IN VIVO ACTIVATION OF THE ENDOPLASMIC RETICULUM UNFOLDED PROTEIN RESPONSE WITHOUT DISTURBED PROTEOSTASIS

by

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Abstract

The Mediator is a conserved transcriptional co-factor complex required for eukaryotic gene expression. In *C. elegans*, the Mediator subunit *mdt-15* is essential for the expression of genes involved in fatty acid metabolism and ingestion-associated stress response. *mdt-15* loss-of-function causes defects in reproduction and mobility and shortens lifespan. In the present study, we find that *mdt-15* depletion or mutation specifically decreases membrane phospholipid unsaturation. Accordingly, *mdt-15* worms exhibit disturbed ER homeostasis indicated by a constitutively activated ER unfolded protein response (UPR<sub>ER</sub>). This stress response is only partially the consequence of reduced membrane lipid unsaturation, implicating other *mdt-15*–regulated processes in the protection against ER stress. Interestingly, *mdt-15* inactivation or depletion of lipid metabolism enzymes *SCD* or *sams-1* activates the UPR<sub>ER</sub> without promoting misfolded protein aggregates in the ER. Moreover, these worms all exhibit wild-type sensitivity to chemically induced protein misfolding, and they do not display synthetic lethality with *ire-1*, whose inactivation causes protein misfolding. Therefore, the constitutive UPR<sub>ER</sub> in *mdt-15*, *SCD*, or *sams-1* worms is not the consequence of disturbed proteostasis, but likely the direct result from altered properties of the ER membrane. Altogether, our data suggest that the UPR<sub>ER</sub> can be directly induced by membrane disequilibrium and thus acts as a circuit that comprehensively monitors ER homeostasis.
Preface

I designed, conducted and analyzed all experiments in this project in consultation with my supervisor Dr. Stefan Taubert.

Section 2.5 and subsection 2.10.2 were conducted in collaboration with Dr. J. Watts. Lipid extraction and GC-MS were performed by the Watts lab. I was responsible for sample collection and data analysis.
The worm strain used in subsection 2.9.3 was cloned and constructed by Aljona from the Thorsten Hoppe’s lab.

A version of chapter 2 and 3 has been submitted for publication. In consultation with my supervisor, I designed, conducted and analyzed all the experiments and wrote the manuscript.
Table of Contents

Abstract ........................................................................................................................................ ii
Preface ........................................................................................................................................... iii
Table of Contents .......................................................................................................................... iv
List of Tables .................................................................................................................................. ix
List of Figures ............................................................................................................................... x
List of Abbreviations .................................................................................................................... xii
Acknowledgements ....................................................................................................................... xiii
Dedication ....................................................................................................................................... xiv

Chapter 1: Introduction .................................................................................................................. 1
  1.1 Transcriptional regulation in eukaryotes ................................................................................. 1
  1.2 The Mediator complex is a key eukaryotic co-factor ............................................................. 2
    1.2.1 Molecular actions of the Mediator in transcriptional regulation ......................................... 2
    1.2.2 Structure and composition of the Mediator complex .......................................................... 3
    1.2.3 Mediator subunits and their functional specificity ............................................................... 4
      1.2.3.1 MED1 is a selectively acting Mediator subunit required for nuclear hormone
              receptor function .................................................................................................................. 5
      1.2.3.2 MED15/MDT-15 acts in conserved physiological and developmental signalling
              pathways 6
  1.3 Function of lipids in the cell ................................................................................................... 10
  1.4 The ER unfolded protein response ....................................................................................... 12
    1.4.1 The IRE-1-dependent UPR_{ER} is universal and ancient .................................................. 13
1.4.2 The PERK UPR\textsuperscript{ER} reprograms translation ................................................................. 14
1.4.3 The ATF-6 UPR\textsuperscript{ER} functions differently among metazoans ................................. 15
1.5 The UPR\textsuperscript{ER} is directly involved in human disease pathogenesis ................................. 16
1.5.1 The UPR\textsuperscript{ER} is a key player in the pathogenesis of diabetes ........................................ 17
1.5.2 Diabetes and UPR\textsuperscript{ER} stimulation ........................................................................ 18
1.6 Molecular mechanisms leading to the activation of the UPR\textsuperscript{ER} .................................. 18
1.6.1 Lipid metabolism and UPR\textsuperscript{ER} activation ................................................................. 19
1.6.2 Membrane lipid aberrancy indirectly activates the UPR\textsuperscript{ER} ...................................... 20
1.6.3 Membrane lipid aberrancy directly activates the UPR\textsuperscript{ER} ....................................... 20
1.7 \textit{C. elegans}: a model system to study lipid metabolism and the UPR\textsuperscript{ER} ......................... 22
1.8 Activating the UPR\textsuperscript{ER} \textit{in vivo} without disturbed proteostasis ................................. 23

Chapter 2: Results ............................................................................................................................ 25
2.1 The transcript levels of multiple lipid metabolic genes are affected in \textit{mdt-15} worms .................. 25
2.2 \textit{mdt-15} is required to maintain normal membrane lipid unsaturation ..................................... 28
2.3 \textit{mdt-15} depletion or mutation activates the UPR\textsuperscript{ER} .................................................... 33
2.4 \textit{mdt-15}-regulated lipid unsaturation is essential for ER homeostasis .................................. 36
2.5 Fatty acid uptake and incorporation is not affected upon \textit{mdt-15} depletion ............................ 40
2.5.1 Alterations in C18:0, C20:3, and C20:4 fatty acyl contents or relative PC level do not contribute to the UPR\textsuperscript{ER} activation .................................................................................. 40
2.6 Normal PC production is essential for ER homeostasis but is not under \textit{mdt-15}’s direct regulation .................................................................................................................................. 45
2.7 \textit{mdt-15} itself is not part of the UPR\textsuperscript{ER} ......................................................................... 46
2.8 \textit{mdt-15} is not required for total glutathione synthesis ............................................................. 48
2.9 Genetic inactivation of *mdt-15* and lipid metabolism genes induces the UPR\textsuperscript{ER} without disturbed proteostasis ................................................................. 49

2.9.1 Depletion of *mdt-15*, *SCD*, or *sams-1* does not hypersensitize animals to proteostatic stresses .............................................................................................................................. 50

2.9.2 Depletion of *mdt-15*, *SCD*, or *sams-1* does not cause synthetic interaction with a UPR\textsuperscript{ER} mutant .................................................................................................................. 54

2.9.3 Alterations in membrane lipid compositions do not lead to misfolded protein aggregation ........................................................................................................................................ 55

2.10 Defects in PC production but not in membrane lipid unsaturation affect mitochondrial homeostasis and activate the UPR\textsuperscript{mito} ............................................................................................................................... 58

2.10.1 *sams-1* but not *mdt-15* or *SCD*, is required to prevent UPR\textsuperscript{mito} activation .... 59

2.10.2 Depletion of *mdt-15* or *SCD* does not affect the fatty acid composition or the relative level of cardiolipin .................................................................................................................... 61

Chapter 3: Discussion and Conclusion .............................................................................. 63

3.1 Membrane lipid disequilibrium activates the UPR\textsuperscript{ER} without disturbed proteostasis .. 63

3.2 *mdt-15*-mediated processes are essential to maintain ER homeostasis ...................... 63

3.2.1 The reduction in relative PC level does not contribute to *mdt-15* induced UPR\textsuperscript{ER} .. 65

3.2.2 Downregulation of C20:3 and C20:4 does not contribute to *mdt-15* induced UPR\textsuperscript{ER} 65

3.2.3 Substrate build-up of C18:0 does not contribute to *mdt-15* induced UPR\textsuperscript{ER} ........ 66

3.2.4 Additional *mdt-15*-mediated pathways for ER homeostasis may be both lipid-dependent and -independent ....................................................................................................................... 66
3.2.5 Additional mdt-15–regulated pathways for ER homeostasis can be identified using unbiased genetic screen.................................................................................................................. 67

3.3 mdt-15 induced UPR**ER** occurs due to metabolic defects, not transcriptional defects... 69

3.4 UPR**ER** activation and ER proteostasis.................................................................................................................. 70

3.4.1 Proteostatic disturbance may be dependent upon the severity of membrane lipid aberrancy........................................................................................................................................... 71

3.4.2 Protein degradation may not be activated in our mutant contexts............................... 72

3.4.3 Activation of the UPR**ER** in mdt-15 worms is not due to general sickness ............ 72

3.5 Activation of the UPR**ER** does not correlate to stress resistance or longevity........... 73

3.6 Defects in PC production trigger the UPR**mito**........................................................................................................ 73

3.7 The fatty acid composition of cardiolipin is distinctive from that of PC and PE ........ 74

3.8 PC production, UPR**mito**, and longevity.................................................................................. 75

3.9 Conclusions.................................................................................................................................................. 76

Chapter 4: Materials and Methods .................................................................................................................. 78

4.1 Worm strains.................................................................................................................................................. 78

4.2 Worm growth conditions ............................................................................................................................... 79

4.3 Feeding RNA interference ............................................................................................................................ 79

4.4 Thin layer chromatography and gas chromatography-mass spectrometry ..................... 80

4.5 RNA isolation and real-time quantitative PCR......................................................................................... 80

4.6 DIC, fluorescence microscopy and confocal microscopy......................................................... 80

4.7 Protein extraction and immunoblots ................................................................................................. 81

4.8 Pharyngeal pumping assay....................................................................................................................... 82

4.9 Glutathione assay........................................................................................................................................ 82
4.10 Statistical analysis .......................................................................................................................... 82

References ......................................................................................................................................... 84

Appendices ......................................................................................................................................... 95
List of Tables

Table 1. p-values representing the significance of the changes in lipid profiles of unsupplemented and supplemented control(RNAi) and mdt-15(RNAi) worms. ........................................ 95

Table 2. p-values representing the significance of the changes in lipid profiles between wild-type (WT) and mdt-15(tm2182) worms. ......................................................................................................................... 97

Table 3. p-values representing the significance of the changes in lipid profiles of unsupplemented and supplemented control(RNAi) and SCD(RNAi) worms............................................ 98

Table 4. Primer sequences used for RT-qPCR........................................................................................................ 99
List of Figures

Figure 1. The Mediator is a conserved co-factor complex required for eukaryotic transcriptional regulation. .......................................................... 2

Figure 2. mdt-15 is required for fatty acid desaturation.......................................................... 8

Figure 3. The UPR\textsuperscript{ER} signaling pathways.......................................................... 13

Figure 4. Inactivation of mdt-15 alters the expression of multiple lipid metabolism genes. ...... 27

Figure 5. mdt-15 is required to maintain normal membrane lipid unsaturation. ..................... 32

Figure 6. mdt-15 inactivation leads to activation of the UPR\textsuperscript{ER}. .................................. 35

Figure 7. mdt-15-mediated lipid unsaturation is required for maintaining ER homeostasis. ..... 39

Figure 8. Fatty acid uptake and incorporation is intact in lipid desaturation-defective worms... 44

Figure 9. Normal PC synthesis is required for ER homeostasis. .............................................. 46

Figure 10. mdt-15 does not participate in the UPR\textsuperscript{ER}. ................................................ 48

Figure 11. The glutathione level is not altered upon mdt-15 depletion. .................................. 49

Figure 12. Inactivation of mdt-15 and essential lipid metabolism genes does not compromise ER proteostasis................................................................. 53

Figure 13. Alterations in membrane lipid biosynthesis do not compromise ER protein quality control. .................................................................................. 55

Figure 14. Defects in lipid unsaturation or PC synthesis do not enhance misfolded protein aggregation.............................................................................................................. 58

Figure 15. Defects in lipid unsaturation do not disrupt mitochondrial homeostasis whereas reduction in PC production does......................................................... 60
Figure 16. The FA composition and the relative level of cardiolipin are not altered by \textit{mdt-15} or \textit{SCD} depletion. ........................................................................................................................................ 62

