolog of the mammalian PERK) that phosphorylates eIF2α, thereby interfering with the formation of the ternary complex and diminishing translation initiation (Harding et al., 1999; Hinnebusch, 1994). Another dedicated eIF2α kinase in *C. elegans* is GCN-2. Interestingly, in response to mitochondrial stress GCN-2 has been shown to phosphorylate eIF2α thereby inhibiting cytosolic translation (Baker et al., 2012). Therefore, we decided to test whether induction of *mtss-1$_{pr}$ ::gfp* expression by *rps-22* knockdown integrates eIF2α activity. Levels of phosphorylated eIF2α were strongly reduced in *sptf-3*; *rps-22* double RNAi condition and almost absent when exposed to *rps-22* RNAi only, suggesting that translation initiation is rather favored upon *rps-22* knockdown (Figure 3.17 A). Unfortunately, we could not quantify the levels of total eIF2α, as the antibody raised against mammalian homolog recognizes epitopes not conserved in nematodes. Detection of the phosphorylated eIF2α was possible as the region containing the phosphorylation site is highly conserved among different species, including *C. elegans*. Nevertheless, this result suggests a possibility of eIF2α contribution to the *rps-22* knockdown induced *mtss-1$_{pr}$::gfp* expression.

Potentially reduced levels of P-eIF2α upon *rps-22* knockdown prompted us to retest, whether reduction of eIF2α phosphorylation would be sufficient to induce *mtss-1$_{pr}$::gfp* expression. One possibility to reduce eIF2α phosphorylation is to inhibit *gcn-2* function, which we have done in the context of aforementioned experiment (Table 3.6). However, this time we used *gcn-2(ok871)* 1.481 bp deletion mutant in order to completely prevent GCN-2 mediated eIF2α phosphorylation. Assuming that lack of GCN-2 decreases levels of phosphorylated eIF2α, we tested *mtss-1$_{pr}$::gfp* expression in *gcn-2(ok871)* animals. Additionally, we tested *mtss-1$_{pr}$::gfp* expression in *ife-2(ok306)* 1.628 bp deletion mutant with reduced accumulation of newly synthesized proteins (Hansen et al., 2007). The effect on translation is caused by the function of *ife-2* encoding the translation initiation factor 4, subunit eIF-4E, which is involved in cap-dependent mRNA translation initiation. Again, instead of RNAi mediated knockdown we used a mutant strain in order to be sure

that animals did not retain residual IFE-2 activity. Both mutant backgrounds, *gcn-2(ok871)* and *ife-2(ok306)*, significantly reduced *mtss-1$_{pr}$ ::gfp* expression in worms raised at 20°C (Figure 3.17 B). As we have shown previously, temperature increase induces *mtss-1$_{pr}$ ::gfp* expression (Figure 3.1 A, 3.17 B). Strikingly, both mutations prevented enhanced GFP expression at 25°C (Figure 3.17 B). Eventually, if our assumption that lack of GCN-2 decreases levels of phosphorylated eIF2α was correct, then dephosphorylated eIF2α *per se* does not induce *mtss-1$_{pr}$::gfp* expression. Although lack of *gcn-2* does not necessarily mean predominantly dephosphorylated form of eIF2α, as it still can be phosphorylated by PEK-1.

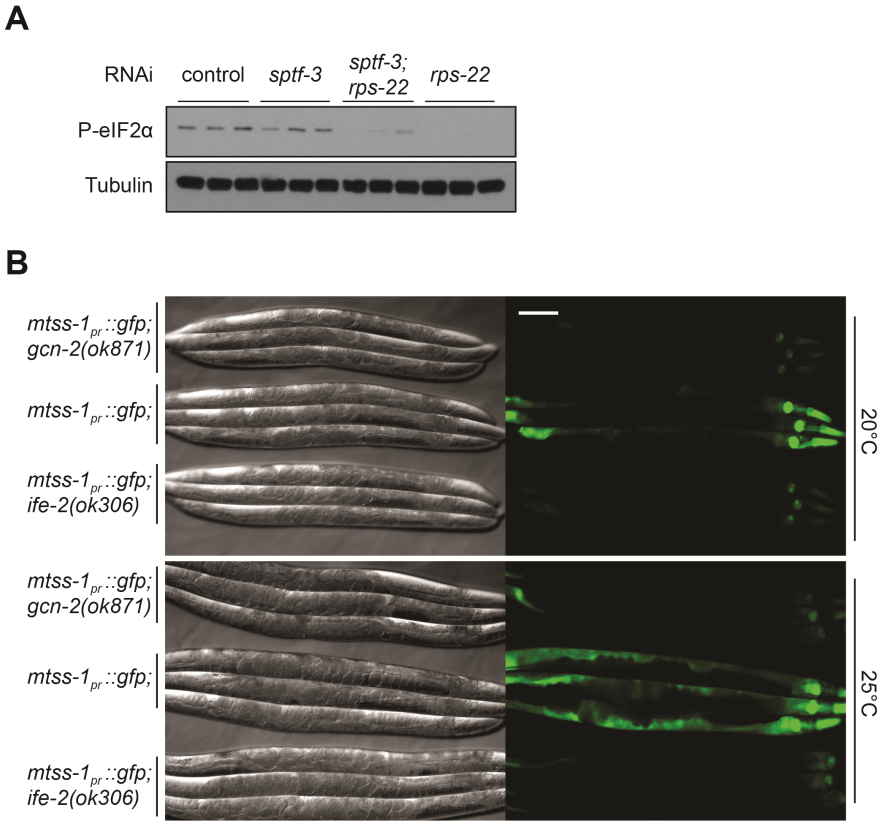

Figure 3.17 *gcn-2(ok871)* and *ife-2(ok306)* impact on *mtss-1$_{pr}$::gfp*
(A) Western blot analysis of P-eIF2α in total protein extracts from worms raised on control, *sptf-3*, *rps-22* or *sptf-3*; *rps22* double RNAi at 20°C and collected at day 1 of adulthood. Tubulin used as loading control. (B) Representative photomicrographs of *mtss-1$_{pr}$ ::gfp* transgenic worms and *mtss-1$_{pr}$::gfp* crossed to *gcn-2(ok871)* and *ife-2(ok306)* mutants, raised at 20°C/25°C. Scale bar, 0.1 mm.

We then tested the impact of *rps-22* inhibition on *mtss-1$_{pr}$ ::gfp* in *gcn-2(ok871)* and *ife-2(ok306)* backgrounds. To overcome the developmental delay caused by RPG knockdown, we diluted *rps-22* RNAi with control bacteria in 1:1 ratio. Silencing *rps-22* robustly induced GFP expression in both mutants (Figure 3.18 A). Nevertheless, both

mutations prevented increase of GFP expression to the level observed in control animals (Figure 3.18 B). Quantitative PCR analysis revealed clear increase of *gfp* transcripts in both mutants upon *rps-22* knockdown (Figure 3.18 C). However, *mtss-1* transcripts were mildly increased in *gcn-2(ok871)* and unchanged in *ife-2(ok306)* background upon *rps-22* knockdown, indicating that changes are more pronounced in the expression of the transgenic reporter than endogenous *mtss-1*.

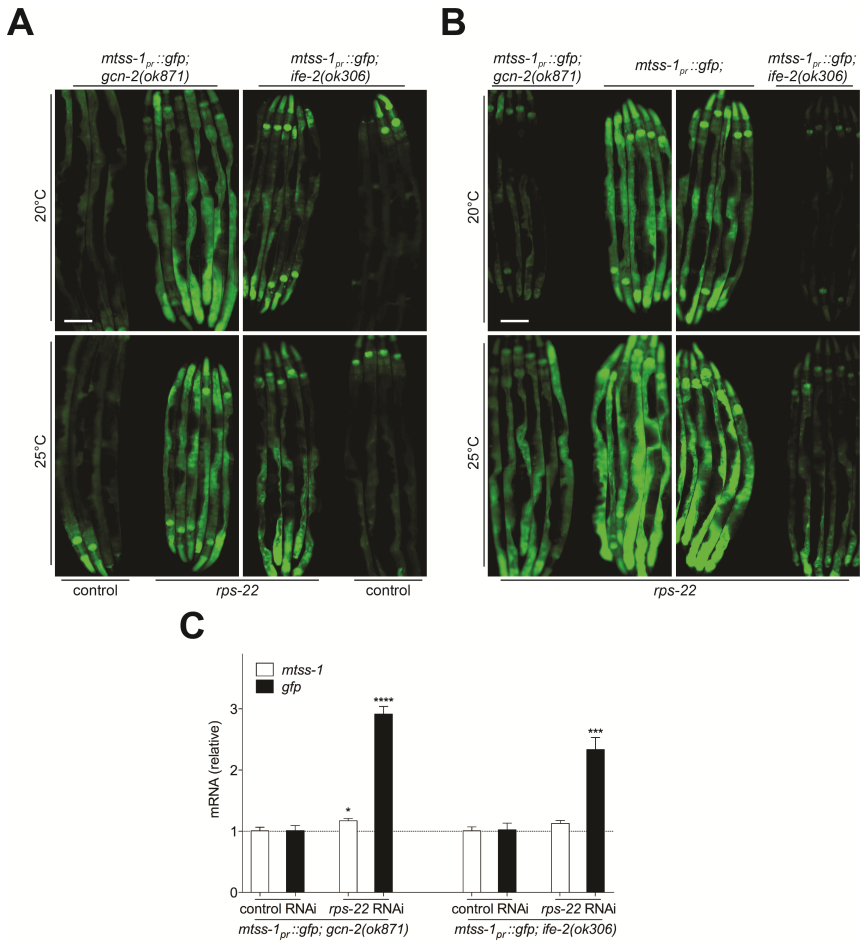

Figure 3.18 *mtss-1$_{pr}$::gfp* induction in *gcn-2(ok871)* and *ife-2(ok306)* mutants
(A) Representative photomicrographs of *mtss-1$_{pr}$::gfp* transgenic worms crossed to *gcn-2(ok871)* and *ife-2(ok306)* mutants, raised at 20°C/25°C on control or *rps-22* RNAi. *rps-22* diluted 1:1 with control RNAi. Scale bar, 0.1 mm. **(B)** Representative photomicrographs of *mtss-1$_{pr}$::gfp* transgenic worms and *mtss-1$_{pr}$::gfp* crossed to *gcn-2(ok871)* and *ife-2(ok306)* mutants, raised at 20°C/25°C on *rps-22* RNAi. *rps-22* diluted 1:1 with control RNAi. Scale bar, 0.1 mm. **(C)** Relative transcript levels of *mtss-1* and *gfp* in *mtss-1$_{pr}$::gfp; gcn-2(ok871)* and *mtss-1$_{pr}$::gfp; ife-2(ok306)* worms raised at 20°C and exposed to control or *rps-22* diluted 1:1 with control RNAi. Bars represent mean ± SEM, [Student's t test], p*<0.05, p***<0.001, p****<0.0001, n=6.

Collectively these data indicate that *rps-22* knockdown induces *mtss-1$_{pr}$::gfp* expression largely independent of *gcn-2* function. It is not clear which role the

phosphorylation state of eIF2α plays upon *rps-22* inhibition but presumably translation initiation is favored though total eIF2α levels have to be determined. Furthermore, reduced GFP protein levels and unchanged *mtss-1* and *gfp* transcript levels in *gcn-2(ok871)* and *ife-2(ok306)* mutants support the assumption that reduction of general translation does not induce *mtss-1*$_{pr}$*::gfp* expression, consistent with conclusions from RNAi experiments in 3.12. Reduction of global translation and knockdown of *rps-22* have rather contrary effects on *mtss-1*$_{pr}$*::gfp* expression, inhibitory and activating respectively.