Figure 17. Outline for an unbiased genetic screen to identify additional \textit{mdt-15}–mediated processes that are required for ER homeostasis......................................................................................................................... 69

Figure 18. Working model proposed by this study......................................................................................................................... 77
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>TF</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>Pol II</td>
<td>RNA polymerase II</td>
</tr>
<tr>
<td>SREBP</td>
<td>Sterol regulatory element binding protein</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>SCD</td>
<td>Stearyl-CoA Desaturase</td>
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<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
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<td>PE</td>
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<td>Phosphatidylserine</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>UPR&lt;sub&gt;ER&lt;/sub&gt;</td>
<td>ER unfolded protein response</td>
</tr>
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<td>IRE1/IRE-1</td>
<td>Inositol requiring enzyme</td>
</tr>
<tr>
<td>XBP-1/HAC1</td>
<td>X-box binding protein</td>
</tr>
<tr>
<td>PERK</td>
<td>Protein Kinase RNA-like ER Kinase</td>
</tr>
<tr>
<td>ATF-6</td>
<td>Activating transcription factor 6</td>
</tr>
<tr>
<td>ERAD</td>
<td>ER associated protein degradation</td>
</tr>
<tr>
<td>eIF2α</td>
<td>Eukaryotic Translation Initiation Factor 2α</td>
</tr>
<tr>
<td>UPR&lt;sub&gt;mt&lt;/sub&gt;</td>
<td>Mitochondrial unfolded protein response</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas chromatography-mass spectrometry</td>
</tr>
<tr>
<td>SAMS-1</td>
<td>S-adenosyl methionine synthase</td>
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To my lovely family: 💗
Dedication

I dedicate this thesis to my beloved and loving grandfather, the hero of my life.

Thank you for your lifetime encouragement and optimism. You had been here with me during every single step of my adventure, and I hope you would be proud of me.
Chapter 1: Introduction

1.1 Transcriptional regulation in eukaryotes

Transcription is required to implement the genetic information contained in every organism’s DNA; therefore, transcriptional control is crucial for organism survival and development. Transcriptional regulation involves transcription factors (TFs), co-factors, chromatin modifications, and the general transcriptional machinery [1]. TFs recognize particular DNA sequences called the response elements and recruit co-factors, which typically do not possess DNA-binding properties [2]. TFs and their co-factors are important during at least two steps of transcription: initiation and elongation [2].

During transcription initiation, TFs bind to specific response elements and co-factors, which then recruit RNA Polymerase II (Pol II). Pol II in turn interacts with general initiation factors (e.g. TFIIB, TFIID, TFIIE, TFIIF and TFIIH), which allow Pol II to selectively bind to promoters. Once Pol II starts transcribing, it only does so for initially ~20-50 bases, and then pauses [2]. At this point, the switch between transcription initiation and elongation occurs: pause factors such as the negative elongation factor (NELF) physically stall Pol II, which requires further action of TFs and co-factors to overcome the pause and begin transcription elongation [2]. In eukaryotes, both transcription initiation and elongation activation require a conserved co-factor complex called the Mediator [2, 3]. With its ability to connect diverse TFs and other regulators directly to the Pol II apparatus, the Mediator is essential for the expression of most protein-encoding genes (Figure 1) [3].
1.2 The Mediator complex is a key eukaryotic co-factor

Initially thought to serve simply as an adaptor between TFs and Pol II, the Mediator is now widely understood to be a centre for signal integration, where various signalling inputs are converted into transcriptional outcomes.

1.2.1 Molecular actions of the Mediator in transcriptional regulation

The Mediator is involved in transcription initiation, elongation, and potentially even termination [3, 4]. During initiation, the Mediator recruits the Pol II apparatus upon binding to TFs and facilitates assembly of the pre-initiation complex. In this context, the Mediator helps increase the
local concentration of Pol II during transcription initiation, and thus serves as a general regulator for transcriptional activation of almost all genes [5-8]. However, the Mediator/Pol II ratio at certain loci is not necessarily kept at one at all times. As Pol II is required for the transcription of all genes, this means that there are genes whose transcriptional initiation is independent of the Mediator at least during certain phases [9-12].

During the transition between transcription initiation and elongation, the Mediator is recruited to the pre-elongation complex (PEC), within which Pol II is already bound to promoters [2, 3]. In this context, the Mediator regulates PEC activity and helps re-activate the paused Pol II [3]. Therefore, the Mediator does not function to recruit Pol II machinery during elongation activation; rather, it modulates the activity of the general transcription apparatus.

During both transcription initiation and elongation activation, the essential function of the Mediator is to transmit specific signals elicited from the interactions between response elements and TFs to the Pol II apparatus.

1.2.2 Structure and composition of the Mediator complex

In line with its fundamental role in transcriptional regulation, the Mediator complex has been preserved throughout eukaryotes [3]. In all organisms studied to date, the Mediator is composed of a core which includes a head module, a middle module, and a tail module. Additionally, some forms of Mediator contain a kinase module, which is dissociable (Figure 1) [3]. The head and middle modules provide structural integrity and contact sites for the Pol II apparatus, whereas the tail module functions primarily as a docking site for different TFs [3]. Indeed, subunits that
interact with TFs are almost exclusively located within or near the tail module (e.g. MED15, MED16, MED23, MED24, and MED1) [3]. Therefore, the tail module is considered the “signal-receiving” end of the Mediator complex [3, 13]. This may explain why individual subunits in the tail module have diverged considerably from yeast to mammals [3]. High divergence of the tail subunits allows eukaryotes to evolve distinct and specific signals, meeting the need for more sophisticated and complicated systems in higher organisms.

Although the Mediator is generally considered a co-activator, the kinase module allows the Mediator to negatively regulate transcription [13]. Transcriptional repression by the kinase module is accomplished through stabilization of the compact core Mediator and therefore sterically hindering Mediator binding to Pol II [13-15]. However, increasing evidence suggests that the kinase module is also capable of activating transcription [13, 16-19]. For example, the kinase subunit cdk-8 is a co-activator for the tumor suppressor p53 and it facilitates TF β-catenin to activate target genes [18, 19]. Importantly, as it is capable of reversely binding to the Mediator core, the kinase module provides an extra dimension for fine-tuning eukaryotic gene expression.

1.2.3 Mediator subunits and their functional specificity

The requirement of individual subunits for structural and functional integrity of the entire Mediator is subunit-specific. Not surprisingly, loss of subunits important for the architectural organization of the complex can lead to general loss of Mediator function [3]. For instance, the middle module subunits MED7 and MED21 provide essential structural domains for interactions with other subunits [20]. Similarly, MED6 serves as the bridge that links the middle and the head modules together [20]. Therefore, loss of any one of these subunits disrupts overall Mediator
structure and impairs the expression of most, if not all, Mediator-dependent genes, to an effect mimicking the loss of Pol II itself.

In contrast to such structural functions and the consequent non-specific requirement for gene transcription, many individual Mediator subunits are involved in specific biological processes. Below are two examples for functional specificity of Mediator subunits that are relevant for this thesis; several excellent reviews provide additional detail on selective actions of other Mediator subunits [3, 13].

1.2.3.1 MED1 is a selectively acting Mediator subunit required for nuclear hormone receptor function

MED1 is an excellent example of a gene-specific Mediator subunit. This Mediator subunit resides at the junction between the tail and the middle modules [3]. In mice and in mammalian cells, genetic ablation of MED1 selectively blocks nuclear hormone-receptor (NHR) mediated gene activation [3]. NHRs are TFs that are allosterically regulated through the binding of small molecular ligands [3]. Examples of NHRs include the thyroid hormone receptor (TR), Vitamin D receptor (VDR), peroxisome proliferator-activated receptor γ (PPAPγ), hepatocyte nuclear factor 4α (HNF4α), glucocorticoid receptor (GR), and estrogen receptor (ER), all of which interact physically with MED1 [5, 21-26]. The interaction between the NHR activation domain and the NHR-binding domain in MED1 is both necessary and sufficient for recruiting the Mediator complex to the NHRs [23].

Interestingly, some NHRs such as GR and PPAPγ additionally interact with other Mediator subunits such as MED14; in vivo, MED1 binding is not strictly required for e.g. PPAPγ-driven
adipogenesis; this suggests that TF interactions with Mediator are complex and involve multiple, perhaps partially redundant, molecular partnerships [5, 24, 27]. Nevertheless, MED1 implements specific activator-driven transcriptional programs without being required for Mediator complex integrity or Mediator action as a whole.

1.2.3.2 MED15/MDT-15 acts in conserved physiological and developmental signalling pathways

MED15 (referred to as Gal11 or Med15 in yeast, MDT-15 in C. elegans, and PCQAP/TIG-1/MED15 in mammals) locates to the tail module of the Mediator [3, 28]. Like MED1, MED15 is a selectively acting Mediator subunit that impacts several physiological functions in eukaryotes, particularly in the regulation of metabolism. Notably, these functions, and at least in part the underlying molecular mechanisms, are evolutionarily conserved throughout eukaryotes.

MED15 regulates lipid metabolism across eukaryotes. In yeasts, Med15 interacts with the TF Oaf1 to regulate fatty acid β-oxidation [28]. This interaction is mediated by Med15’s N-terminal KIX-domain, a three-helix bundle that is both structurally and functionally conserved in metazoans [28]. A similar regulation occurs in mammalian cells: mammalian MED15 uses its KIX-domain to physically interact with the Sterol-Regulatory-Element-Binding Protein 1α (SREBP-1α), a master transcriptional regulator for lipogenesis and adipogenesis [29]. In conditions of low cholesterol, MED15 co-activates SREBP targets such as the fatty acid synthase [29]. In C. elegans, MDT-15 uses its KIX-domain to physically interact with the SREBP-1α ortholog SBP-1 [29]. In addition to SBP-1, MDT-15 interacts with several HNF4α-like NHRs, notably NHR-49, a known regulator of fatty acid metabolism [30]. Together with SBP-1 and NHR-49, MDT-15 co-activates fatty acid metabolic genes [30]. Clearly, the existence of TF-
MED15-fatty acids metabolic axis, although involving distinct TFs, has been conserved from yeast to human.

In *C. elegans*, depletion of *mdt-15* by feeding RNA interference (RNAi) results in phenotypes such as developmental arrest, sterility, defective locomotion, a shortened lifespan and stress sensitivity [30, 31]. Similarly, a reduction-of-function mutation, which causes an in-frame deletion in *mdt-15* and results in a protein truncated with a central region of MDT-15, results in delayed development, a shorter lifespan, and low brood size [31, 32]. These phenotypes occur in part because *mdt-15* is required to co-activate three genes encoding the fatty acid ∆9-desaturases (*fat-5*, -6, and -7) [29, 30]. Fatty acid desaturases are enzymes that introduce carbon-carbon double bonds into saturated fatty acids and therefore change the secondary structure of a fatty acid, by decreasing its saturation level (Figure 2) [33]. In line with ∆9-desaturases as MDT-15 targets, dietary supplementation with the ∆9-desaturase downstream products partially rescues the fertility, mobility, and lifespan phenotypes of *mdt-15(RNAi)* worms [29, 30]. As the rescue is only partial, there exist other *mdt-15*-regulated pathways that are also involved in development, survival and reproduction.
In line with this notion, MED15 regulates processes other than lipid metabolism in various organisms. Yeast Med15 interacts with the TFs Gal4 and Gcn4 to regulate carbohydrate metabolism [34], and with the TFs Pdr1/Pdr3 to promote multidrug/xenobiotic resistance [35]. Additionally, Med15 also participate in the yeast oxidative stress response [36], though the underlying mechanisms remain unclear. Similarly, C. elegans mdt-15 is required for inducible gene expression programs. Relevant stimuli include fasting (mdt-15 co-activates genes required for fatty acid β-oxidation) [30], heavy metal and xenobiotic stress (mdt-15 is required to induce

Figure 2. mdt-15 is required for fatty acid desaturation.
In C. elegans, mdt-15 is required for the expression of the Δ9 desaturases, fat-6 and fat-7 (collectively called SCDs). Fatty acid desaturases add carbon-carbon double bonds into a particular saturated fatty acid and thus change its secondary structure. The 3D-ball-and-stick figures illustrate the structural differences of saturated and unsaturated fatty acids. Black sticks and balls represent the carbon-carbon backbones; white balls represent hydrogen molecules; red balls represent oxygen molecules. FAT-2 inserts a carbon-carbon double bond in the monounsaturated oleic acid and produces linoleic acid. Further desaturation and elongation of the linoleic carbon chain produces polyunsaturated fatty acids (PUFAs). Showing here is a representative omega-3 polyunsaturated fatty acid.
detoxification genes) [32], as well as exogenous oxidative stress (mdt-15 is important to induce anti-oxidative gene expression) [31]. Interestingly, the hypersensitivity of mdt-15-depleted worms to oxidative stress cannot be rescued by supplementation with dietary polyunsaturated fatty acids [31]. This demonstrates that mdt-15–regulated fatty acid desaturation and oxidative stress responses can be separated mechanistically.