3.15 *sptf-3* knockdown impairs UPR^{mt} induction

After discovering that SPTF-3 plays critical role in *mtss-1* expression, we then asked what is the consequence of *sptf-3* knockdown for mitochondrial function. When *mtss-1* expression rate does not match mitochondrial requirements it might lead to functional disturbance of the organelle, which in turn possibly activates stress pathways in order to restore functionality. Thus, we decided to investigate *sptf-3* function in activation of stress responses. A number of transcriptional fusion reporters have been used to monitor expression of genes required to cope with compartment specific perturbations. First we examined whether downregulation of *sptf-3* leads to activation of stress response pathways. For this we knocked down *sptf-3* in worms expressing *gst-4*$_{pr}$*::gfp* as oxidative stress reporter (Figure 3.19 A), *hsp-4*$_{pr}$*::gfp* as endoplasmatic reticulum unfolded protein response (UPR^{er}) reporter (Figure 3.19 B) and *hsp-6*$_{pr}$*::gfp* as UPR^{mt} reporter (Figure 3.19 C). Inhibition of *sptf-3 per se* did not induce any stress response, suggesting that loss of *sptf-3* does not generate compartment specific stress (Figure 3.19 A, B, C). Slight induction of *gst-4*$_{pr}$*::gfp* and *hsp-4*$_{pr}$*::gfp* at 25°C is rather caused by increased temperature and not by *sptf-3* knockdown (Figure 3.19 A, B).

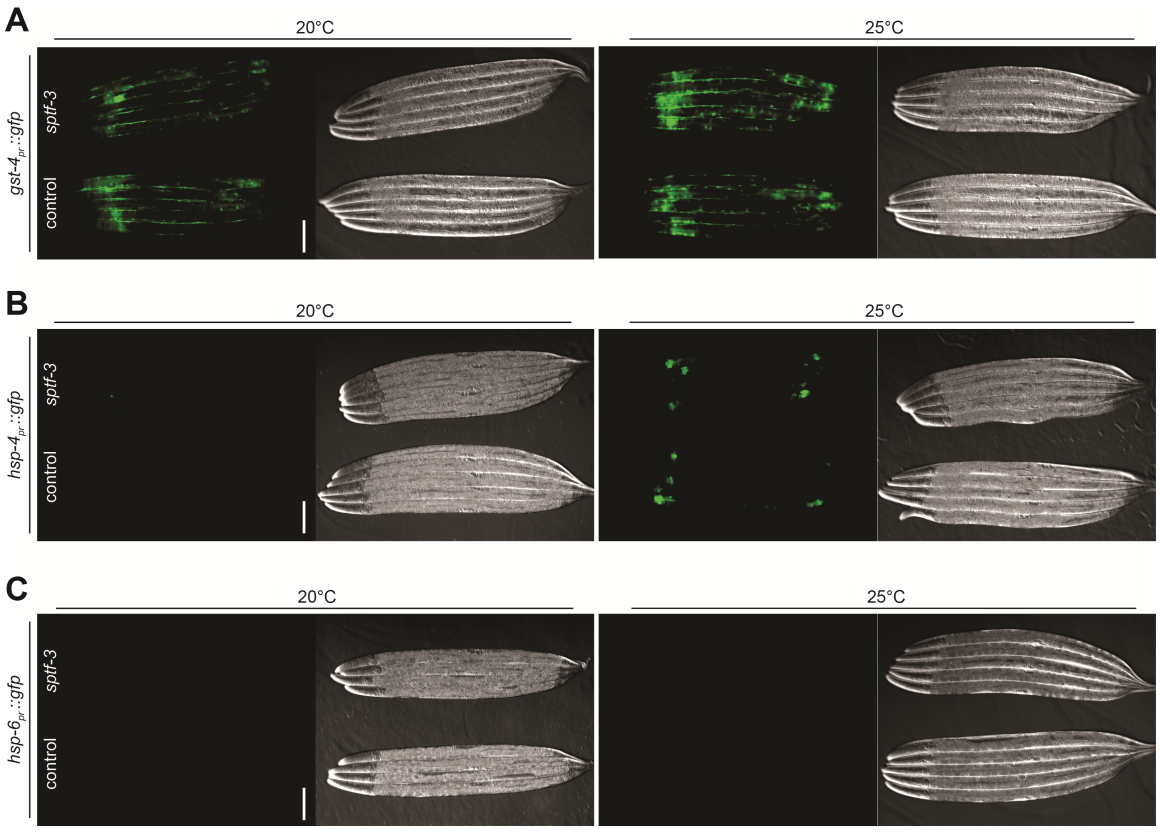

Figure 3.19 Effect of *sptf-3* knockdown on stress reporters
Representative photomicrographs of **(A)** *gst-4*$_{pr}$ *::gfp*, **(B)** *hsp-4*$_{pr}$ *::gfp* and **(C)** *hsp-6*$_{pr}$ *::gfp* transgenic worms raised at 20°C/25°C on control or *sptf-3* RNAi. Scale bar, 0.1 mm.

ATFS-1 and CLPP-1 were identified as two central players in the *C. elegans* UPR^{mt} (Haynes et al., 2010). In the recently published study they appear among potential transcriptional targets of SPTF-3 (Hirose and Horvitz, 2013). Thus we hypothesized that SPTF-3 function might be required for the UPR^{mt} activation. To test this we knocked down *spg-7*, whose loss is known to induce mitochondrial unfolded protein response (Benedetti et al., 2006). Induced response is reflected by expression of mitochondrial chaperones such as *hsp-6* (ortholog of human HSPA9/mtHSP70). Therefore we exposed *hsp-6*$_{pr}$ *::gfp* transgenic worms to *spg-7* RNAi only and in combination with *atfs-1* or *sptf-3* RNAi (Figure 3.20). Downregulation of *spg-7* successfully activated UPR^{mt}, as reflected by induced *hsp-6*$_{pr}$ *::gfp* expression. ATFS-1 has been previously shown to regulate UPR^{mt} activation (Nargund et al., 2012). Consequently *spg-7* knockdown failed to induce UPR^{mt} when *atfs-1* function was impaired, which served as control for the inhibition of activated UPR^{mt} (Figure 3.20). Remarkably, *sptf-3* knockdown also prevented *spg-7* knockdown mediated UPR^{mt} activation. Notably, sptf-3 knockdown did not fully suppress

hsp-6$_{pr}$ *::gfp* expression, as more residual GFP could be detected in *sptf-3*; *spg-7* than in *atfs-1*; *spg-7* knockdown condition (Figure 3.20). This indicates that either the role of SPTF-3 in UPR^{mt} is marginal or it regulates the activity of the *hsp-6* promoter rather indirectly, *e.g.* through expression control of another transcription factor such as *atfs-1*.

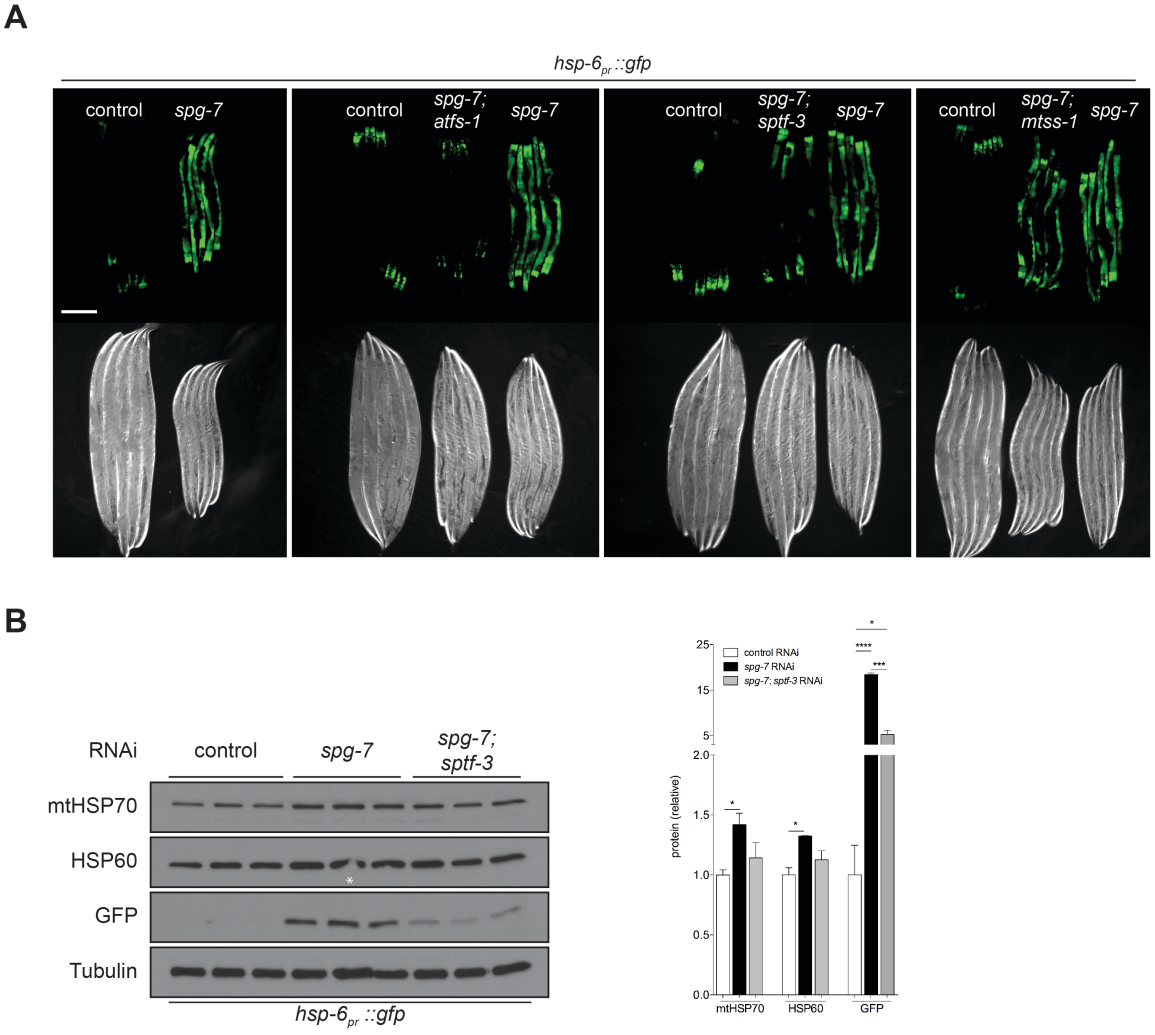

Figure 3.20 Effect of *sptf-3* knockdown on induced *hsp-6*$_{pr}$ *::gfp* expression
(A) Representative photomicrographs of *hsp-6*$_{pr}$ *::gfp* transgenic worms raised on control, *spg-7, spg-7; atfs-1, spg-7; sptf-3* or spg-7; *mtss-1* double RNAi at 20°C. Scale bar, 0.2 mm. **(B)** Western blot analysis and quantification of mtHSP70, HSP60 and GFP levels in total protein extracts from worms raised on control, *spg-7* or *spg-7; sptf-3* double RNAi at 20°C. Tubulin used as loading control (n=3). Asterisk indicates the band excluded from analysis.