Recently, the role of mdt-15 in adult worm lifespan has also been explored. The findings suggest that mdt-15 is required for the normal longevity of long-lived mutants [37]. mdt-15 impinges on longevity likely through generating lipid-derived signals that improve age-related defects such as protein homeostasis in a cell non-autonomous fashion [37]. However, mdt-15 depletion or mutation also strongly shortens the lifespan of wild-type worms [30]; as such, it is not clear that this gene provides a selective and specific function in promoting pro-longevity transcriptional programs in the above mutant contexts.

Lastly, in Xenopus laevis, MED15 is required for normal dorsal-ventral axis formation by acting in the TGF-β/Nodal signalling pathway [38]. Specifically, ectopic expression of MED15 RNA in embryos induces axis duplication whereas depletion of MED15 inhibits axis formation [38]. MED15 interacts physically and functionally with the TGF-β-regulated Smad-type transcription factors [38]. The link between TGF-β-signalling and MED15 is also present in a cancer model in mice, whereby MED15 is required for metastasis formation of transplanted cancer cells due to its essential action in TGF-β-signalling [39]. Interestingly, MED15 is overexpressed in castration-resistant prostate cancer, and this parallels the increased TGF-β-signalling activity [40].
Clearly, MED15/mdt-15 is of great relevance to eukaryotic, metazoan, and human biology, as reflected by its capacity to interact with several central physiological and developmental pathways. This is especially highlighted by the MED15/mdt-15-mediated regulation of lipid metabolism.

1.3 Function of lipids in the cell

Eukaryotic cells employ approximately 5% of their genes to synthesize thousands of lipid species [41]. This diversity allows lipids to perform three main functions that are fundamental for all organisms: energy storage, signalling, and membrane formation. Because they are enriched in carbon-carbon bonds, lipids have a relatively reduced biochemical status. Thus, energy can be stored stably in the form of neutral lipids, such as triglycerides or steryl esters, which are then incorporated into lipid droplets [41]. Additionally, some lipids such as diacylglyceride and ceramide are signalling molecules [41]. Acting as first or secondary messengers, they facilitate signal transduction across cellular barriers. Lastly, perhaps as the most ancient and vital function, lipids are the main constituents of cellular membranes. Polar lipids such as phospholipids contain both a hydrophobic and hydrophilic moieties [41]. The former tend to self-associate whereas the latter favors interactions with aqueous surroundings [41]. These intrinsic properties of membrane lipids enable barrier formation and thus allow cells and organelles to separate their internal contents from the external environment [41]. Organelle formation is key for intracellular compartmentalization and thus for functional separation within each cell.
One key property of membrane lipids is their ability to influence the fluidity of the membrane lipid layers they constitute. For example, the bigger the polar head group of a phospholipid, or the more unsaturated the fatty acyl chain of a phospholipid, the more space there is between neighbouring molecules, and thus the more fluidity there is in a membrane [41, 42]. Therefore, the biophysical property of a membrane is determined by the composition of its lipid components. As such, to maintain the proper functions of different membranous systems, the biosynthesis of membrane lipids is locally restricted [41].

A classic example of locally maintained membrane composition is the endoplasmic reticulum (ER). As the major lipid biosynthetic site, the ER produces most of the structural lipids including phospholipids, cholesterol, and complex sphingolipids [41]. However, little sterol or sphingolipid is found in the ER membrane, suggesting that these lipids are being rapidly transported out of the ER to their relevant destinations [41]. Instead, the ER membrane is enriched in the less compact phospholipids and is therefore, loosely packed [41].

The high fluidity of the ER membrane is crucial for its central function in the biosynthesis, insertion, and transportation of another elemental biomolecule, protein. The ER is the major site for secretory and membrane protein synthesis, modification, and translocation; protein concentration inside the ER can be as high as 100 mg/ml [43]. In addition to lipid and protein metabolism, the ER also serves as an intracellular Ca^{2+} storage compartment; Ca^{2+} concentration in the ER can reach thousands of times of that in the cytosol [44].
1.4 The ER unfolded protein response

Due to ER’s key functions in lipid, protein and calcium homeostasis, cells constantly monitor ER homeostasis. This is particularly true for cells that are specialized in protein secretion (e.g. plasma cells), lipid synthesis (e.g. hepatocytes), or Ca\(^{2+}\) signalling (e.g. sarcoplasmic reticulum in myocytes). The machinery that has evolved for these needs is termed the unfolded protein response (UPR\(^{ER}\)), a surveillance mechanism that ensures ER homeostasis. The UPR\(^{ER}\) consists of three parallel signalling branches that each center on a conserved stress sensors/signal transducer: the Inositol-Requiring-Enzyme 1 (IRE-1), the protein kinase RNA-like ER kinase (PERK), and the Activating-Transcription-Factor (ATF-6) [45] (Fig. 3) [46]. Upon ER stress, signalling through these pathways induces transcriptional and translational reprogramming, which mitigate stress inside the ER and improve its overall functional capacity [47].
The IRE-1-dependent UPR\textsuperscript{ER} is universal and ancient

The IRE-1 signalling pathway is the most ancient and conserved branch of the UPR\textsuperscript{ER}, as it is present from yeast to human [48]. Originally identified in yeast, Ire1 is a transmembrane protein
that possesses kinase and endonuclease activities [49]. The Ire1 endonuclease unconventionally splices a small number of mRNAs inside the ER, including the mRNA encoding the TF Hac1 [50-52]. Spliced Hac1 mRNA then produces the active Hac1 TF, which in turn activates the expression of genes involved in protein folding, protein modification, ER-Golgi transport, lipid/inositol metabolism, and cell wall biosynthesis [49, 51, 53]. In parallel to inducing genes that facilitate ER protein folding, Ire1/Hac1 signalling also triggers the ER-associated protein degradation pathway (ERAD), which promotes the degradation of misfolded proteins [49, 54]. Not surprisingly, inactivation of Ire1 or Hac1 compromises yeasts’ ability to survive ER stress, though neither Ire1 nor Hac1 is essential for survival under normal conditions [49].

In metazoans, Hac1 is replaced by the X-box binding protein 1 (xbp-1), which remains as the target of IRE-1 endonuclease [49, 55, 56]. Like Hac1, XBP-1 activates genes that improve ER protein folding capacity and promote misfolded protein degradation [55, 57, 58]. In line with this function, ire-1 and xbp-1 are required for survival under ER stress conditions in C. elegans [59]. Additionally, ire-1 is important for normal larval but not embryonic development in worms [60]. In contrast, in flies and in mice, loss of ire1 results in embryonic lethality. Curiously, loss of ire-1 does not hypersensitize mammalian cells to exogenous ER stress stimuli [61]; this initially confusing finding ultimately led to the identification of additional UPR_{ER} pathways that act in parallel to IRE1.

### 1.4.2 The PERK UPR_{ER} reprograms translation

In higher eukaryotes, the number of the UPR_{ER} pathways has increased. Absent in yeast, the PERK-mediated UPR_{ER} signalling is present in worms (C. elegans), flies (D. melanogaster), and
mammals [50, 60, 62, 63]. PERK mainly functions to attenuate general protein synthesis in times of proteostatic stress. This function is achieved through the intrinsic kinase activity of PERK, which phosphorylates and inhibits the eukaryotic translation initiation factor eIF2α, causing general translational attenuation [46, 49]. This reduces protein synthesis in the ER and therefore effectively lowers the ER’s proteotoxic burden. Interestingly, certain transcripts, such as the mRNA encoding the TF atf-4, escape this translational downregulation; instead, the atf-4 transcript produces the ATF-4 TF, which activates genes that help improve ER luminal conditions, such as redox homeostasis [64]. In line with its distinctive regulatory function in the UPRER, depletion of perk in worms and mammalian cells increases sensitivity to ER insults [60, 65]. Like ire-1, perk is non-essential for worms [57, 60], but essential in flies and in mice [50, 66-68].

1.4.3 The ATF-6 UPRER functions differently among metazoans

In addition to ire-1 and perk, a third UPRER sensor/transducer gene is present in metazoans, atf-6 [49]. Like IRE-1 and PERK, ATF-6 is an ER-resident membrane protein. Unlike IRE-1 and PERK, whose activation induces downstream transcription factors, ATF-6 is itself a transcriptional activator. Upon ER stress, ATF-6 translocates from the ER to the Golgi, where it undergoes proteolytic modification [69]. This allows its transcriptional activation domain to be released, and promote gene activation in the nucleus [46].

In worms and flies, the function of the ATF-6 UPRER pathway remains somewhat unclear, as inactivation of atf-6 does not compromise development or survival under normal or ER stress conditions [57]. This is consistent with the fact that atf-6 is not required for the induction of most
UPR\textsuperscript{ER} genes [57]. However, \textit{C. elegans ire-1 :atf-6} loss-of-function double mutant display developmental arrest and larval lethality, and ATF-6 is required for the transcription of a subset of genes that are constitutively expressed [57]; thus, \textit{atf-6} may play redundant roles in the development with the other UPR\textsuperscript{ER} pathways in \textit{C. elegans}.

Contrary to its role in \textit{C. elegans}, ATF6 is a crucial factor in the mammalian UPR\textsuperscript{ER}, as it is responsible for the expression of major ER chaperones [70]. ATF6 functions in partnership with the IRE1/XBP1 signalling pathway [49]. In particular, proteolytically activated ATF6 dimerizes with XBP1 and together they activate the ERAD pathway [61, 67, 70]. This may explain why \textit{ire1/xbp1} is not essential for mammalian cell survival under ER stress conditions, as ATF6 has taken over the major role of promoting ER protein folding capacity from the IRE/XBP1 UPR\textsuperscript{ER} in higher metazoans.

Overall, the three branches of the UPR are of different relevance dependent on species. PERK UPR\textsuperscript{ER} signalling provides an extra regulatory dimension in metazoans to overcome both external and physiological ER stress conditions. In contrast, the burden of the IRE-1 UPR\textsuperscript{ER} under ER stress has shifted to the ATF-6-mediated pathway from flies to mammals [49]. The pressures that caused these precise evolutionary changes remain to be unveiled.

1.5 \textbf{The UPR\textsuperscript{ER} is directly involved in human disease pathogenesis}

Disruption of ER homeostasis and the UPR\textsuperscript{ER} can affect a wide spectrum of biological processes and it apparently is linked to various diseases [71]. In general, there are three scenarios of UPR\textsuperscript{ER} involvement in disease pathogenesis: 1) Cells have decreased or lost the ability to fold or secret proteins, causing UPR\textsuperscript{ER} activation. 2) Cells can no longer properly respond to misfolded
proteins or to an increased load of proteins inside the ER. 3) The UPR\textsuperscript{ER} is activated inappropriately, compromising cell survival. The pathogenesis of diabetes is an excellent example of how important the UPR\textsuperscript{ER} is for human health [71].

1.5.1 The UPR\textsuperscript{ER} is a key player in the pathogenesis of diabetes

Type 1 diabetes (T1D) is mainly caused by an autoimmune destruction of the pancreatic β-cells; as a result, the pancreas can no longer synthesize or secret a sufficient amount of insulin. Currently, several proposed mechanisms of T1D pathogenesis involve the UPR\textsuperscript{ER} [71]. For example, the release of cytokines or nitric oxide (due to autoimmune response) leads to a constitutively activated UPR\textsuperscript{ER}, which results in apoptosis of β-cells in T1D patients [72, 73]. In addition, PERK is required for pancreatic β-cell differentiation, and \textit{perk} expression during fetal and neonatal stage is specifically required for postnatal glucose homeostasis [74].

The UPR\textsuperscript{ER} is also involved in the other major form of diabetes, Type 2 diabetes (T2D). Unlike T1D, T2D is a metabolic disorder that arises due to insulin resistance. In this case, pancreatic β-cells remain initially capable of secreting sufficient insulin; however, the peripheral tissues display insulin resistance and therefore reduced effectiveness of the insulin-signalling cascade [71]. To compensate for insulin resistance, the β-cells overproduce and secret insulin until they reach exhaustion [71]. Insulin over-production caused sustained UPR\textsuperscript{ER} activation, which ultimately commits the pancreatic β-cells to apoptosis, further exacerbating the vicious cycle.
1.5.2 Diabetes and UPR\textsuperscript{ER} stimulation

In β-cells, stimuli such as glucose deprivation and free fatty acids trigger the UPR\textsuperscript{ER} and downstream apoptosis [75-77]. Interestingly, relief of the UPR\textsuperscript{ER} activation in peripheral tissues, rather than β-cells themselves, can alleviate diabetic syndromes [71]. This has been shown by multiple studies in the obesity-induced T2D mouse models. For instance, rescue of ER protein folding capacity in the liver by small chemical chaperones normalizes blood glucose level and improves insulin sensitivity in the livers of obese mice, a T2D model [78]. Liver-specific overexpression of a single ER chaperone is also able to rescue glucose tolerance and insulin sensitivity in these animals [79]. These suggest that the UPR\textsuperscript{ER} induction in peripheral tissue, rather than that in the β-cells is critical in T2D. However, why and how the UPR\textsuperscript{ER} sensors are activated in peripheral tissues, and why improving proteostasis in these tissues improves β-cell health, still remains unclear.

1.6 Molecular mechanisms leading to the activation of the UPR\textsuperscript{ER}

What is the molecular input that triggers the UPR\textsuperscript{ER}, and how do UPR\textsuperscript{ER} signal transducers sense these inputs? Glucose starvation, inhibition of protein glycosylation, overexpression of misfolded proteins, and disruption of the ER’s Ca\textsuperscript{2+} pool all induce the ER resident chaperone BiP (also known as GRP48 and HSP-4) [44, 80]. BiP binds to misfolded proteins and corrects protein misfolding [81]. Several studies suggested that the association of misfolded proteins with BiP activates the UPR\textsuperscript{ER} by reducing the concentration of free BiP, implying that free BiP can directly repress UPR\textsuperscript{ER} sensor activation [43, 82-85]. In line with this model, BiP binds to the luminal domains of IRE-1 and PERK and stabilizes them in their monomeric and inactive state [43, 86]. In the presence of misfolded proteins, BiP dissociates from the UPR\textsuperscript{ER} sensors, which
then dimerize and/or form high-order oligomers [87-89]. Oligomerization of IRE-1 or PERK activates their enzymatic activities [87-89].

In parallel to this BiP-driven mechanism, misfolded proteins serve as direct ligands for IRE-1 [43]. In particular, binding of misfolded proteins to the IRE-1 luminal domain destabilizes the monomeric and inactive IRE-1 and promotes IRE-1 oligomerization and activation [90, 91]. In summary, as implied by the term Unfolded Protein Response implies, misfolded proteins can indirectly (through binding to BiP) and directly act as the stimuli for the activation of the UPR\(^\text{ER}\) sensors.

A key finding from the above studies is that the IRE-1 and PERK enzymatic domains are highly cooperative. In other words, high-order oligomerization must take place for downstream signalling to occur. This biophysical requirement for the activation of IRE-1 and PERK provides a strong support for another candidate of UPR\(^\text{ER}\) inducer that has recently come into the spotlight: the ER lipid membrane.

### 1.6.1 Lipid metabolism and UPR\(^\text{ER}\) activation

Alterations in lipid metabolism can adversely affect ER homeostasis. For instance, exogenous saturated fatty acids can trigger the UPR\(^\text{ER}\) [75]. Free fatty acids are constantly being incorporated into membrane phospholipids [92]. This means that lipid metabolism affects membrane lipid compositions. Therefore, abnormal lipid metabolism can affect ER homeostasis by manipulating ER membrane lipid composition. Indeed, changes in membrane unsaturation, phospholipid ratios, and membrane cholesterol accumulation can activate the UPR\(^\text{ER}\) [93-96]. This is consistent with the fact that the ER demands a flexible membrane; as such, a decrease in
l lipid unsaturation, a reduction in phospholipid headgroup size (e.g. through a change of the phosphatidylcholine (PC)/phosphatidylethanolamine (PE) ratio), and tighter spacing between fatty acyl tails (e.g. as caused by cholesterol accumulation) are all expected to reduce ER membrane fluidity \[97\]. In turn, ER membrane flexibility may modulate the UPR\textsuperscript{ER} by influencing membrane protein insertion, soluble protein translocation, membrane anchoring, and the functionality of ion channels and transporters, such as the ER Ca\textsuperscript{2+} ATPase \[98\].

**1.6.2 Membrane lipid aberrancy indirectly activates the UPR\textsuperscript{ER}**

Whether loss of ER membrane integrity induces the UPR\textsuperscript{ER} indirectly through misfolded proteins remains ambiguous. Chemical chaperones suppress the UPR\textsuperscript{ER} induction by saturated fatty acids and steroid lipid in yeast and in obese mice \[78, 79, 99\]. This implies that membrane lipid abnormalities activate the UPR\textsuperscript{ER} through impaired ER protein folding. Alternatively, the UPR\textsuperscript{ER} could be triggered indirectly through disruption of ER Ca\textsuperscript{2+} homeostasis. For instance, the ER from obese mice has a higher PC/PE ratio, disrupted Ca\textsuperscript{2+} pump activity and an activated UPR\textsuperscript{ER} \[95\]. Restoration of the PC/PE ratio rescues Ca\textsuperscript{2+} ATPase activity and suppresses the induction of the UPR\textsuperscript{ER} \[95\]. As Ca\textsuperscript{2+} strongly influences the ER luminal protein folding machinery, it is highly likely that changes in Ca\textsuperscript{2+} levels ultimately causes UPR\textsuperscript{ER} induction through its effect on proteostasis \[100, 101\]. Together, these studies thus suggest that lipid aberrancy can activate the UPR\textsuperscript{ER} indirectly through introducing misfolded proteins.

**1.6.3 Membrane lipid aberrancy directly activates the UPR\textsuperscript{ER}**

In contrast to the above studies, several recent reports hint at UPR\textsuperscript{ER} activation without the involvement of misfolded proteins. Kitai and colleagues demonstrated that IRE-1 does not
undergo oligomerization when the UPR\textsuperscript{ER} is induced by exogenous saturated fatty acids [102]. Specifically, an IRE-1 mutant defective for oligomerization retained the capacity to activate the UPR\textsuperscript{ER} in these conditions [102]. This suggests that IRE-1 oligomerization is not required for downstream signal activation in this context; and perhaps, that misfolded proteins may not be involved in such UPR\textsuperscript{ER} induction, as the association of misfolded proteins and/or BiP disassociation are thought to occur upstream of IRE-1 oligomerization [43, 102].

Another study proposed that IRE-1 activation can take place in the absence of its misfolded-protein-sensing domains, as long as the stimuli are restricted to membrane perturbations, such as inositol depletion [103]. In this study, inositol deprivation did not cause BiP-associated protein aggregation in wild-type yeast cells [103]. This implies that inositol depletion activates the IRE-1 UPR\textsuperscript{ER} without BiP binding to misfolded proteins and by extension, misfolded proteins.

An elegant recent study by Volmer \textit{et al} showed that, in a mammalian cell systems where the luminal domains of IRE-1 and PERK are genetically deleted, these two sensors retain the ability to activate downstream UPR\textsuperscript{ER} signalling in response to increased membrane lipid saturation [104]. The authors thus suggested that increased saturation of ER membrane lipids triggers the UPR\textsuperscript{ER} by favoring IRE-1 and PERK oligomerization, which is facilitated through the transmembrane domains of IRE-1 and PERK [104]. Therefore, IRE-1 and PERK can sense membrane lipid disequilibrium independently of their ability to detect luminal proteostasis [104].

However, the model put forward by Volmer \textit{et al} contradicts the findings by Kitai \textit{et al.}, which indicate that membrane-related ER stimuli trigger the UPR\textsuperscript{ER} signalling without oligomerization of the UPR\textsuperscript{ER} sensors. Additionally, in all of these studies, the proteostatic states of the ER was
not directly addressed, i.e., although these studies demonstrate that misfolded protein-dependent IRE-1 and PERK signalling mechanism can be disconnected from lipid membrane aberrancy induced UPR$^{\text{ER}}$, they do not address whether the lipid imbalance is accompanied by proteostatic disturbances.

Furthermore, although all of the above studies provide novel insights into a misfolded protein-independent mechanism of UPR$^{\text{ER}}$ activation, the interpretations of the results are limited to reconstituted systems – whereas the available in vivo studies implicate disturbed proteostasis as a key event in lipid disequilibrium induced UPR$^{\text{ER}}$. Hence, more comprehensive experiments are needed for a better understanding on how the UPR$^{\text{ER}}$ activation occurs in response to membrane lipid aberrancy.

1.7 *C. elegans*: a model system to study lipid metabolism and the UPR$^{\text{ER}}$

What events take place in a live organism when the UPR$^{\text{ER}}$ is activated by ER perturbation other than misfolded proteins? How is the physiology affected at the whole-organism level? Finally, when membrane disequilibrium triggers the UPR$^{\text{ER}}$, are there misfolded proteins present at all? To answer these questions, we turned to the model organism *C. elegans*, an excellent animal to study lipid biology and stress response pathways.

First, many essential lipid metabolism genes and regulators such as *SREBP* and the Δ9-desaturases (*SCD*, or *fat-6* and *-7*)) are conserved from worms to mammals [105]. Although *C. elegans* does not have an adipose tissue equivalent of that in metazoans, it uses similar pathways to store energy in form of lipid droplets, and similar neuronal circuits to regulate lipid metabolism (e.g. insulin, adiponectin, and NHR signalling) [33]. In particular, as described
above, the Mediator subunit *mdt-15*, its key targets (the three *SCD* genes), and UPR\textsuperscript{ER} signalling are conserved in *C. elegans* [3, 48].

Moreover, *C. elegans* provides outstanding genetic and genomic tractability, and the physiological impact of a given gene can be examined by monitoring through phenotypes such as locomotion, lifespan, and brood size. Furthermore, anatomy of *C. elegans* is simple and well characterized, which often allow observations at a single-cell resolution. Altogether, these features make *C. elegans* an excellent choice for the current study, as it enables integration of functional, genetic, and metabolic approaches in one *in vivo* system.