Previously, it was shown that transgenic reporters are much more sensitive to mitochondrial perturbations than endogenous genes (Yoneda et al., 2004). This prompted us to determine protein amounts of mtHSP70 and GFP in order to explore to what extent reporter based GFP levels reflect internal mtHSP70 levels (Figure 3.20 B).

While GFP protein levels clearly supported our visual observations, also mtHSP70 followed the pattern of GFP levels, although in a much milder fashion: *spg-7* knockdown induced the expression of mtHSP70 and *sptf-3*; *spg-7* double knockdown diminished this activation. In the same samples we checked the levels of HSP60, another marker of activated UPR^{mt}, and found similar changes as in the case of mtHSP70 (Figure 3.20 B). Additionally we tested the effect of *sptf-3* knockdown on *hsp-60$_{pr}$ ::gfp* induction. Again, *sptf-3* knockdown clearly inhibited *spg-7* mediated UPR^{mt} activation (Figure 3.21). Taken together these data demonstrate that *sptf-3* function is required for UPR^{mt} activation. Protein levels of endogenous *hsp-6* and *hsp-60* follow the expression pattern of respective reporter, though to a much milder extent.

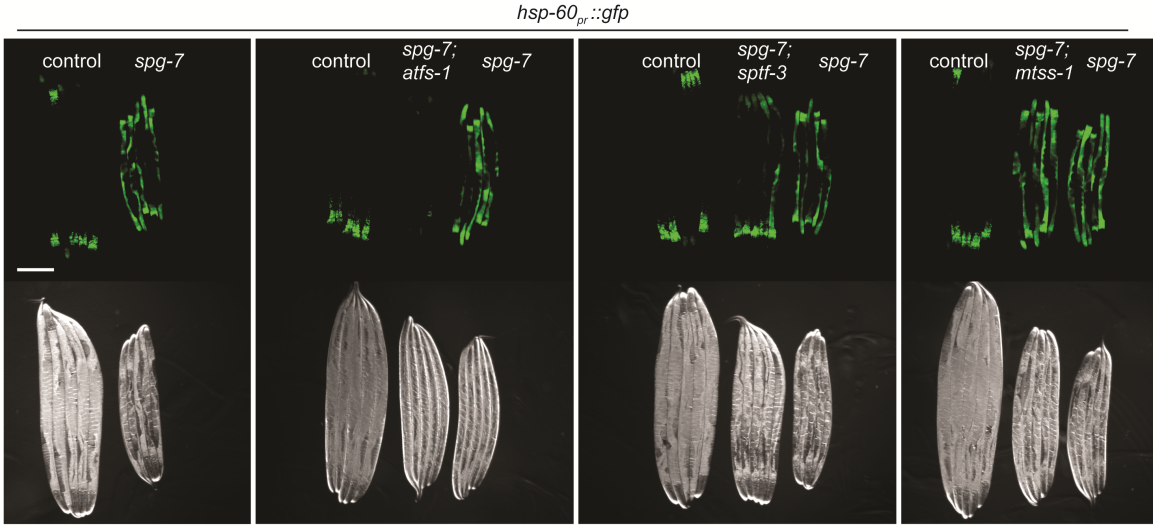

Figure 3.21 Effect of *sptf-3* knockdown on induced *hsp-60$_{pr}$ ::gfp* expression
Representative photomicrographs of *hsp-60$_{pr}$ ::gfp* transgenic worms raised on control, *spg-7, spg-7; atfs-1, spg-7; sptf-3* or spg-7; *mtss-1* double RNAi at 20°C. Scale bar, 0.2 mm.

In order to test *sptf-3* role in a condition where UPR^{mt} is constitutively active and does not depend on RNAi efficacy as in the case of *spg-7* knockdown, we used *hsp-6$_{pr}$ ::gfp* reporter in *isp-1(qm150)*; *ctb-1(qm189)* background, leading to a permanent activation of UPR^{mt} as reported previously (Liu et al., 2014). When we knocked down *sptf-3*, induction of the *hsp-6$_{pr}$ ::gfp* reporter was robustly inhibited, validating our previous observations that *sptf-3* function is essential for full UPR^{mt} activation (Figure 3.22).

Recently it has been suggested that mammalian mitochondrial SSBP1 potentiates heat-shock factor 1 (HSF1) transcription activity (Tan et al., 2015). It has

been proposed that under stress conditions SSBP1 can be found in the nucleus where it interacts with HSF1. At least in murine cells, the presence of SSBP1 enhanced HSF1 mediated expression of HSP60, HSP10 and mtHSP70 in response to mitochondrial proteotoxic stress caused by heat shock (Tan et al., 2015). To investigate the role of *mtss-1* in activation of UPR^{mt} in *C. elegans*, we tested induction of *hsp-6$_{pr}$ ::gfp* and *hsp-60$_{pr}$ ::gfp* in worms with inhibited *mtss-1* function (Figure 3.20 A, Figure 3.21, Figure 3.22). In all tested conditions *mtss-1* knockdown did not attenuate GFP expression suggesting that MTSS-1 is not involved in UPR^{mt} activation in *C. elegans*. In contrast to enhanced expression of mitochondrial chaperones in murine cells, heat shock in nematodes does not induce UPR^{mt} (Tan et al., 2015; Yoneda et al., 2004). This indicates distinct activation modes of UPR^{mt} between nematodes and mammals.

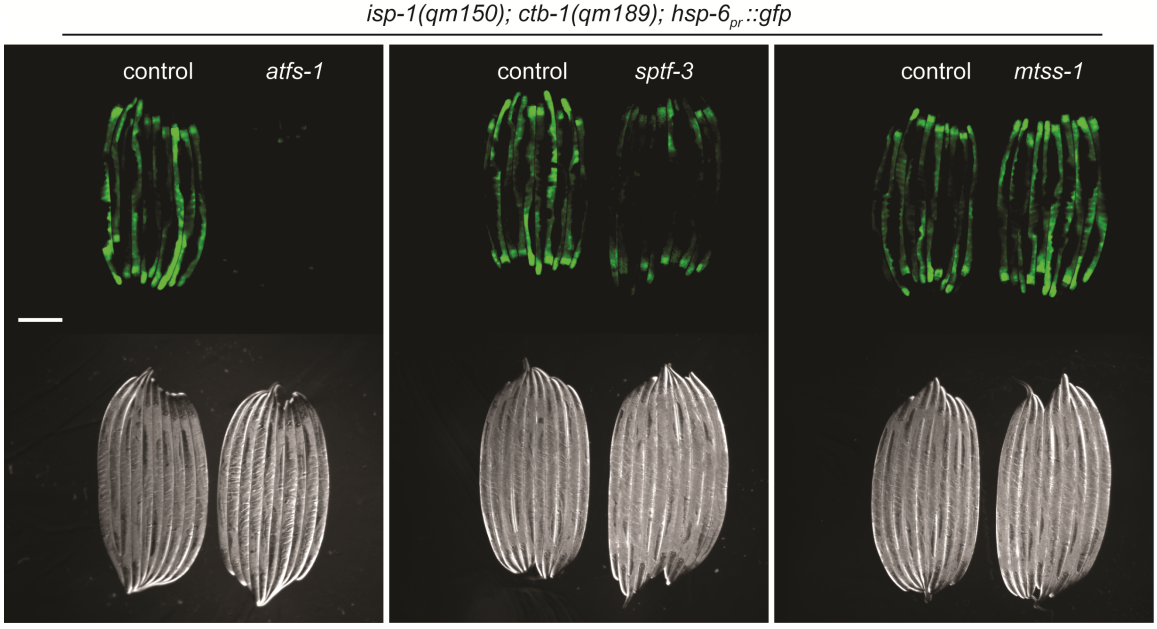

Figure 3.22 Effect of *sptf-3* knockdown on constitutively induced *hsp-6$_{pr}$ ::gfp* expression in *isp-1(qm150); ctb-1(qm189)* background
Representative photomicrographs of *isp-1(qm150)*; *ctb-1(qm189)*; *hsp-6$_{pr}$ ::gfp* transgenic worms exposed to control, *atfs-1, sptf-3* or *mtss-1* RNAi at 20°C. Scale bar, 0.2 mm.

3.16 *sptf-3* function is required specifically for UPR^{mt} induction

As SPTF-3 was shown to feature around 2.500 potential DNA binding sites, we wanted to investigate the specificity of *sptf-3* mediated UPR^{mt} induction. To do this we used a negative control, *hsp-4$_{pr}$ ::gfp* reporter of endoplasmatic reticulum unfolded protein response (UPR^{er}). With low basal level GFP expression, it can be activated

by *pdi-3* knockdown, a gene encoding ER protein disulfide isomerase (Yoneda et al., 2004). One possibility to abolish UPR^{er} is to decrease IRE-1 levels, a stress-activated endonuclease resident in the ER (Calfon et al., 2002). Knockdown of *pdi-3* massively activated UPR^{er} whereas exposure to *pdi-3* RNAi in combination with inactivation of *ire-1* function blocked *hsp-4$_{pr}$ ::gfp* expression (Figure 3.23 A). After finding conditions, which activate or prevent activation of UPR^{er}, we tested whether *sptf-3* knockdown affects UPR^{er} induction. Inhibition of *sptf-3* function did not alter *hsp-4$_{pr}$ ::gfp* expression, induced by *pdi-3* knockdown (Figure 3.23 A). Together with the observation that also *sptf-3* inhibition *per se* does not induce UPR^{er} (Figure 3.19 B) these data suggest that SPTF-3 is not involved in UPR^{er} activation.

To test *sptf-3* role in mediating oxidative stress response we used worms carrying *gst-4$_{pr}$ ::gfp* reporter construct, which is constitutively expressed in the *isp-1(qm150)* and *isp-1(qm150)*; *ctb-1(qm189)* mutant background (Khan et al., 2013). Glutathione S-transferase 4 (GST-4) is a detoxifying enzyme responsible for conjugation of glutathione to electrophiles. Expression of *gst-4* is induced in response to oxidative stress, *e.g.* paraquat (Kahn et al., 2008; Tawe et al., 1998). While *klf-1* (Kruppel-like transcription factor) knockdown was previously shown to reduce *gst-4$_{pr}$ ::gfp* expression in *isp-1(qm150)*; *ctb-1(qm189)* mutants (unpublished data), *sptf-3* knockdown did not reduce but rather increased GFP expression, suggesting that SPTF-3 is not required for oxidative stress response induction (Figure 3.23 B). Collectively these data suggest that *sptf-3* function is specifically required for full UPR^{mt} activation, while UPR^{er} and oxidative stress response are completely inducible when *sptf-3* function is impaired.