### 1.8 Activating the UPR\textsuperscript{ER} *in vivo* without disturbed proteostasis

Two independent studies demonstrated that the Mediator subunit MDT-15 is essential for unsaturated fatty acid synthesis and thus the development, survival, and reproduction of *C. elegans* [29, 30]. However, dietary supplementation with unsaturated fatty acids fails to fully rescue the phenotypes induced upon *mdt-15* depletion [29, 30]. This leads to several interesting questions. First, why is *mdt-15*-mediated fatty acid biosynthesis so important for the normal physiology of *C. elegans*? Second, what are the cellular consequences of *mdt-15* inactivation?

Notably, the ER specific chaperone BiP/hsp-4 is upregulated in *mdt-15*-depleted worms [106]. This is interesting as it suggests that the UPR\textsuperscript{ER} signalling may be constitutively activated in worms with a dysfunctional *mdt-15*. Therefore, we set out to address the following specific questions:

1) How does *mdt-15* regulate the fatty acyl compositions of individual lipids and how does it affect their overall levels?
2) Does *mdt-15* influence ER homeostasis?

3) If so, how does it affect the UPR\(^{\text{ER}}\) and ER homeostasis?

4) What are the additional *mdt-15*-mediated pathways that enable homeostasis, organismal health, and longevity?

By answering these questions, this study revealed unexpected findings, which led to more comprehensive evidence supporting a novel mechanism that links lipid metabolism and the activation of the UPR\(^{\text{ER}}\).
Chapter 2: Results

2.1 The transcript levels of multiple lipid metabolic genes are affected in *mdt-15* worms.

In *C. elegans*, MDT-15-mediated unsaturated fatty acid biosynthesis is required for development, survival and reproduction [29, 30]. However, the defects that result from *mdt-15* inactivation cannot be completely rescued by polyunsaturated fatty acid supplementation [29, 30]. What are the other *mdt-15*-regulated pathways that contribute to the phenotypes in *mdt-15* worms? In 2011, Walker *et al* showed that *sbp-1* is required for the expression of genes involved in phosphatidylcholine (PC) biosynthesis [107]. As MDT-15 interacts with SBP-1, we hypothesized that *mdt-15* might also be required for PC production.

To test this hypothesis, we first used real-time quantitative PCR (RT-PCR) to examine the expression of genes in the PC biosynthetic pathways. As shown in Fig. 4A and B, depletion of *mdt-15* by RNA interference (RNAi) caused a significant downregulation of genes required for PC biosynthesis, such as *sams-1*, *pmt-2*, *pcyt-1*, and *cept-1* (compare to control(RNAi) worms exposed to empty RNAi vector); *sams-1* is especially noteworthy as it is the gene responsible for the production of methyl-group in the PC headgroup. (Fig. 1D) [107]. Although these same genes were not significantly affected in the *mdt-15(tm2182)* reduction-of-function mutant (compared to wild-type) [32], *pmt-2* and *cept-1*, which are important enzymes in the PC de novo biosynthetic pathways are substantially downregulated in both *mdt-15(RNAi)* and *mdt-15(tm2182)* worms (Fig. 4B and D). Together, these results suggest that PC levels may be reduced in worms without a functional *mdt-15*. 
Because the expression of a metabolic gene can influence the expression of its related genes [108], we wanted to test whether the downregulation of the PC genes is the indirect consequence of the reduced expression of mdt-15-dependent fatty acid metabolism genes, specifically two Δ9 Stearyl-CoA desaturases (SCDs; *fat*-6 and -7 in *C. elegans*) that are key mdt-15 targets. Interestingly, SCD depletion also resulted in downregulation of *sams*-1 (Fig. 4C). However, many other genes involved in PC synthesis (e.g. *pmt*-2, *pcyt*-1, *cept*-1) that are downregulated in the mdt-15(RNAi) worms were not affected upon SCD depletion. Therefore, mdt-15 mutation or depletion may directly cause defects in PC biosynthesis.
Figure 4. Inactivation of mdt-15 alters the expression of multiple lipid metabolism genes. (A-C) Bars indicate the average fold change of mRNA levels (relative to control(RNAi) worms or wild-type worms as appropriate). mRNA was isolated from L4 stage control(RNAi), mdt-15(RNAi), SCD(RNAi), wild-type (WT), or mdt-15(tm2182) worms (n=3); mRNA levels were normalized to the average expression of act-1, tba-1 and ubc-2 to yield relative mRNA levels; error bars represent SEM, and * indicates p<0.05 (two-tailed student t-test). Genes in red boxes are essential for PC production [107]. (D) The diagram depicts the pathways for PC biosynthesis (adapted from [107]).
2.2 *mdt-15* is required to maintain normal membrane lipid unsaturation

To determine whether *mdt-15* is required to maintain normal levels of PC or other phospholipids, and to gain comprehensive insight into the role of *mdt-15* in regulating lipid fatty acyl compositions, we extracted lipids from *mdt-15(RNAi)* worms and the hypomorph *mdt-15(tm2182)* mutant (compared to (control)RNAi worms and wild-type N2 worms, respectively). We separated lipids by thin layer chromatography and quantified the fatty acid compositions of individual lipids using gas chromatography-mass spectrometry (GC-MS).

Consistent with previous findings [29, 30], we observed a global increase in saturated and an overall decrease in polyunsaturated fatty acids in *mdt-15* worms (Fig. 5A). The upregulation in lipid saturation and the downregulation in lipid polyunsaturation both arose from membrane phospholipids and triglyceride (Fig. 5B and C). On the contrary, the abundance of monounsaturated fatty acids was not altered upon *mdt-15* depletion or mutation (Fig. 5D). This seems to contradict the fact that *mdt-15* is required to express *SCD*, which synthesizes monounsaturated fatty acids such as C18:1n-9. However, it is mostly likely a substrate build-up effect due to the massively downregulated C18:2 content (Fig. 2).

Interestingly, although both PC and phosphatidylethanolamine (PE) are major membrane phospholipids, PC unsaturation was more substantially altered than PE in *mdt-15* worms (Fig. 5C, see Table 1 and 2 for p-values). The severe changes in PC were also evident in the relative abundance of individual fatty acid species. For instance, in *mdt-15(RNAi)* worms, the longest and most unsaturated fatty acid C20:5 was strongly reduced in PC, but was not affected in PE (Fig. 5E and F). On the contrary, most other fatty acyl chains were significantly affected in both phospholipids. For example, the C18:2 contents were strongly reduced in both PC and PE; and
the saturated fatty acids C16:0 and C18:0 were both highly upregulated in PC and PE upon (Fig. 5E and F).

In contrast to the fatty acid compositions, the relative levels of membrane phospholipids and triglyceride were not substantially affected by mdt-15 depletion or mutation (Fig. 5G). We only observed a small change in PC level relative to total phospholipids in the mdt-15(RNAi) worms, and no significant change of PC levels in the mutant (Fig. 5G). Moreover, the slight reduction in the mdt-15(RNAi) worms was suppressed by unsaturated fatty acid supplementation (similar to the results in SCD(RNAi) worms; Fig. 8B-D and see below). This lack of change in PC levels was somewhat unexpected, as the transcript profiles of mdt-15(RNAi) and mdt-15(tm2182) worms showed substantial downregulation of several crucial PC synthesis genes (section 2.1), which implies potential downregulation in overall PC production (Fig. 4A and B).

Together, these data suggest that mdt-15 is not essential to maintain the relative levels of phospholipids and triglyceride; rather, it is specifically required for maintaining normal fatty acid polyunsaturation in the major phospholipids, particularly PC.
Figure 5. *mdt-15* is required to maintain normal membrane lipid unsaturation.

(A) Bars indicate the average abundance of FAs relative to total lipids (TLs) extracted. “Saturated” represents the average abundance of TLs that contain C16:0 and/or C18:0 fatty acyl chains. “Polyunsaturated” represents the average abundance of TLs that contain C18:2, C20:3, C20:4, C20:4n-3, and/or C20:5 fatty acyl tails. (B) Bars indicate the average abundance of PC, PS, PI, PE, and triglyceride with C16:0 and/or C18:0 acyl tails relative to TLs. (C) Bars represent the average abundance of PC, PS, PI, PE, and triglyceride with C18:2, C20:3, C20:4, C20:4n-3 and/or C20:5 fatty acyl tails relative to TLs. (D) Bars represent the average abundance of PC, PS, PI, PE and triglyceride (TG) with C18:1n-7 and/or C18:1n-9 fatty acyl tails relative to TLs. (E) Bars indicate the relative abundance of individual FAs in PC. (F) Bars indicate the relative abundance of individual FAs in PE. (G) Bars represent the relative levels of individual lipid species relative to total TLs extracted (left panel) and total phospholipids extracted (right panel). In all panels, lipids were isolated from L4 stage control(RNAi), *mdt-15*(RNAi), wild-type (WT), or *mdt-15*(tm2182) worms (n=3); error bars represent SEM, and * indicates p<0.05 (two-tailed student t-test).
2.3  *mdt-15* depletion or mutation activates the UPR\textsuperscript{ER}

The saturation degree and the chain length of fatty acyl tails strongly influence membrane phospholipid structure and thus the functions of cellular organelles [97]. For instance, the ER has a more fluidic membrane as it needs to accommodate constant biosynthesis of proteins and lipids [97, 109]. Interestingly, *mdt-15*(RNAi) worms display constitutive upregulation of the ER chaperone BiP/hsp-4 [32]. This suggests that the UPR\textsuperscript{ER} may be activated upon *mdt-15* depletion. Taken together with the fact that the ER membrane lipid PC is profoundly altered in *mdt-15* worms, we thus hypothesized that *mdt-15* is required for ER homeostasis.

To test this hypothesis, we first determined whether *mdt-15* depletion or mutation induces the UPR\textsuperscript{ER}. In metazoans, activation of the IRE-1 branch of the UPR\textsuperscript{ER} results in increased splicing of the mRNA encoding the transcription factor X-box-Binding-Protein-1 (XBP-1), which activates UPR\textsuperscript{ER} target genes such as *hsp-4* [46]. Using *hsp-4* and spliced *xbp-1* (*xbp-1s*) as markers, we found that *mdt-15* depletion or mutation results in constitutive activation of the UPR\textsuperscript{ER}, as indicated by an increase in the *xbp-1s* and *hsp-4* transcript levels (Fig. 6A). Similarly, a transcriptional *hsp-4* reporter (*hsp-4p::gfp* [56]) was strongly upregulated in *mdt-15*(RNAi) and *mdt-15(tm2182)* worms (Fig. 6B). The elevated expression of *hsp-4p::gfp* was abrogated by concurrent loss of *xbp-1* (and *ire-1*, Fig. 14 and see below) (Fig. 6C), demonstrating that *hsp-4* induction in *mdt-15* worms reflects canonical UPR\textsuperscript{ER} signalling [56].

Activation of the UPR\textsuperscript{ER} PERK arm attenuates general protein translation by phosphorylating and inhibiting the eukaryotic translation initiation factor eIF2α [46]. Using immunoblot analysis with antibodies specific for phosphorylated eIF2α, we found that depleting or mutating *mdt-15*
substantially increased eIF2α phosphorylation (Fig. 6D). Thus, *mdt-15* inactivation perturbs ER homeostasis and activates at least two branches of the canonical UPR^ER^. 
Figure 6. 

(A) Bar graphs represent the average fold change of xbp-1, spliced xbp-1 (xbp-1s), and hsp-4 mRNA levels in control(RNAi) and mdt-15(RNAi) worms (p=0.098, 0.049, and 0.00049, respectively) (left panel) or wild-type (WT) and mdt-15(tm2182) worms (p=0.19, 0.063, and 0.0064, respectively) (right panel) (n=3). Error bars represent SEM, and * indicates p < 0.05 (two-tailed student t-test). 