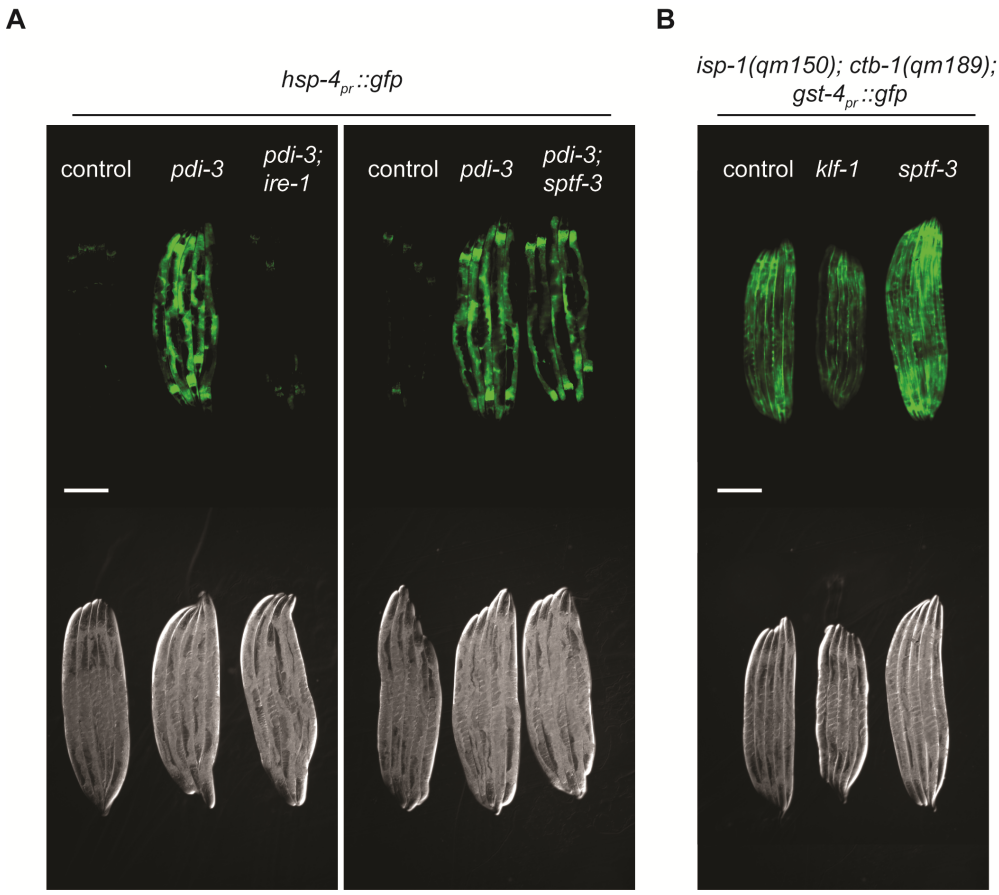

Figure 3.23 *sptf-3* knockdown effect on induced UPR^{er} and oxidative stress response
(A) Representative photomicrographs of *hsp-4$_{pr}$::gfp* transgenic worms raised on control, *pdi-3, pdi-3; ire-1 or pdi-3; sptf-3* double RNAi at 20°C. Scale bar, 0.2 mm. **(B)** Representative photomicrographs of *isp-1(qm150)*; *ctb-1(qm189)*; *gst-4$_{pr}$::gfp* transgenic worms raised on control, *klf-1* or *sptf-3* RNAi at 20°C. Scale bar, 0.2 mm.

Previously it has been shown that knockdown of cytosolic ribosomal protein genes leads to activation of the cellular surveillance system, which in turn prevents activation of UPR^{mt}. Notably, reduction of the KGB-1 kinase, essential component of the surveillance system, partially released the inhibitory effect of RPG knockdown on UPR^{mt} activation (Runkel et al., 2013). In this study we confirmed that the effect of RPG inhibition is highly specific to UPR^{mt}, as *rps-22* knockdown almost completely inhibited expression of *hsp-6$_{pr}$::gfp* and *hsp-60$_{pr}$::gfp* but only partially of *hsp-4$_{pr}$::gfp,* in conditions when the respective UPR branch was active (Figure 3.24 A-C). We also have shown that *rps-22* knockdown mediated *mtss-1$_{pr}$::gfp* induction was dependent on *sptf-3* function (Figure 3.16). Further, we showed that *sptf-3* function is critical for full UPR^{mt} activation (Figure 3.20, Figure 3.21, Figure 3.22). Therefore, we investigated the effect of *sptf-3* and *rps-22* double knockdown on *hsp-6$_{pr}$::gfp* induction in *isp-1(qm150)*; *ctb-1(qm189)* background. As

expected, the UPR^{mt} was inhibited by double RNAi treatment but interestingly the inhibitory effect was additive. Single *rps-22* (Figure 3.24 D) and *sptf-3* knockdowns (Figure 3.22) still exhibited low levels of *hsp-6$_{pr}$ ::gfp* expression, although upon *rps-22* inhibition GFP signal was barely detectable. Nevertheless, *rps-22*; *sptf-3* double knockdown completely abolished UPR^{mt} induction (Figure 3.24 D). Taken together, our results indicate that *sptf-3* and cytosolic ribosomes specifically modulate UPR^{mt} activity in presumably independent manner. Notably, *rps-22* knockdown leads to increased levels of SPTF-3 (Figure 3.16 D), which theoretically would promote UPR^{mt} induction. Therefore downregulation of ribosomal proteins might play a dual role in modulating UPR^{mt}: more dominating, inhibitory role via the surveillance system and inducing role through *sptf-3*.

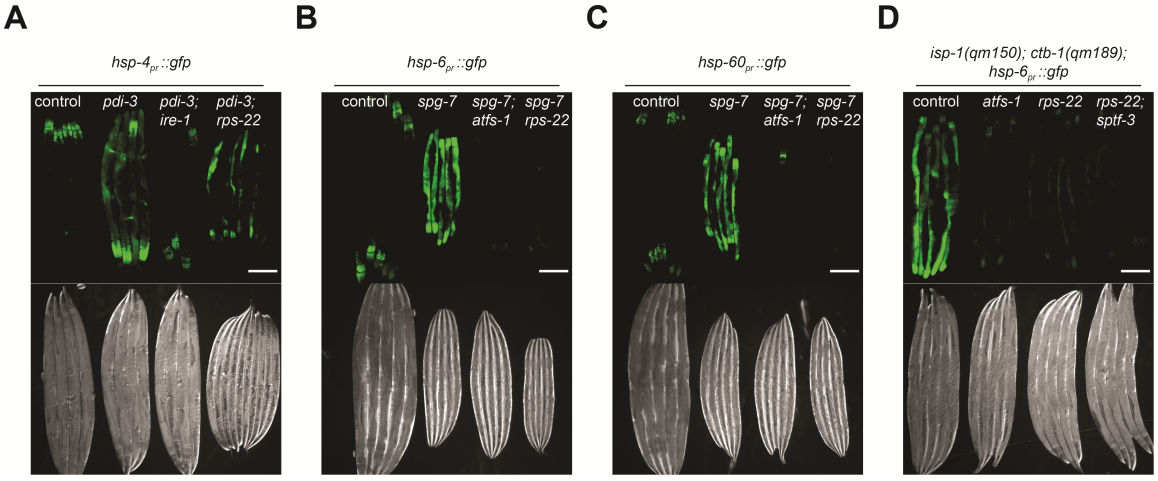

Figure 3.24 Effect of *sptf-3*; *rps-22* double knockdown on UPR^{mt} induction
(A) Representative photomicrographs of **(A)** *hsp-4$_{pr}$ ::gfp*, **(B)** *hsp-6$_{pr}$ ::gfp*, **(C)** *hsp-60$_{pr}$ ::gfp* and **(D)** *isp-1(qm150)*; *ctb-1(qm189)*; *hsp-6$_{pr}$ ::gfp* transgenic worms exposed to control, *pdi-3, pdi-3; ire-1, pdi-3; rps-22, spg-7, spg-7; atfs-1, spg-7; rps-22, atfs-1, rps-22 and rps-22; sptf-3* double RNAi at 20°C. Scale bar, 0.2 mm.

4 Discussion

Almost 60 years ago the free radical theory of aging was born and ever since mitochondrial function, or rather dysfunction, emerged as one of the driving forces of aging associated diseases and the aging process in general. Moreover, mitochondrial dysfunction has been linked to a wide range of disorders that are remarkably tissue specific and differ in the time point of disease onset (for review, see (DiMauro and Schon, 2008; Schapira, 2012). To better understand and eventually develop therapeutic strategies, numerous studies were targeted at finding the Holy Grail of mitochondrial regulation. However, we still do not know exactly how the expression of the mitochondrial and nuclear genomes is coordinated. Despite the fact that factors involved in these processes differ between species, tissues within one organism have different energetic requirements leading to the possibility of correspondingly distinct fine-tuning programs for mitochondrial regulation. Moreover, mitochondrial biogenesis might imply diverse sets of players depending on the current physiological state of the organism. Indeed the most prominent, stigmatized "master regulator" of mitochondrial biogenesis in mammals - PGC-1α - is dispensable under resting conditions (Lin et al., 2004). Therefore we established a *C. elegans* model, which allowed us to perform RNAi screens looking for genes facilitating or suppressing activation of mitochondrial biogenesis. As one of three proteins essential for mtDNA replication (Korhonen et al., 2004; Ruhanen et al., 2010; Sugimoto et al., 2008) mitochondrial single-stranded DNA binding protein is a valuable marker for changes in mtDNA replication rate, which predominantly mirror changes in mitochondrial biogenesis (Chow et al., 2007; Civitarese et al., 2007; Lentz et al., 2010). Importantly *C. elegans* maintenance temperature is designated as between 15°C and 25°C, whereas mild heat stress of 25°C is sufficient to elevate mtDNA replication (Bratic et al., 2009; Stiernagle, 2006; Vilchez et al., 2012). Hence our model allowed us to identify factors important for on demand regulation of mitochondrial biogenesis.

4.1 *C. elegans* specific regulation of mitochondrial biogenesis is largely unknown

In mammals, NRF-1 and NRF-2 regulate the expression of a variety of genes in the respiratory chain as well as genes involved in mtDNA expression (for review, see (Kelly and Scarpulla, 2004)). Whereas these factors maintain the basal mitochondrial biogenesis, their activity is fine-tuned by cofactors like PGC-1α and β in response to external physiological stimuli. How changes in mitochondrial functionality are communicated to these factors is still to be resolved. Potential signaling factors are calcium, cAMP, AMP/ATP levels or redox state (NAD+/NADH) (for review, see (Vega et al., 2015), yet they converge on PGC-1α, thus addressing predominantly stress induced signaling but not basal adaptations in expression of genes related to mitochondria. In *Saccharomyces cerevisiae*, expression of nDNA-encoded mitochondrial genes can be regulated via the RTG system in response to alterations in mitochondrial function, known as retrograde signaling (Liao and Butow, 1993). The central role of RTG genes is glutamate homeostasis. During mitochondrial stress the RTG system controls the expression of *CIT1*, *ACO1*, *IDH1* and *IDH2,* enzymes facilitating first three steps of the TCA cycle, and induces expression of genes encoding enzymes involved in anaplerotic pathways that provide metabolites required for α-ketoglutarate production (Liu and Butow, 1999). So far, no comparable discoveries have been made in *C. elegans*. To our knowledge, there are no RTG genes in nematodes, nor there are homologs of NRFs or PGC-1s (Knutti et al., 2000). Interestingly, Mediator subunit MDT-15 (mammalian MED15) functionally overlaps with PGC-1α in terms of regulation genes involved in fatty acid metabolism (Taubert et al., 2006; Vega et al., 2000). Still, no effect of MDT-15 on other mitochondria-related genes has been reported.

As mitochondrial biogenesis also has to be regulated in worms, there might be mechanisms shared between species, previously not linked or neglected concerning regulation expression of genes with mitochondrial function. One possibility is that already known mammalian factors have extended or newly acquired functions in nematodes. On the other hand, another regulation system could have developed involving *C. elegans* specific factors. Along this line, we investigated regulation of *mtss-1$_{pr}$ ::gfp* reporter expression by knocking down transcription factors and genes residual on chromosome III. A genome wide RNAi screen approach will enable the discovery of further factors involved in mitochondrial

biogenesis. Transcriptional and translational fusion will help to unveil factors affecting transcription, translation and localization. To cover all facets of mtDNA expression, *hmg-5$_{pr}$::gfp* (mammalian TFAM) reporter can be used next to *mtss-1$_{pr}$ ::gfp* in order to additionally monitor mtDNA transcription. Moreover, a mutant background that upregulates expression of the reporters, has to be determined if search for factors involved in stress mediated mitochondrial adaptation is intended.

While some factors modulating mitochondrial biogenesis are dispensable for organismal survival, others are essential. One study on mice demonstrated that one functional copy of *Nrf-1* is sufficient to ensure viability and fertility of the animals. However, homozygous *Nrf-1* mutants die between embryonic days 3.5 and 6.5 due to massive loss of mtDNA (Huo and Scarpulla, 2001). Loss of NRF-1 function via siRNA in a rat hepatoma cell line resulted in reduction of TFAM and mtDNA levels (Piantadosi and Suliman, 2006). Knockdowns of *gei-17, sptf-3* and W*04D2.4*, all reduced lifespan, whereas all three mutants with large deletions in coding regions are lethal/sterile, underlying the importance of these genes. In the case of *sptf-3* knockdown the lifespan reducing effect was even stronger at 25°C and normally long-lived *isp-1(qm150); ctb-1(qm189)* animals were undistinguishable from wild type animals raised on *sptf-3* RNAi. This suggests that under even mild stress as 25°C *sptf-3* knockdown effect is robust; suppressing lifespan extending effect of deployed mutants. This might be also a hint for the involvement of *sptf-3* in *isp-1(qm150); ctb-1(qm189)* mediated lifespan extension. That *sptf-3* and *W04D2.4* knockdowns changed transcript levels of different genes, suggests that they target different group of genes, partially overlapping. Due to the size and intensity of the project we decided to focus on W04D2.4 and SPTF-3 function.

4.2 W04D2.4 a novel, nematode specific regulator of mitochondrial biogenesis?

Almost as a rule, transcription factors are ornamented with post-translational modifications (PTMs) determining their localization and activity (for review, see (Filtz et al., 2014)). One example of regulating mitochondrial biogenesis through transcription factor control was shown in rat cells, when under low oxidant stress level

NRF-1 is phosphorylated by AKT and translocates to the nucleus (Piantadosi and Suliman, 2006). In this study we show that W04D2.4 preferentially localizes to the nucleus at 25°C. Yet there are four major questions to be answered, if W04D2.4 is truly a transcription factor: (1) which genes are regulated by W04D2.4 (2) at which site W04D2.4 is modified (3) which protein facilitates the modification and (4) is temperature the direct cause or is W04D2.4 modified in response to changes in *e.g.* mitochondrial biogenesis caused by increased temperature. In other words, can we trigger W04D2.4 nuclear translocation by generating cellular stress (*e.g.* using RNAi) or is this response temperature specific?

The most important question concerns targets of W04D2.4. The RNA-seq data suggest that a big portion of genes with mitochondrial function might be transcriptionally regulated by W04D2.4. Strikingly, eleven out of twelve mtDNA encoded genes and 28 out of 65 mitochondrial RPGs were less abundant upon W04D2.4 knockdown. Most likely, mtDNA-encoded genes are indirectly regulated by W04D2.4, since next to mitochondrial RPGs, transcripts of *mtss-1* and *polg-1*, representing mitochondrial replication machinery, were downregulated upon *W04D2.4* knockdown. Notably, in mammals NRF-1 was shown to directly regulate expression of OXPHOS genes and furthermore of TFAM, involved in mtDNA expression and maintenance (Evans and Scarpulla, 1990; Satoh et al., 2013; Virbasius et al., 1993). Our data indicate that W04D2.4 is involved in direct regulation of genes with mitochondrial function and indirect regulation of mitochondrial genes by controlling expression of genes responsible for mitochondrial replication and translation processes. For further characterization it is important to determine direct transcriptional targets of W04D2.4 using ChIP-seq.

4.3 SPTF-3 regulates expression of genes with mitochondrial function in *C. elegans*

Mammalian Specificity Protein/ Kruppel-like factor (SP/KLF) family of C2H2-type zinc-finger transcription factors family consists of nine Sp and 16 KLF subfamily members (for review, see (Suske et al., 2005)). Despite their similarities in primary structure and in affinity to bind GC rich boxes, Sp1-4 have distinct, mainly non-redundant functions *in vivo*. Whereas Sp4 is expressed in neurons, Sp1-3 are expressed ubiquitously (for

review, see (Suske, 1999)). More strikingly, Sp1 (Marin et al., 1997) and Sp2 (Baur et al., 2010) null mice die during embryonic development. Sp3 null mice die at birth due to respiratory failure (Bouwman et al., 2000), while two-thirds of Sp4 null mutants die within first four weeks after birth and the once which survive are smaller in size and fail to breed (Addo et al., 2010; Gollner et al., 2001).

In mammals, proximal promoters of several mitochondria-related genes contain NRF-1 and NRF-2 recognition sites, indicating nuclear respiratory factor mediated regulation of mitochondrial biogenesis (for review, see (Kelly and Scarpulla, 2004)). Next to NRF-1 and NRF-2, at least one Sp1 recognition site can be found within the promoter region of TFAM, TFB1M, TFB2M and POLγ, factors essential for mtDNA replication/transcription. Importantly, Sp1 recognition sites function as negative and positive elements in the context of TFB1M and TFB2M promoter respectively, suggesting dual activity for other Sp transcription factors (Gleyzer et al., 2005). Moreover, Sp3 was suggested to act as a transcriptional repressor, due to its low transcription stimulation capacity and competitive binding of Sp1 recognition elements (Kennett et al., 2002).

In Na2 neurons, Sp1 was shown to induce expression of all 13 COX subunits (Dhar et al., 2013). The 10 nDNA-encoded COX genes are regulated directly, whereas the mtDNA-encoded ones indirectly via regulating expression of genes encoding TFAM, TFB1M and TFB2M. Knockdown of Sp1 by shRNA convincingly reduced transcript and protein levels of COX subunits by at least 40%. Strikingly, in primary neurons Sp4 and not Sp1 was shown to regulate COX gene expression (Johar et al., 2013). However in *C. elegans* the role of Sp transcription factors in mitochondrial biogenesis have not been investigated so far. The nematode Sp family includes *sptf-1*, *sptf-2*, *sptf-3* and *tlp-1* genes (Ulm et al., 2011; Zhao et al., 2002b). Under our experimental conditions using *mtss-1*$_{pr}$ *:gfp* reporter, we suggest that in somatic cells only *sptf-3* is involved in the regulation of *mtss-1*, though the role of *tlp-1* still has to be tested. Notably, consistent with findings that Sp1 overexpression eventually leads to apoptosis (Deniaud et al., 2006), we could not establish a line transmitting the *sptf-3* overexpression array beyond F1 generation. Protein blast algorithms do not reveal clear human or murine ortholog of SPTF-3. Though it is highly speculative to conclude homology relations based on expression profiles, nevertheless, SPTF-3 is ubiquitously expressed which is least overlapping with neuronal specific Sp4 expression profile in mice (Hirose and Horvitz, 2013).

Additional studies targeting mammalian mitochondrial aconitase and citrate synthase, two TCA cycle enzymes, revealed Sp1 sites in their proximal promoters. Deletion/mutation of Sp1 recognition sites led to decreased transcript levels of *ACO2* and *CS* in reporter constructs (Kraft et al., 2006; Yu et al., 2006). Consistent with the described findings we observed decreased expression of *aco-2*$_{pr}$ *::gfp*, *cts-1*$_{pr}$ *::gfp* and *hmg-5*$_{pr}$ *::gfp* reporters upon *sptf-3* knockdown, though with certain restrictions. In *C. elegans* neither *aco-2* nor *hmg-5*, but *cts-1* appear to possess SPTF-3 binding sites in predicted promoter regions (Hirose and Horvitz, 2013). This supports our observation concerning minor changes in *aco-2*$_{pr}$ *::gfp* and strong changes in *cts-1*$_{pr}$ *::gfp* expression levels. Still *hmg-5* is possibly regulated by SPTF-3, as we detected decreased *hmg-5* transcript levels upon *sptf-3* RNAi. One possible explanation, why in the ChIP-seq data (Hirose and Horvitz, 2013) *hmg-5* does not appear, is that it resides downstream of *nrde-4* gene in the CEOP4236 operon. In turn, *nrde-4* proximal promoter contains one SPTF-3 binding site, suggesting *sptf-3* mediated *hmg-5* regulation by distal promoter elements.

Further, only four nuclear and one mtDNA-encoded COX subunits appear to have a SPTF-3 binding site in their promoters. Testing the transcript and protein levels of the subunit encoded by mtDNA, we detected a decrease in the mRNA expression but not protein levels upon *sptf-3* knockdown. Testing protein levels of other COX subunits would require generation of *C. elegans* specific antibodies. Taken together, these data indicate that under tested conditions the effect of *sptf-3* knockdown on *ctc-1* (homolog of human COXIV subunit 1) is moderate, supported by unchanged respiration rates. Possibly general Sp mediated regulation of COX genes in *C. elegans* is distinct from that of mammalian systems. Nevertheless our experiments suggest that SPTF-3 is involved in mitochondrial DNA maintenance, as mtDNA content was reduced by *sptf-3* knockdown, presumably via reducing *hmg-5, mtss-1* and *polg-1* levels. Eventually a broader range of genes with mitochondrial function has to be tested for *sptf-3* dependent regulation, ideally under conditions when mitochondrial biogenesis is robustly upregulated. Moreover, future studies should test involvement of other Sp genes in the expression of mitochondria–related genes, although we showed that only *sptf-3* is involved in *mtss-1* regulation under tested conditions.

In mouse embryonic fibroblasts (MEFs) mitochondria elongate under starving conditions in order to maintain ATP levels (Gomes et al., 2011). In murine beta cells (β-cells, pancreas) increased levels of glucose and fatty acids result in changing

mitochondrial morphology towards fragmentation (Molina et al., 2009). Moreover, knockdown of COX scaffold protein SCO1 in *C. elegans* and mammalian cells induced mitochondrial hyperfusion suggesting transient a compensatory mechanism for maintaining ATP levels (Rolland et al., 2013). Interestingly, neither respiratory chain complex I, nor mitochondrial polymerase (*mrpo-1*) or mitochondrial transcription factors (*mtfb-1* or *hmg-5*) deficiency lead to hyperfusion, suggesting hyperfused phenotype specificity to COX deficiency. Upon knockdown of *sptf-3* we also observed a moderate shift of mitochondrial networks towards the hyperfused state. This could be an indicator for *sptf-3* knockdown mediated COX activity impairment, which has to be addressed in future experiments by determining ATP levels in *sptf-3* deficient worms. While COXIV-1 protein levels and respiration were unchanged, transcript levels and mitochondrial morphology were slightly altered upon *sptf-3* knockdown. This contradiction might result from low sensitivity of used assays under given parameters and should be addressed in future experiments.