(B) Micrographs depict control(RNAi), mdt-15(RNAi), WT, and mdt-15(tm2182) worms expressing the hsp-4p::gfp transcriptional reporter; one of four independent experiments is shown. 

(C) Micrographs show hsp-4p::gfp reporter expression in WT and xbp-1(zc12) worms on control or mdt-15 RNAi. 

(D) Immunoblots depict the levels of phospho-Ser51 eIF2α (P-eIF2α) and actin in control(RNAi), mdt-15(RNAi), WT, and mdt-15(tm2182) worms. The numbers represent the intensity of the P-eIF2α bands relative to the corresponding actin bands. One of four independent experiments is shown.
2.4 *mdt-15*-regulated lipid unsaturation is essential for ER homeostasis

Decreased membrane lipid unsaturation induces the UPR\textsubscript{ER} in cultured mammalian cells and in yeast [110-112]. As *mdt-15* is required to maintain normal membrane phospholipid unsaturation (Fig. 5), we hypothesized that *mdt-15* confers ER homeostasis by maintaining unsaturated fatty acid contents.

To test this hypothesis, we supplemented *mdt-15*(RNAi) and *mdt-15*(tm2182) worms with dietary C18:1n-9, C18:2 and C20:5, as these fatty acids are downstream products of the key *mdt-15* target *SCD* and are strongly downregulated following *mdt-15* depletion or mutation (Fig. 5E, Table 1). Using the *hsp-4p::gfp* reporter as a marker for UPR\textsubscript{ER} activation, we found that in *mdt-15*(RNAi) and *mdt-15*(tm2182) worms, oleic acid (C18:1n-9) alone and combinations of unsaturated fatty acids substantially but only partially suppressed *hsp-4p::gfp* induction and morphological phenotypes (e.g. small and clear body morphology) (Fig. 7A). Similarly, unsaturated fatty acid supplementation effectively but incompletely suppressed the induced *hsp-4* and *xbp-1s* mRNA levels in *mdt-15*(RNAi) and *mdt-15*(tm2182) worms (Fig. 7B).

To test whether the incomplete rescue simply reflects an intrinsic flaw of the dietary supplementation protocol, we attempted a phenocopy experiment with *SCD*(RNAi). As expected, depletion of the *mdt-15* target *SCD* by RNAi also induced the UPR\textsubscript{ER}, as indicated by both an induced *hsp-4p::gfp* reporter (Fig. 7A) and the upregulated *hsp-4* and *xbp-1s* transcript levels (Fig. 7B).

However, in contrast to *mdt-15* worms, the UPR\textsubscript{ER} activation in the *SCD*(RNAi) worms was fully suppressed by either oleic alone or combination of downstream polyunsaturated fatty acids (Fig. 7B).
2, Fig. 7A and B, see table 1 and 3 for p-values). This indicates that 1) fatty acid supplementation was effective and 2) SCD synthesized monounsaturated fatty acid (oleic acid) is important for normal downstream polyunsaturated fatty acid production, which is required for ER homeostasis. Together, these data show that mdt-15-mediated fatty acid unsaturation is crucial for ER homeostasis.
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Figure 7. *mdt-15*-mediated lipid unsaturation is required for maintaining ER homeostasis.

(A) Micrographs show control(RNAi), *mdt-15*(RNAi), WT and *mdt-15*(tm2182) worms expressing the hsp-4p::gfp reporter (left panel) grown without dietary supplement (right panel); with oleate (C18:1n-9); with linoleate (C18:2) and EPA (C20:5) (PUFAs); or with oleate and PUFAs (O+PUFAs), respectively. (B) Bar graphs represent the average fold change of hsp-4 and spliced *xbp-1* (*xbp-1s*) mRNA levels in control(RNAi), SCD(RNAi), and *mdt-15*(RNAi) worms with or without oleate supplement (n=3). p-values: *hsp-4*: p=0.048, 0.059, 0.96, 0.40, 0.022, *xbp-1s*: 0.087, 0.25, 0.42, 0.66, 0.060, respectively. The bar graphs in the right panel represent the average fold change of expression in WT or *mdt-15*(tm2182) hypomorph worms, with or without supplements (oleate and PUFAs). p-values: *xbp-1*(s): p=0.32, 0.11, 0.32, *hsp-4*: p=0.24, 0.080, 0.0076, *pdi-3*: p=0.46, 0.31, 0.20, F07A11.2: p=0.43, 0.25, 0.96, *srp-7*: p=0.36, 0.025, 0.034. Error bars represent SEM, and * indicates p<0.05 (two-tailed unequal variant student t-test).
2.5 Fatty acid uptake and incorporation is not affected upon mdt-15 depletion

The phenocopy experiment with SCD(RNAi) worms shows that the dietary rescue protocol can be effective, but it might not succeed if mdt-15 worms are defective for fatty acid uptake or incorporation into lipids. Indeed, the pharyngeal pumping rate (pharynx is a neuromuscular pump C. elegans use to ingest food sources and it directly connects to the intestine) was significantly reduced in mdt-15 worms; and it was only partially rescued by unsaturated fatty acid supplementation (Fig. 8A).

To determine whether fatty acid uptake or incorporation is defective in mdt-15 worms, we used GC-MS to profile the lipid contents of control(RNAi), SCD(RNAi), and mdt-15(RNAi) worms grown in the absence or presence of dietary unsaturated fatty acids (C18:1n-9, C18:2 and C20:5). As shown (see relative abundance of C18:1n-9, C18:2 and C20:5), depletion of SCD or mdt-15 did not impair fatty acid dietary uptake or their incorporation into PC (Fig. 8B-C), suggesting effective exogenous fatty acid complementation.

2.5.1 Alterations in C18:0, C20:3, and C20:4 fatty acyl contents or relative PC level do not contribute to the UPR\textsuperscript{ER} activation

Although the uptake and incorporation of the dietary fatty acid supplements were not affected, alterations in other PC fatty acyl contents may contribute to the activated UPR\textsuperscript{ER}. Indeed, the relative abundance of other polyunsaturated fatty acids such as C20:3, C20:4 and C20:4n-3 was also reduced upon mdt-15 or SCD depletion in PC and even in PE; however, the reduction in these polyunsaturated fatty acids was not rescued by fatty acid supplementation (Fig. 8B and E). As fatty acid supplementation fully repressed the UPR\textsuperscript{ER} induction in SCD (RNAi) worms,
downregulation in the C20:3 and C20:4 polyunsaturated fatty acids cannot explain the remaining UPR\textsuperscript{ER} in the \textit{mdt}-15 worms. Similar argument also holds for the saturated fatty acid C18:0, as its upregulation was not suppressed by fatty acid supplementation in either \textit{SCD}(RNAi) or \textit{mdt}-15(RNAi) worms (Fig. 8B and C). This suggests that the increase in phospholipid saturated C18:0 alone does not induce the UPR\textsuperscript{ER} in worms.

Interestingly, the PC C16:0 (palmitic acid) content was only upregulated in \textit{mdt}-15(RNAi) worms, but not in the \textit{SCD}(RNAi) animals. This alteration was not suppressed upon unsaturated fatty acid supplementation. As free palmitic acid triggers the UPR\textsuperscript{ER} in mammalian cells and in yeast [42, 75, 111], this may contribute to the remaining UPR\textsuperscript{ER} activation in the \textit{mdt}-15 worms. Like \textit{mdt}-15 RNAi, \textit{SCD} RNAi significantly reduced the relative PC level; concomitantly, it also increased the relative level of PE (Fig. 8D). Importantly, this change in PC and PE production was completely suppressed upon unsaturated fatty acid supplementation, both in \textit{mdt}-15 and \textit{SCD} depleted worms (Fig. 8D). This suggests that the reduction in polyunsaturated fatty acid contents affects PC and PE levels and further supports the notion that \textit{mdt}-15 is not directly required for maintaining the relative PC levels.

Overall, as the fatty acid profile of polyunsaturated fatty acid supplemented \textit{mdt}-15(RNAi) worms is very similar to that of supplemented \textit{SCD}(RNAi) worms (Fig. 8E), we concluded that the induction of UPR\textsuperscript{ER} in \textit{mdt}-15 worms is partly the consequence of imbalanced membrane phospholipid unsaturation. Additionally, \textit{mdt}-15 must also regulate other unidentified pathways that contribute to ER homeostasis.
Figure 8. Fatty acid uptake and incorporation is intact in lipid desaturation-defective worms.

(A) Dot plots represent pharyngeal pumping rates of control(RNAi), mdt-15(RNAi) worms with or without oleate supplement (left panel), or WT and mdt-125(tm2182) worms with or without PUFA supplements (right panel). The data are pooled from four independent experiments. Error bars represent SEM, and * indicates p<0.05. (B) The bar graphs in the left panel represent the relative abundance of individual FAs in PC with or without unsaturated FA supplementation. The bar graphs in the right panel indicate the average levels of individual lipids relative to total phospholipids with or without unsaturated FAs supplements. (C) Bar graphs show the relative abundance of individual FAs in PC (left panel) or PE (right panel) with or without unsaturated FAs supplements. (D) Bar graphs average levels of lipids relative to total phospholipids extracted (left panel) or TLs extracted (right panel) with or without unsaturated FA supplements. (E) Bars represent the average abundance of PC, PS, PI, PE, and triglyceride containing saturated or polyunsaturated FA tails relative to total lipids. “Saturated” represents the sum of the corresponding lipids that contain C16:0 and/or C18:0 fatty acyl tails. “Polyunsaturated” represent the sum of the corresponding lipids that contain C18:2, C20:3, C20:4n-3, and/or C20:5 fatty acyl tails. For B-E, lipids were extracted from L4 stage control(RNAi), mdt-15(RNAi), and SCD(RNAi) worms supplemented with or without unsaturated FA supplements (n=3). Error bars are SEM, and *indicates p<0.05.
2.6 Normal PC production is essential for ER homeostasis but is not under mdt-15’s direct regulation

Alterations in PC/PE ratio induce the UPR$^{ER}$ [113, 114]. Although the above GC-MS analysis indicates that the reduction in PC level is entirely due to downregulation in polyunsaturated fatty acid synthesis, it does not rule out the possibility that PC deficiency contributes to the UPR$^{ER}$ activation in the mdt-15 worms.

To test this possibility, we examined the effects of choline supplementation on mdt-15(RNAi) worms, using hsp-4p::gfp induction to assess UPR$^{ER}$ activation. The combination of unsaturated fatty acids and choline supplementation would be expected to rescue the UPR$^{ER}$ if mdt-15 RNAi caused low PC levels due to sams-1 downregulation (Fig. 4A), in addition to defective fatty acid desaturation. However, neither choline supplementation alone nor the combination of choline and fatty acid supplements had any additional effect on the activated UPR$^{ER}$ in mdt-15 depleted worms, as compared to the fatty acid-supplemented sample (Fig. 9).

To ensure choline supplementation efficiency, we used worms depleted of sams-1 as positive control. SAMS-1 synthesizes S-adenosyl methionine, a critical precursor for one of the two PC biosynthetic pathways (Fig. 4D) [107]. Interestingly, sams-1 RNAi also activated hsp-4 in worms; importantly, this UPR$^{ER}$ activation was fully suppressed by dietary supplementation with the sams-1 downstream product choline, but not by dietary supplementation with its upstream metabolite ethanolamine (Fig. 4D and Fig. 9). This is in agreement with the published studies, which suggest that alteration in PC/PE ratio and reduction in PC biosynthesis activate the UPR$^{ER}$ [107, 114].
Together, these data indicate that 1) both PC polyunsaturation and normal PC level are crucial for maintaining ER homeostasis and 2) *mdt-15* regulates specifically the unsaturation of lipids, but not their relative levels.