4.4 Knockdown of cytosolic ribosomal protein genes induces *mtss-1* expression in specific, *sptf-3* mediated manner

Changing environmental conditions and accessibility of nutrients affect organismal growth and survival. Many studies investigated molecular mechanisms of lifespan extension mediated by caloric restriction (CR) or cytosolic translation inhibition. In CR rats, Electron Microscopy (EM) reveals increased number of mitochondria in liver tissue (Lopez-Lluch et al., 2006). Moreover, CR on HeLa cells elevated transcript and protein levels of NRF-1 and NRF-2. Interestingly, increased insulin levels appear to diminish the CR effect on the expression levels of mitochondrial biogenesis factors. We tested the CR effect in our model by using the *eat-2(ad1116)* mutant. Mutations in *eat* genes partially disrupt pharyngeal function, thereby decreasing food uptake. This genetic CR model increases *C. elegans* lifespan by up to 50% (Lakowski and Hekimi, 1998). In our hands, *eat-2(ad1116)* mutation did not cause elevated expression of *mtss-1*$_{pr}$*::gfp* (data not shown), suggesting that either CR is not affecting mitochondria-related genes at transcriptional level or *mtss-1* is not part of CR mediated alterations.

Further, in different studies cytosolic translation appears to be the focal process coupling nutrient deprivation, mitochondrial biogenesis and longevity (Pan et al., 2007; Rogers et al., 2011; Syntichaki et al., 2007; Wang et al., 2008). Moreover, contrary to transcription, translational regulation of selective mRNAs displays a sophisticated way to quickly adjust protein concentration in response to stress stimuli. In *Drosophila melanogaster*, CR was shown to enhance translation of genes with mitochondrial function leading to the eukaryotic translation initiation factor 4E binding protein (4E-BP) mediated lifespan extension (Zid et al., 2009). Whereas global translation rate is reduced, mRNAs of nDNA-encoded mitochondrial genes are preferentially translated due to less structured 5' UTR. In turn, reduction of ETC subunits diminishes the lifespan extension effect of CR. The pivotal role in the regulation of translation events is attributed to the nutrient-sensing serine/threonine protein kinase TOR (for review, see (Dowling et al., 2010; Fonseca et al., 2014)). Acting in two distinct multi-protein complexes TOR modulates translation through its downstream effectors. To name but a few, mammalian target of rapamycin complex 1 (mTORC1) promotes selective translation of genes involved in mitochondrial function by phosphorylating 4E-BP (Morita et al., 2013) and mediates formation of the translation preinitiation complex via S6 kinase (S6K) (Holz et al., 2005). Remarkably, in our study only direct reduction of cytosolic ribosomes induced *mtss-1*$_{pr}$*::gfp* expression, leading to increased transcript and protein levels of both, MTSS-1 and GFP. Notably, GFP protein increase indicates that promoted translation of *mtss-1* is most likely not caused by mRNA structural features but rather by increased *mtss-1* mRNA abundance. Neither knockdown of *Ce*TOR components nor inhibition of translation factors induced reporter expression. Most likely the induction is largely dependent on the *mtss-1* promoter activity, that we suggest to be regulated by SPTF-3.

In response to a wide range of stress conditions, eukaryotic initiation factor 2 subunit alpha, eIF2α can be phosphorylated by GCN2, PERK, HRI and PKR, reducing availability of ternary complex, which is critical for translation initiation (for review, see (Proud, 2005; Sonenberg and Hinnebusch, 2009)). In *C. elegans* only two kinases are known to phosphorylate eIF2α, namely GCN-2 and PEK-1 (homolog of mammalian PERK). In addition to UV and nutrients, mitochondrial stress has also been shown to induce GCN-2 mediated eIF2α phosphorylation, slowing general translation (Baker et al., 2012). In our study, we observed decreased levels of phosphorylated eIF2α upon RPG knockdown.

Importantly, we were able to determine only the levels of the phosphorylated eIF2α, missing out on the information about the ratio between the phosphorylated and dephosphorylated forms. Thus, generating *C. elegans* specific pan-eIF2α antibody will help to clarify whether phosphorylated or simply total eIF2α levels are decreased upon *rps-22* knockdown. Given that knockdown of a single ribosomal protein gene leads to impaired ribosome assembly, dephosphorylated eIF2α could possibly serve as a compensatory mechanism releasing "the brake" from translation initiation, allowing maximum translational capacity under the present circumstances. Our results suggest, that *mtss-1$_{pr}$ ::gfp* expression is independent of GCN-2 function, as the reporter was inducible upon RPG knockdown in *gcn-2(ok871)* null-mutation background. Nevertheless, the induction was lower than in wild type background, suggesting that other factors affect global protein synthesis in the absence of functional GCN-2. Possibly, phosphorylation of eIF2α is induced in *gcn-2(ok871)* background, potentially via PEK-1, resulting in impaired initiation, subsequently leading to reduced translation. Loss of *ife-2*, homolog of mammalian eIF4E (eukaryotic initiation factor 4E), reduces global protein synthesis in *C. elegans* (Syntichaki et al., 2007). Animals carrying *ife-2(ok306)* deletion allele reproduced reporter induction pattern of *gcn-2(ok871)* animals, indicating reduced global translation upon *gcn-2* knockdown.

In eukaryotes most mRNAs initiate translation in a cap-dependent manner (for review, see (Thompson, 2012)). The presence of a 5' cap is recognized by eIF4E, subunit of the eIF4F cap-binding complex, enabling the initiation factors to load the mRNA on the 40S ribosomal subunit, previously recruited as 43S pre-initiation complex, consisting of 40S ribosomal subunit, eiF3, eIF1, eIF4A, eIF5 and the ternary complex (eIF2GTPmet-tRNA). Once the 40S subunit reaches the start codon, eIF5B recruits the 60S subunit to facilitate the formation of the 80S ribosome and allow translation start. During certain cellular stress conditions cap-dependent translation is reduced and the alternative cap-independent protein synthesis is exerted. Around 3% of human cellular mRNAs are heavily associated with translational apparatus, despite nonfunctional cap-binding eIF4F initiation complex (Johannes et al., 1999). Cap-independent translation requires an internal ribosome entry site (IRES), which first were found in viral RNAs. Presently, there is still discrepancy in the understanding of well-studied viral and cellular IRES mechanisms (for review, see (Komar and Hatzoglou, 2011; Thompson, 2012)). Importantly, under normal growth conditions IRES mediated translation utilizes the ternary complex dependent translation

initiation, whereas during stress IRES RNAs are translated using eIF5B dependent mechanism (Thakor and Holcik, 2012). IRES is found in the 5' UTR of *Sp1* and in rat neurons Sp1 protein levels are increased in an early response to ischemia-induced H_2O_2 stress. Importantly, at later stages higher levels of Sp1 increased *Sp1* transcripts in a feedback manner (Yeh et al., 2011). The follow up study showed that also during tumorigenesis Sp1 was activated in IRES dependent manner (Hung et al., 2014). Our results suggest that cellular stress as reduction of cytosolic ribosomes likewise enhances SPTF-3 translation, most probably through an IRES mediated mechanism. Subsequently elevated SPTF-3 levels lead to higher MTSS-1 transcript and protein levels. SPTF-3 binding sites can be found within its own proximal promoter region explaining the upward trend of *sptf-3* transcript levels, which might get significantly different in animals of later stages.

Taken together, our findings suggest that MTSS-1 expression is induced exclusively upon ribosomal protein gene knockdown, as RNAi mediated knockdown of TOR signaling components and translation factors failed to induce reporter expression. This induction appears to be mediated via SPTF-3, most likely through IRES dependent translation. Ribosomal profiling and computational sequence analysis will clarify whether *sptf-3* possesses IRES and whether more mitochondria-related genes are regulated in a similar manner. Nevertheless, in *C. elegans* this intriguing IRES mechanism might offer an alternative to the 4E-BP mediated translational modification under stress conditions. It has to be tested whether other physiological relevant stimuli can induce *mtss-1$_{pr}$::gfp* expression. Antimycin and pathogen infection both generate mitochondrial stress, thus are appealing conditions to be tested in the future (Liu et al., 2014; Pellegrino, 2014). Additionally, W04D2.4 knockdown not only abolished *mtss-1$_{pr}$::gfp* induction at 25°C but also abrogated reporter expression upon RPG knockdown (data not shown). This raises the possibility that SPTF-3 and W04D2.4 interaction is essential for *mtss-1* transcription. Moreover, SPTF-3 and W04D2.4 might regulate specifically nDNA-encoded mitochondrial genes when acting in combination.

Further, adulthood specific reduction of translation associated proteins, including *rps-22* knockdown, increased lifespan in *C. elegans*, suggesting general translation inhibition induces lifespan extension (Hansen et al., 2007). Notably, in our hands permanent exposure to *sptf-3* RNAi reduced life span of wild type and long-lived worms, causing developmental defects as reported previously (Ulm et al., 2011).

Consequently it is to be tested, whether upon RPG knockdown during adulthood only, SPTF-3 mediates the lifespan extension effect. SPTF-3 binding sides can be found in proximal promoter regions of many ribosomal protein genes (Hirose and Horvitz, 2013). Thus increased SPTF-3 levels caused by RPG knockdown might subsequently lead to elevated transcription of RPGs. Regulating expression of ribosomal protein genes and/or genes with mitochondrial function in a feedback manner are two potential mechanisms for SPTF-3 mediated lifespan extension upon RPG knockdown.

4.5 SPTF-3 function is required for the UPR^{mt} activation

Proteins are involved in almost every biological process. Newly synthesized polypeptides have to fold into their 'native state' to gain functional activity. Principally, biophysical features of the amino acid sequence determine the three-dimensional structure of proteins. However, the precise protein folding mechanism remains largely a conundrum, as no algorithm was found yet to accurately predict 3D structures. Proper function has to be assured in spatio-temporal manner, which is challenging in a crowded cellular environment. Moreover, protein function mostly depends on conformation flexibility presupposing relatively low degree of thermodynamic stability of the native state. Mutations, interactions with other proteins or intrinsic physical forces of partially folded proteins might lead to kinetically trapped molecules, potentially forming aggregates. Molecular chaperones were identified as proteins assisting in *de novo* folding, refolding of denaturated proteins and assistance in proteolytic degradation (for review, see (Hartl et al., 2011)). Their basal expression is upregulated in order to regain the protein-folding environment, and can be induced in an endoplasmatic reticulum, cytosol and mitochondria specific manner (Yoneda et al., 2004). The role of chaperones becomes obvious in the light of diseases associated with aggregation of misfolded proteins, like neurodegenerative Alzheimer's (AD) and Parkinson's diseases (PD) to name the most prominent ones (for review, see (Valastyan and Lindquist, 2014)). Notably, various studies indicate involvement of dysfunctional mitochondria in the pathogenesis of these diseases (for review, see (Lin and Beal, 2006; Rugarli and Langer, 2012)). Therefore, coordinated expression of chaperones

might be essential for cell protection from potential consequence of malfunctioning mitochondria.

Mitochondrial unfolded protein response (UPR^{mt}) displays a retrograde response mechanism when changes in mitochondria induce expression of nuclear genes in order to reestablish full functionality of the organelle. Though initially mitochondrial specific stress response was identified in mammals (Zhao et al., 2002a), much more insight was gained from studies in *C. elegans*. Accumulation of misfolded proteins within mitochondria leads to upregulated expression of mitochondrial chaperones HSP-6 and HSP-60 (mammalian HSPA9 and HSP60 respectively) (Yoneda et al., 2004). ATFS-1 was identified as the central player in this pathway, with possible dual targeting within cells, as it possesses mitochondrial targeting sequence and nuclear localization signal (Haynes et al., 2010; Nargund et al., 2012). Normally, ATFS-1 is imported into mitochondria, while during mitochondrial stress the import efficiency is impaired, so ATFS-1 can translocate to the nucleus, thus activating expression of specific mitochondrial protective genes, including mitochondrial chaperones *hsp-6* and *hsp-60*. Moreover ATFS-1 directly binds promoters of OXPHOS genes in both, nDNA and mtDNA, thus coordinating gene expression between the two genomes (Nargund et al., 2015). Our data suggest that SPTF-3 is indispensable for full UPR^{mt} induction. Most likely, SPTF-3 regulates *hsp-6* and *hsp-60* indirectly, via regulation of *atfs-1*, as a SPTF-3 binding site can be found only in the proximal promoter of *atfs-1* and not of the chaperones (Hirose and Horvitz, 2013). While knockdown of *spg-7* increases *atfs-1* mRNA levels (Nargund et al., 2012), future studies will have to address whether *sptf-3* is responsible for this upregulation and how SPTF-3 activity is specifically regulated upon mitochondrial stress. Posttranslational modifications like phosphorylation or sumoylation have been found in Sp1, yet the consequences regarding the function have to be dissected (for review, see (Waby et al., 2008)). Another study in *C. elegans* suggests that upon disruption of core activities like translation, cellular surveillance system is activated, eventually preventing UPR^{mt} induction (Runkel et al., 2013). Our knockdown experiments confirm that knockdown of ribosomal protein genes inhibits UPR^{mt} induction, whereas still some residual level is detectable in the reporter strains. Given that RPG knockdown induces SPTF-3 translation, it appears counterintuitive that UPR^{mt} is inhibited through the surveillance system. Possibly, in the case of simultaneous mitochondrial impairment and impaired ribosomal function, increased SPTF-3 levels are necessary to preserve basal levels of

mitochondrial chaperones; otherwise inhibition of UPR^{mt} would be total, eventually leading to critical level of mitochondrial dysfunction. SPTF-3s role in the basal level maintenance is supported by our *sptf-3*; *rps-22* double knockdown experiment, where the reporter induction is completely abolished.

4.6 Conclusions

Mitochondrial integrity is one of the defining aspects in the course of aging and onset of neurodegenerative diseases. Whereas much progress has been achieved in understanding transcriptional regulation of genes with mitochondrial function in mammals, there is still a gap of knowledge about how the bigenomic expression is coordinated. Moreover, little is known about the regulation of mitochondria-related gene expression in *C. elegans*, particularly in the absence of pivotal transcription factors identified in mammals. Therefore, in this study we aimed to identify determinants of mitochondrial biogenesis, particularly required upon mild stress in terms of moderate temperature increase. Due to the lack of NRF-1, NRF-2 and PGC-1α in *C. elegans*, we initially focused our investigations on transcription factors, finding W04D2.4 and SPTF-3. SPTF-3 is potentially involved in the regulation of a subset of OXPHOS subunits and a variety of genes with mitochondrial function, due to the presence of SPTF-3 binding sites in respective proximal promoters. Its specificity might be dependent on the interaction with W04D2.4, thus together, these two transcription factors, offer a potential regulatory complex for mitochondria-related genes in *C. elegans*. Extending the RNAi screen to the genome wide level will help to identify the players, communicating mitochondrial state to the transcription factors. Further, it will be crucial to investigate the role of SPTF-3 dependent UPR^{mt} induction possibly via control of *atfs-1* expression and whether SPTF-3 might be the connecting node between UPR^{mt} and mitochondrial biogenesis. Though Sp1 coordinates expression of COX genes in mammals (Dhar et al., 2013; Johar et al., 2013), cytochrome *c* (*CYCS*) (Li et al., 1996b) and adenine nucleotide translocase 2 (*ANT2*) (Li et al., 1996a), attention has mainly been given to NRFs, PPARs, ERRs and PGC-1s with regard to the regulation of genes with mitochondrial function. Our results indicate that Sp1 family transcription factors may play a much bigger role in mammalian mitochondrial biogenesis, than assumed thus far.

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6 Appendix

WormBase ID	Gene	*W04D2.4* 25°C/ control 25°C	*W04D2.4* 25°C/ control 20°C	control 25°C/ control 20°C
WBGene00014224	*mrps-23*	-2.97	-3.60	-0.66
WBGene00019800	*mtss-1*	-2.50	-2.10	0.38
WBGene00010967	*ND5*	-1.19	-1.52	-0.36
WBGene00004954	*spd-3*	-1.18	-1.69	-0.54
WBGene00194707	*C53A5.17*	-1.13	-0.89	0.21
WBGene00002025	*hsp-60*	-1.09	-1.16	-0.11
WBGene00000229	*atp-2*	-0.95	-1.19	-0.28
WBGene00000550	*clu-1*	-0.93	-1.21	-0.31
WBGene00010963	*ND4*	-0.76	-1.13	-0.40
WBGene00006565	*tfg-1*	-0.61	-0.82	-0.24
WBGene00020993	*W03F8.3*	-0.61	-0.27	0.31
WBGene00000877	*cyn-1*	-0.52	-0.63	-0.15
WBGene00004015	*phb-2*	-0.51	-0.68	-0.21
WBGene00010015	*atad-3*	-0.50	-0.88	-0.41
WBGene00016844	*sucg-1*	-0.49	-0.55	-0.09
WBGene00021920	*mrps-25*	-0.45	-0.65	-0.23
WBGene00010419	*H28O16.1*	-0.43	-0.53	-0.13
WBGene00007385	*atp-5*	-0.43	-0.55	-0.15
WBGene00003214	*mel-32*	-0.43	-0.51	-0.11
WBGene00010303	*cri-3*	-0.43	-0.54	-0.14
WBGene00019900	*R05G6.7*	-0.42	-0.50	-0.11
WBGene00006574	*tin-13*	-0.42	-0.58	-0.20
WBGene00003162	*mdh-2*	-0.40	-0.53	-0.16
WBGene00007686	*tomm-40*	-0.39	-0.53	-0.17
WBGene00000206	*asb-1*	-0.39	-0.43	-0.07
WBGene00010794	*dld-1*	-0.38	-0.55	-0.20
WBGene00002879	*let-754*	-0.36	-0.53	-0.20
WBGene00002045	*icd-1*	-0.34	-0.76	-0.45
WBGene00015413	*pdhb-1*	-0.34	-0.45	-0.14
WBGene00022722	*ZK370.8*	-0.34	-0.53	-0.22
WBGene00007684	*C18E9.4*	-0.33	-0.61	-0.32
WBGene00018963	*ucr-1*	-0.32	-0.45	-0.16

WormBase ID	Gene	*W04D2.4* 25°C/ control 25°C	*W04D2.4* 25°C/ control 20°C	control 25°C/ control 20°C
WBGene00001028	*dnj-10*	-0.32	-0.14	0.15
WBGene00000041	*aco-2*	-0.32	-0.42	-0.14
WBGene00002001	*hars-1*	-0.30	-0.56	-0.30
WBGene00006439	*ant-1.1*	-0.28	-0.53	-0.28
WBGene00012315	*immt-2*	-0.28	-0.15	0.09
WBGene00020347	*T08B2.7*	-0.27	-0.37	-0.13
WBGene00009664	*idha-1*	-0.27	-0.37	-0.13
WBGene00004931	*sod-2*	-0.27	-0.10	0.13
WBGene00001134	*eat-3*	-0.26	-0.42	-0.19
WBGene00007918	*sphk-1*	-0.26	-0.45	-0.22
WBGene00009082	*dlat-1*	-0.25	-0.45	-0.24
WBGene00011273	*R53.4*	-0.24	-0.40	-0.19
WBGene00000765	*coq-5*	-0.24	-0.41	-0.20
WBGene00007993	*idhb-1*	-0.22	-0.39	-0.19
WBGene00015391	*sdha-1*	-0.22	-0.42	-0.23
WBGene00021562	*nuo-5*	-0.22	-0.40	-0.21
WBGene00000829	*CYTB*	-0.22	-0.58	-0.39
WBGene00020348	*mrpl-23*	-0.21	-0.43	-0.25
WBGene00020275	*atp-4*	-0.21	-0.34	-0.16
WBGene00009187	*CELE_F27D4.1*	-0.21	-0.35	-0.17
WBGene00013376	*Y62E10A.6*	-0.21	-0.39	-0.22
WBGene00020636	*T20H4.5*	-0.20	-0.29	-0.12
WBGene00008505	*F01G4.6*	-0.20	-0.43	-0.26
WBGene00003831	*nuo-1*	-0.20	-0.31	-0.14
WBGene00017982	*hpo-18*	-0.19	-0.34	-0.18
WBGene00010624	*mrps-15*	-0.19	-0.41	-0.25
WBGene00007880	*CELE_C33A12.1*	-0.19	-0.30	-0.14
WBGene00002162	*isp-1*	-0.19	-0.24	-0.09
WBGene00000209	*asg-1*	-0.19	-0.38	-0.22
WBGene00022336	*Y82E9BR.3*	-0.18	-0.36	-0.20
WBGene00020511	*immt-1*	-0.18	-0.28	-0.13
WBGene00004014	*phb-1*	-0.17	-0.30	-0.16
WBGene00017261	*acl-6*	-0.17	-0.27	-0.13
WBGene00020417	*nuo-2*	-0.17	-0.26	-0.12
WBGene00011510	*pdha-1*	-0.17	-0.26	-0.12

WormBase ID	Gene	*W04D2.4* 25°C/ control 25°C	*W04D2.4* 25°C/ control 20°C	control 25°C/ control 20°C
WBGene00009246	*CELE_F29C12.4*	-0.16	0.13	0.26
WBGene00015186	*misc-1*	-0.16	-0.32	-0.19
WBGene00000869	*cyc-1*	-0.16	-0.22	-0.09
WBGene00020679	*ogdh-1*	-0.16	-0.21	-0.08
WBGene00007122	*CELE_B0250.5*	-0.15	-0.39	-0.27
WBGene00018491	*mdh-1*	-0.15	-0.39	-0.27
WBGene00007630	*har-1*	-0.14	-0.31	-0.20
WBGene00044305	*rad-8*	-0.14	-0.26	-0.16
WBGene00020181	*T02H6.11*	-0.13	-0.22	-0.12
WBGene00004930	*sod-1*	-0.13	-0.19	-0.09
WBGene00022170	*Y71H2AM.5*	-0.12	-0.23	-0.14
WBGene00010809	*M01F1.3*	-0.11	0.40	0.48
WBGene00017121	*cyc-2.1*	-0.11	-0.16	-0.08
WBGene00010780	*K11H3.3*	-0.10	-0.16	-0.09
WBGene00000230	*atp-3*	-0.09	-0.18	-0.12
WBGene00000833	*cts-1*	-0.09	-0.30	-0.24
WBGene00015814	*C16A3.10*	0.18	-0.22	-0.43
WBGene00001794	*gta-1*	0.20	-0.12	-0.35
WBGene00000787	*cps-6*	0.22	0.49	0.24
WBGene00017659	*F21C10.10*	0.23	0.98	0.72
WBGene00008980	*tag-299*	0.29	0.73	0.40
WBGene00000114	*alh-8*	0.37	0.12	-0.28
WBGene00004932	*sod-3*	0.42	1.13	0.69
WBGene00004113	*pqn-24*	0.42	1.25	0.80
WBGene00001149	*bcat-1*	0.43	-0.52	-0.98
WBGene00001564	*icl-1*	0.45	0.36	-0.13
WBGene00012608	*Y38F1A.6*	0.62	-0.49	-1.14
WBGene00010957	*ND6*	-1.20	-0.21	-
WBGene00010959	*ND1*	-0.97	-0.15	-
WBGene00010961	*ND2*	-0.88	-0.20	-
WBGene00010966	*ND3*	-0.82	-0.22	-
WBGene00010960	*ATP6*	-0.75	-0.28	-
WBGene00013433	*Y66D12A.7*	-0.74	-0.28	-
WBGene00014172	*clpp-1*	-0.72	-0.22	-
WBGene00022862	*CELE_ZK1236.1*	-0.58	-0.23	-