**Figure 9. Normal PC synthesis is required for ER homeostasis.** Micrographs show *control*(RNAi), *sams-1*(RNAi), and *mdt-15*(RNAi) worms expressing the *hsp-4p::gfp* transcriptional reporter grown without dietary supplements or with ethanolamine or choline supplements, respectively. Fluorescence micrographs depict activation of the *hsp-4* reporter, whereas the DIC micrographs reveal morphological phenotypes.

### 2.7 *mdt-15* itself is not part of the UPR\textsuperscript{ER}

What are the additional pathway(s) that contribute to ER homeostasis and under the control of *mdt-15?* The UPR\textsuperscript{ER} is required for normal development and for survival under ER stress induced by exogenous stimuli. Failure to activate UPR\textsuperscript{ER} genes compromises ER protein folding and hypersensitizes cells and organisms to ER stress. Thus, the activation of UPR\textsuperscript{ER} in *mdt-15*
worms could arise from defective response to ER insults due to a requirement of \( mdt-15 \) in the UPR\(^{ER}\).

To test this hypothesis, we compared the genes that are induced by a chemical protein folding inhibitor in \( ire-1/xbp-1-, perk-, \) or \( aft-6-\) dependent fashion (inducible UPR\(^{ER}\) genes [57]) to genes that are downregulated or upregulated in \( mdt-15(RNAi) \) or the \( mdt-15(tm2182) \) mutant worms [32]. We found that the genes downregulated in \( mdt-15(RNAi) \) or \( mdt-15(tm2182) \) worms did not overlap at all with inducible UPR\(^{ER}\) genes (i.e. 0 shared genes). In contrast, genes upregulated by \( mdt-15 \) depletion significantly overlapped with inducible UPR\(^{ER}\) genes (Fig. 10A, not significant overlap in the \( mdt-15(tm2182) \) mutant); this supports the notion that loss of \( mdt-15 \) function causes UPR\(^{ER}\) activation.

To corroborate these data, we used RT-qPCR analysis to test whether \( mdt-15 \) is required to induce select UPR\(^{ER}\) genes in response to chemically induced ER stress. Specifically, we quantified the transcript levels of three UPR\(^{ER}\) genes in control\((RNAi) \) and \( mdt-15(RNAi) \) worms in the absence or presence of tunicamycin, a protein glycosylation inhibitor that induces the UPR\(^{ER}\) in many experimental systems, including \( C. \) elegans [59]. We found that the induction of these UPR genes by tunicamycin was not impaired in \( mdt-15(RNAi) \) worms; instead, they were constitutively upregulated upon \( mdt-15 \) depletion (Fig. 10B). Thus, \( mdt-15 \) is not required for the activation of the UPR\(^{ER}\) but its depletion provokes an ER homeostatic response.
As the folding of proteins requires an oxidizing environment, the ER lumen is enriched with the cellular reducing agent glutathione. *mdt-15* is required for transcriptional inductions and for survival in response to exogenous oxidative stress. Accordingly, *mdt-15* might also be necessary to maintain endogenous redox balance. qPCR analysis showed that several genes required for glutathione biosynthesis are downregulated by *mdt-15* RNAi (e.g. *metr-1*, *mthf-1*, *cth-1*, and *cth-2*; Fig. 4A [107]). Therefore, we decided to test whether the cellular glutathione level is

**Figure 10. *mdt-15* does not participate in the UPR\(_{ER}\).*

(A) The Venn diagram shows the overlap between genes upregulated in *mdt-15*(RNAi) worms and *ire-1/xbp-1*–dependent inducible UPR\(_{ER}\) genes. The p-value was calculated as described [31]. (B) Bar graphs represent the average fold change of mRNA levels in control(RNAi) and *mdt-15*(RNAi) worms treated with DMSO or tunicamycin; fold change is relative to DMSO-treated control(RNAi) worms. Error bars represent SEM (n=3), and * indicates p < 0.05 (two-tailed student t-test).

### 2.8 *mdt-15* is not required for total glutathione synthesis

As the folding of proteins requires an oxidizing environment, the ER lumen is enriched with the cellular reducing agent glutathione. *mdt-15* is required for transcriptional inductions and for survival in response to exogenous oxidative stress. Accordingly, *mdt-15* might also be necessary to maintain endogenous redox balance. qPCR analysis showed that several genes required for glutathione biosynthesis are downregulated by *mdt-15* RNAi (e.g. *metr-1*, *mthf-1*, *cth-1*, and *cth-2*; Fig. 4A [107]). Therefore, we decided to test whether the cellular glutathione level is
altered by \textit{mdt-15} depletion. As shown by Fig. 11, the total glutathione level was not affected by \textit{mdt-15} depletion. Thus, we presumed that endogenous antioxidant environment mediated by glutathione is not affected by \textit{mdt-15} genetic inactivation.

![Figure 11. The glutathione level is not altered upon \textit{mdt-15} depletion.](image)

(A) The bar graph indicates the total glutathione levels in \textit{control(RNAi)} and \textit{mdt-15(RNAi)} worms normalized to total protein levels. Error bars represent SEM, n=3.

\textbf{2.9 Genetic inactivation of \textit{mdt-15} and lipid metabolism genes induces the UPR\textsubscript{ER} without disturbed proteostasis}

Activation of the UPR\textsubscript{ER} is considered the consequence of unfolded protein accumulation in the ER lumen. However, increased membrane lipid saturation triggers the UPR\textsubscript{ER} in cells expressing IRE-1 and PERK mutants that are incapable of sensing misfolded proteins [104]. Is the activated UPR\textsubscript{ER} following genetic inactivation of \textit{mdt-15}, \textit{SCD} or \textit{sams-1} associated with impaired ER protein folding? To address this question, we used pharmacological, genetic and molecular methodologies in parallel.
2.9.1 Depletion of *mdt-15*, *SCD*, or *sams-1* does not hypersensitize animals to proteostatic stresses

If the UPR\textsuperscript{ER} activation in *mdt-15*, *SCD*, or *sams-1* worms were due to the accumulation of unfolded proteins, animals should be hypersensitive to exogenous proteostatic stress. To test this hypothesis, we first used tunicamycin. Consistent with published data [59], tunicamycin shortened the lifespan of adult wild-type worms, indicating that protein misfolding does compromise survival (Fig. 12A-B). Although adult *mdt-15(RNAi)*, *mdt-15(tm2182)*, *SCD(RNAi)*, and *sams-1(RNAi)* worms all display an induced UPR\textsuperscript{ER} (Fig. 6 and Fig. 7), they were not hypersensitive to tunicamycin compared to wild-type or *control(RNAi)* worms (Fig. 12A).

To corroborate these results, we used developmental rates as an alternate readout for tunicamycin sensitivity. Consistent with the survival assay, the dose-dependent developmental arrest (and lethality throughout development; Fig. 12D) caused by tunicamycin was similar in *control(RNAi)*, *mdt-15(RNAi)*, and *SCD(RNAi)* worms (Fig. 12C). In contrast, depletion of *sca-1*, the Ca\textsuperscript{2+} ATPase that is important for ER calcium levels and proteostasis, caused synthetic developmental arrest with tunicamycin when compared to *control(RNAi)* (Fig. 12B). This agrees with the notion that Ca\textsuperscript{2+} homeostatic disturbance induces the UPR\textsuperscript{ER} indirectly by impairing ER protein folding [100, 114].

Besides tunicamycin, we also studied the synthetic interactions of *mdt-15* and *SCD* with thapsigargin, which specifically inhibits SCA-1 and thus disrupts ER Ca\textsuperscript{2+} storage and homeostasis [115, 116]. The data resembled those obtained with tunicamycin: neither *mdt-15* nor *SCD* depletion leads to thapsigargin hypersensitivity as assessed with the developmental assay, whereas *sca-1* RNAi resulted in a strong synthetic interaction with thapsigargin (Fig. 12B) (due
to the small body size of *sams-1(RNAi)* worms, we were unable to use the developmental assays in this context).
Figure 12. Inactivation of \textit{mdt-15} and essential lipid metabolism genes does not compromise ER proteostasis.

(A) Plots represent the first 150 hours of survival of adult control\textit{(RNAi)}, \textit{ire-1(RNAi)}, \textit{mdt-15(RNAi)}, \textit{SCD (RNAi)}, \textit{sams-1(RNAi)}, WT, \textit{mdt-15(tm2182)}, and \textit{ire-1(zc14)} worms on tunicamycin. Micrographs show the morphological phenotypes of control\textit{(RNAi)}, \textit{mdt-15(RNAi)}, \textit{SCD(RNAi)}, and \textit{sams-1(RNAi)} worms and indicate RNAi efficiency. (B) Additional replicates of the tunicamycin survival experiments. (C) Bar graphs represent the portions of L1/L2, L3 or L4 control\textit{(RNAi)}, \textit{mdt-15(RNAi)}, \textit{SCD(RNAi)}, and \textit{sca-1(RNAi)} larvae that are alive after 48 hours on 0, 2, 5 \text{\mu}g/ml tunicamycin (top panel) or on 0, 3, 10 \text{\mu}g/ml thapsigargin (bottom panel). (D) Bar graphs represent the fractions of dead and alive larvae on control, \textit{mdt-15}, or \textit{SCD RNAi}. Error bars are SEM, \textit{n}=3.
2.9.2 Depletion of *mdt-15*, *SCD*, or *sams-1* does not cause synthetic interaction with a UPR\textsuperscript{ER} mutant

The above data suggested that *mdt-15*, *SCD*, and *sams-1* depletion activate the UPR\textsuperscript{ER} without compromising ER protein quality control. To corroborate the pharmacological studies, we next used a genetic approach. If *mdt-15*(RNAi), *SCD*(RNAi), and *sams-1*(RNAi) worms were defective for the UPR\textsuperscript{ER} and/or proteostasis, they should exhibit synthetic lethality or growth defects in combination with a mutation in a UPR\textsuperscript{ER} gene [57, 60, 117].

To test this hypothesis, we studied the genetic interactions of *mdt-15*, *SCD*, and *sams-1* with UPR\textsuperscript{ER} sensor *ire-1*, which is responsible for most of the inducible UPR\textsuperscript{ER} genes in *C. elegans* and whose loss-of-function leads to ER misfolded protein aggregation, as well as proteostatic stress hypersensitivity [59, 118]. Specifically, we quantified the progeny production in wild-type worms and *ire-1(zc14)* loss-of-function mutant [119] grown on control, *mdt-15*, *SCD* or *sams-1* RNAi. As a positive control, we depleted *enpl-1*, which encodes the ER-specific chaperone ENPL-1/GRP94 and therefore should result in synthetic lethality with *ire-1* [117]. As published, *mdt-15*, *SCD*, and *enpl-1* RNAi reduced progeny production in the wild-type background (Fig. 13). However, *mdt-15*, *SCD* or *sams-1* depletion did not result in *ire-1(zc14)* synthetic lethality, whereas *enpl-1* depletion led to almost no progeny production (Fig. 13). Altogether, these data support the hypothesis that loss of *mdt-15*, *SCD*, or *sams-1* triggers the UPR\textsuperscript{ER} without compromising ER proteostasis.
2.9.3 Alterations in membrane lipid compositions do not lead to misfolded protein aggregation

Is the UPR\textsuperscript{ER} induction in \textit{mdt-15}(RNAi), \textit{SCD}(RNAi), or \textit{sams-1}(RNAi) worms accompanied by misfolded proteins? To directly address this question, we used a transgenic strain that carries the \textit{vha-6p::SRP-2\textsuperscript{H302R}}::\textit{GFP} reporter. SRP-2 is a \textit{C. elegans} homolog of mammalian neuroserpin,
and the H302R mutation causes SRP-2 aggregation in the ER lumen. Mutations in UPR\textsuperscript{ER} genes or in the gene encoding heat-shock factor (HSF-1) strongly enhance the aggregation of this misfolded protein reporter [118].