WormBase ID	Gene	*W04D2.4* 25°C/ control 25°C	*W04D2.4* 25°C/ control 20°C	control 25°C/ control 20°C
WBGene00019007	*F57B10.14*	-0.53	-0.19	-
WBGene00007859	*mrps-31*	-0.51	-0.19	-
WBGene00011123	*nuaf-3*	-0.48	-0.30	-
WBGene00002985	*lig-1*	-0.47	-0.32	-
WBGene00010965	*COX2*	-0.46	-0.40	-
WBGene00011247	*mrpl-49*	-0.46	0.48	-
WBGene00021815	*Y53G8AR.8*	-0.41	-0.29	-
WBGene00020366		-0.40	-0.20	-
WBGene00020383	*T09B4.9*	-0.39	-0.28	-
WBGene00009092	*tomm-20*	-0.37	-0.60	-
WBGene00019076	*mrpl-24*	-0.37	-0.16	-
WBGene00012556	*mrps-10*	-0.35	-0.34	-
WBGene00004769	*sel-12*	-0.35	-0.25	-
WBGene00008924	*F17E5.2*	-0.35	1.00	-
WBGene00001740	*gro-1*	-0.35	-0.24	-
WBGene00016391	*C34B2.6*	-0.35	-0.21	-
WBGene00022470	*mrpl-19*	-0.34	-0.35	-
WBGene00011897	*scpl-4*	-0.34	-0.11	-
WBGene00044321	*mrps-30*	-0.33	-0.25	-
WBGene00007444	*CELE_C08F8.2*	-0.33	-0.27	-
WBGene00001093	*drp-1*	-0.33	-0.19	-
WBGene00009559	*mtx-1*	-0.33	-0.72	-
WBGene00010042	*bcs-1*	-0.32	-1.44	-
WBGene00019884	*R05D3.12*	-0.32	-1.12	-
WBGene00002010	*hsp-6*	-0.32	-1.70	-
WBGene00010557	*mspn-1*	-0.32	-1.14	-
WBGene00022045	*mrpl-55*	-0.31	0.47	-
WBGene00013258	*polg-1*	-0.31	-0.20	-
WBGene00010626	*K07C5.3*	-0.30	-0.48	-
WBGene00020604	*T20B12.7*	-0.30	0.66	-
WBGene00018961	*mrps-16*	-0.30	-0.50	-
WBGene00013462	*micu-1*	-0.29	-0.53	-
WBGene00018218	*F40A3.3*	-0.29	-0.18	-
WBGene00007129	*B0272.3*	-0.28	-0.26	-
WBGene00017319	*mrps-9*	-0.28	-0.29	-

WormBase ID	Gene	*W04D2.4* 25°C/ control 25°C	*W04D2.4* 25°C/ control 20°C	control 25°C/ control 20°C
WBGene00010812	*mrpl-35*	-0.27	-0.21	-
WBGene00001509	*fzo-1*	-0.27	-0.18	-
WBGene00008452	*mrps-5*	-0.27	-0.23	-
WBGene00002183	*kat-1*	-0.27	-0.19	-
WBGene00019061	*F58F12.1*	-0.26	-0.33	-
WBGene00017137	*pink-1*	-0.26	-0.43	-
WBGene00001503	*fum-1*	-0.25	0.26	-
WBGene00012094	*T27E9.2*	-0.25	-0.22	-
WBGene00016412	*mrps-26*	-0.24	-0.09	-
WBGene00015125	*B0303.3*	-0.24	0.43	-
WBGene00000423	*ced-9*	-0.24	-0.53	-
WBGene00000941	*ddp-1*	-0.23	-0.21	-
WBGene00001813	*haf-3*	-0.23	-0.36	-
WBGene00014227	*ZK1128.1*	-0.23	-0.08	-
WBGene00001985	*hop-1*	-0.23	-0.82	-
WBGene00015487	*mrps-17*	-0.22	-0.23	-
WBGene00011527	*cchl-1*	-0.22	-0.18	-
WBGene00022054	*Y67D2.4*	-0.21	-0.27	-
WBGene00018967	*F56D2.6*	-0.21	-0.14	-
WBGene00011662	*T09F3.2*	-0.21	-0.15	-
WBGene00009740	*F45H10.3*	-0.20	-0.29	-
WBGene00000933	*dap-3*	-0.20	-0.26	-
WBGene00019544	*miro-1*	-0.20	-0.21	-
WBGene00015248	*mai-2*	-0.19	-0.34	-
WBGene00011740	*mrpl-51*	-0.19	-0.29	-
WBGene00016249	*mrpl-32*	-0.18	0.26	-
WBGene00014054	*ZK669.4*	-0.17	-0.15	-
WBGene00000996	*dif-1*	-0.16	-0.20	-
WBGene00011634	*T09A5.5*	-0.16	-0.23	-
WBGene00017770	*CELE_F25B4.7*	-0.16	-0.55	-
WBGene00011015	*R04F11.2*	-0.16	-0.29	-
WBGene00012553	*cco-2*	-0.16	-0.16	-
WBGene00011883	*mrpl-50*	-0.14	-0.38	-
WBGene00016142	*mrps-18C*	-0.14	-0.16	-
WBGene00015133	*mrpl-11*	-0.13	-0.40	-

WormBase ID	Gene	*W04D2.4* 25°C/ control 25°C	*W04D2.4* 25°C/ control 20°C	control 25°C/ control 20°C
WBGene00022719	*pdhk-2*	-0.13	-0.20	-
WBGene00010052	*CELE_F54D5.7*	-0.12	-0.28	-
WBGene00014202	*mmcm-1*	-0.11	-0.34	-
WBGene00012713	*Y39E4A.3*	0.21	-0.33	-
WBGene00012794	*Y43E12A.2*	0.32	-0.43	-
WBGene00011089	*R07B7.5*	0.34	-0.25	-
WBGene00003124	*mai-1*	0.40	-0.29	-
WBGene00017640	*F20D6.11*	0.43	-0.16	-
WBGene00011105	*R07E3.4*	0.65	-0.47	-
WBGene00010035	*F54C8.1*	1.34	-0.23	-
WBGene00010485	*ant-1.3*	-1.59	-	-0.22
WBGene00010962	*COX3*	-0.39	-	0.22
WBGene00012204	*mfn-1*	-0.33	-	0.19
WBGene00017044	*mrpl-18*	-0.31	-	0.36
WBGene00009812	*suca-1*	-0.30	-	0.22
WBGene00008164	*CELE_C47G2.3*	-0.28	-	-0.21
WBGene00022493	*mrpl-45*	-0.24	-	0.27
WBGene00023487	*mrps-24*	-0.23	-	0.20
WBGene00008147	*C47E12.2*	-0.22	-	0.19
WBGene00000418	*ced-4*	-0.22	-	0.19
WBGene00017777	*F25B5.6*	-0.21	-	0.12
WBGene00019941	*pgam-5*	-0.20	-	-0.17
WBGene00022126	*Y71F9B.2*	-0.18	-	0.08
WBGene00016740	*C48B6.2*	-0.18	-	0.16
WBGene00009051	*nduf-6*	-0.17	-	0.09
WBGene00012031	*T25G3.4*	-0.15	-	0.89
WBGene00020169	*mmaa-1*	-0.12	-	0.15
WBGene00001155	*ech-6*	0.14	-	0.13
WBGene00022380	*Y94H6A.8*	0.15	-	0.14
WBGene00018784	*F54A3.5*	0.19	-	0.17
WBGene00004092	*ppt-1*	0.38	-	-0.43
WBGene00010958	*ND4L*	-1.01	-	-
WBGene00000762	*coq-2*	-0.26	-	-
WBGene00008225	*nuaf-1*	-0.25	-	-
WBGene00000764	*coq-4*	-0.24	-	-

WormBase ID	Gene	*W04D2.4* 25°C/ control 25°C	*W04D2.4* 25°C/ control 20°C	control 25°C/ control 20°C
WBGene00015185	*mrpl-41*	-0.22	-	-
WBGene00013324	*mrps-7*	-0.22	-	-
WBGene00021677	*pgs-1*	-0.21	-	-
WBGene00022516	*mtx-2*	-0.20	-	-
WBGene00022169	*Y71H2AM.4*	-0.20	-	-
WBGene00007622	*CELE_C16C10.1*	-0.19	-	-
WBGene00010077	*F55A11.4*	-0.19	-	-
WBGene00016989	*CD4.3*	-0.16	-	-
WBGene00007113	*B0035.15*	-0.16	-	-
WBGene00010094	*tsfm-1*	-0.15	-	-
WBGene00013878	*atfs-1*	-0.14	-	-
WBGene00019326	*K02F3.2*	-0.13	-	-
WBGene00044325	*tag-321*	-0.11	-	-
WBGene00001662	*gop-3*	-0.10	-	-
WBGene00017864	*pcca-1*	-0.09	-	-

Table 6.1 RNA-seq based list of annotated mitochondria-related genes, with altered transcript levels upon *W04D2.4* knockdown

List of genes graphically represented in Figure3.6 B. The basis of the record are differentially expressed genes with mitochondrial function upon *W04D2.4* 25°C knockdown vs. control at 25°C were and p-value <0.01. The two other comparisons, *W04D2.4*25°C/control20°C and control25°C/control20°C, regard only genes with changed expression in *W04D2.4* 25°Cvs.control25°C comparison.

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Мама, конечно я выражаю тебе огромную благодарность, в первую очередь за то что ты мама, которая переживает за меня и радуется со мной. Я знаю что это было не легко отпускать единственного ребёнка в другую страну, можно сказать навсегда. В какой то степени это может даже и укрепило нашу связь и я надеюсь что из меня вышел человек, которым ты можешь гордиться. Я уверен что всё у нас будет хорошо и мы будем чаще видеться, чем в последние время. Люблю.

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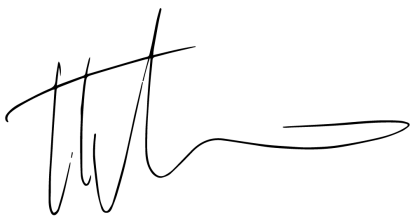
Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Aleksandra Trifunovic betreut worden.

Köln, den 01.10.2015



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