Consistent with published data [118], we found that defects in the canonical UPR\textsuperscript{ER} component IRE-1 and ATF-6 substantially increase the aggregation of SRP-2\textsuperscript{H302R} (Fig. 14A). Similarly, depleting the ER-specific chaperone ENPL-1/GRP94 or the ER Ca\textsuperscript{2+} ATPase SCA-1, known regulators of ER proteostasis, also significantly promoted SRP-2\textsuperscript{H302R} aggregation (and induced the UPR\textsuperscript{ER}, as reported [117, 118]; Fig. 14B). In contrast, mdt-15, SCD, or sams-1 depletion did not increase SRP-2\textsuperscript{H302R}::GFP aggregation (Fig. 14A-B). Similar results were also obtained by the Hoppe lab using the same reporter on the respective RNAi (Hou \textit{et al}, 2014 submitted).

Activation of the \textit{ire-1}/\textit{xbp-1} dependent UPR\textsuperscript{ER} induces the ER-associated degradation (ERAD) pathway, which removes misfolded proteins to lessen ER’s proteotoxic burden [57]. In principle, the absence of enhanced misfolded protein aggregation may be due to the upregulation of the ERAD pathway. To test this possibility, we used RT-qPCR to quantify several ERAD genes. None of these ERAD genes were significantly induced upon \textit{mdt-15} depletion (Fig. 14C). In line with this, our collaborators showed that the protein expression of CPL-1\textsuperscript{W32A,Y35A}::YFP, a mutant of \textit{C. elegans} pro-cathepsin L, which is a substrate for the ERAD machinery [120], did not differ from WT following \textit{mdt-15} or \textit{SCD} depletion (Hou \textit{et al}, 2014 submitted).

Together, these data indicate that the inactivation of genes that directly (\textit{ire-1}, \textit{atf-6}, \textit{enpl-1}) or indirectly (\textit{sca-1}) facilitate protein folding triggers the UPR\textsuperscript{ER} and leads to enhanced misfolded
protein aggregation; in contrast, *mdt-15, SCD, or sams-1* depletion induces the UPR\textsuperscript{ER} without introducing misfolded proteins.
Defects in PC production but not in membrane lipid unsaturation affect mitochondrial homeostasis and activate the UPR\textsuperscript{mito}

Besides the ER, mitochondria also synthesize and modify proteins. Is depletion of \textit{mdt-15}, \textit{SCD} or \textit{sams-1} specifically required for ER homeostasis or does it also affect mitochondria? To address this question, we used mitochondria-specific reporters and analyzed the composition and levels of a lipid specific to the mitochondria.

**Figure 14. Defects in lipid unsaturation or PC synthesis do not enhance misfolded protein aggregation.**

(A) Micrographs indicate the \textit{vha-6::SRP-2\textsuperscript{H302R}::GFP} reporter in worms on control, \textit{ire-1}, \textit{atf-6}, \textit{mdt-15}, \textit{SCD} or \textit{sams-1} RNAi. Green: GFP, Glue: Texas Red captures autofluorescence. Images were pooled from three independent experiments. White arrows indicate the typical GFP signal counted as aggregates in this assay. (B) Dot plot represents quantification of the SRP-2 misfolded-protein reporter in \textit{control(RNAi)}, \textit{ire-1(RNAi)}, \textit{enpl-1(RNAi)}, \textit{mdt-15(RNAi)} and \textit{sca-1(RNAi)} day-1 adult worms. Data points were pooled from three independent experiments. Error bars represent SEM, and * indicates \(p<0.05\) (two-tailed student t-test). (C) Bars represent the average fold change of mRNA levels of select ERAD genes in \textit{control(RNAi)} and \textit{mdt-15(RNAi)} worms (\(n=3\)). Error bars represent SEM.
2.10.1 *sams-1* but not *mdt-15* or *SCD*, is required to prevent UPR\textsuperscript{mito} activation

To determine whether mitochondrial homeostasis is affected in worms with lipid disequilibrium, we examined the mitochondrial UPR (UPR\textsuperscript{mito}). The UPR\textsuperscript{mito} monitors proteostasis in the mitochondrial matrix and its activation and signalling mechanisms are molecularly distinct from the UPR\textsuperscript{ER}. Thus, we studied the expression of the mitochondrial chaperones *hsp-6* and *hsp-60*, specific indicators of the UPR\textsuperscript{mito}\textsuperscript{[121]}.

As shown (Fig. 15A), *mdt-15* and *SCD* RNAi failed to induce the *hsp-6p::gfp* and *hsp-60p::gfp* reporters. To rule out the possibility that the UPR\textsuperscript{mito} is impaired in animals depleted of *mdt-15* or *SCD*, we exposed these worms to the electron transport chain inhibitor antimycin A, which activates the UPR\textsuperscript{mito}\textsuperscript[121]. Neither *mdt-15* nor *SCD* depletion affected the induction of the *hsp-6p::gfp* by antimycin (Fig. 15B). Therefore, *mdt-15* and *SCD* do not influence mitochondrial homeostasis or the UPR\textsuperscript{mito}.

Surprisingly, *sams-1* RNAi caused a substantial induction of *hsp-6::gfp*, and this induction was completely suppressed by dietary supplementation with the SAMS-1 downstream product choline (Fig. 4D and 15C). This suggests that, in addition to the ER, reduced PC levels also disturb homeostasis in the mitochondria.
Figure 15. Defects in lipid unsaturation do not disrupt mitochondrial homeostasis whereas reduction in PC production does.

(A) Micrographs indicate the expression of the hsp-6p::gfp and hsp-60p::gfp reporters in control(RNAi), mdt-15(RNAi), WT, mdt-15(tm2182), and SCD(RNAi) worms. (B) Micrographs illustrate the expression of the hsp-6p::gfp reporter in control(RNAi), mdt-15(RNAi), and SCD(RNAi) worms with or without antimycin A treatment. (C) Micrographs indicate the expression of the hsp-6p::gfp reporter in control(RNAi) and sams-1(RNAi) worms with or without dietary ethanolamine or choline supplements. In all panels, one of three independent experiments is shown.
2.10.2 Depletion of *mdt-15* or *SCD* does not affect the fatty acid composition or the relative level of cardiolipin

To corroborate the above data, we next studied the lipid profile of the mitochondria-specific membrane lipid cardiolipin [97]. As indicated in Fig. 16A, depletion of *mdt-15* or *SCD* did not alter the fatty acid composition of cardiolipin. This is in contrast to the compositions of the two dominant membrane phospholipids PC and PE (Fig. 5E-F and 6C). Moreover, the overall relative levels of cardiolipin in *mdt-15*(RNAi) and *SCD*(RNAi) worms did not differ from that of the control(RNAi) sample (Fig. 16B). Thus, unlike the ER, the membrane lipids of the mitochondria remain relatively unaffected following *mdt-15* or *SCD* depletion. This agrees with the observation that depletion of *mdt-15* or *SCD* did not induce the UPRmito.
Figure 16. The FA composition and the relative level of cardiolipin are not altered by *mdt-15* or *SCD* depletion.

(A) Bar graphs represent the relative abundance of individual fatty acids in cardiolipin.

(B) Bar graphs represent the average cardiolipin level relative to TLs or total phospholipids in *control(RNAi)*, *SCD(RNAi)*, and *mdt-15(RNAi)* worms. n=3, error bars represent SEM.
Chapter 3: Discussion and Conclusion

3.1 Membrane lipid disequilibrium activates the UPR\textsuperscript{ER} without disturbed proteostasis
Recent findings have revealed regulatory relationships between lipid biology, ER homeostasis, and disease pathogenesis. Through the study of a lipid regulator, the Mediator subunit MDT-15, we now show that membrane lipid composition can directly influence the circuit that monitors ER homeostasis. Specifically, we show that defects in phospholipid biosynthesis, either due to altered fatty acyl unsaturation or phospholipid headgroup synthesis, constitutively activate the UPR\textsuperscript{ER} without compromising proteostasis. This expands on the role that lipid disequilibrium plays in UPR\textsuperscript{ER} activation and shows for the first time that UPR\textsuperscript{ER} activation is not strictly linked to a disturbed ER proteostasis \textit{in vivo}.

3.2 \textit{mdt-15}-mediated processes are essential to maintain ER homeostasis
Loss of \textit{mdt-15} function constitutively activates the UPR\textsuperscript{ER} but without disturbing proteostasis. One of these critical ER homeostatic pathways is the \textit{SCD}-dependent lipid unsaturation. \textit{mdt-15} regulates overall fatty acid unsaturation [29, 30], but how \textit{mdt-15} affects the fatty acid compositions in different lipids and whether it influences the overall lipid levels had not yet been examined. In this study, we dissected \textit{mdt-15}’s roles in lipid metabolism in greater detail and found that \textit{mdt-15} inactivation substantially reduces fatty acid polyunsaturation in the major membrane lipid PC; other lipid species, such as the other dominant membrane lipid PE, are much less severely affected. This contrast in the two major phospholipids is especially evident in the highly unsaturated fatty acids such as C20:5, which consists of a greater fraction of the fatty acyl contents in PC than that in PE. (see Fig. 5E-F wild-type). This relatively greater abundance of
C20:5 in PC may be the reason that membranes with high fluidity, such as the ER, are enriched in this particular phospholipid.

The importance of mdt-15- and SCD-PC polyunsaturation in membrane integrity is greatly reflected by a constitutively activated UPR\textsuperscript{ER} in mdt-15 or SCD deficient worms. Multiple markers commonly used to monitor UPR\textsuperscript{ER} activation were upregulated in mdt-15(RNAi) worms, mdt-15(tm2182) mutants and the SCD(RNAi) worms. This constitutively activated UPR\textsuperscript{ER} is partially the consequence of reduced unsaturated fatty acid synthesis, particularly polyunsaturated fatty acids, in the mdt-15 worms. This is because dietary supplementation with unsaturated fatty acids suppressed the defects in membrane lipid unsaturation (Fig. 8), yet failed to fully suppress both the UPR\textsuperscript{ER} induction and the physiological phenotypes of mdt-15 worms. Importantly, this partial rescue of mdt-15-induced UPR\textsuperscript{ER} is not due to ineffective fatty acid uptake or incorporation, as shown by the GC-MS profiles of C18:1n-9, C18:2, and C20:5 fatty acids.

Interestingly, in SCD(RNAi) worms, supplementation with the downstream polyunsaturated fatty acids C18:2 and C20:5 rather than the SCD product C18:1n-9 was sufficient to fully suppress the induced UPR\textsuperscript{ER} (Fig. 7A). This implicates that the C18:2 and C20:5 are likely most critical for ER homeostasis. Furthermore, comparisons between unsupplemented and supplemented mdt-15(RNAi) and SCD(RNAi) worms provided possible explanations for the incomplete rescue of the UPR\textsuperscript{ER} activation upon mdt-15 genetic inactivation, as discussed in the following sections.
3.2.1 The reduction in relative PC level does not contribute to mdt-15 induced UPR^{ER}

The relative PC level (compared to total phospholipids) was slightly but significantly reduced upon mdt-15 depletion (Fig. 8B). SCD(RNAi) worms showed a similar decrease in PC as well as a concomitant increase in PE levels, reflecting a potentially more substantial change in the PC/PE ratio (Fig. 8D). As a reduced PC/PE ratio can activate the UPR^{ER}, this could in principle explain the UPR^{ER} induction in worms depleted of mdt-15 or SCD. However, unsaturated fatty acid supplements fully suppressed these alterations in relative phospholipid levels, but failed to fully suppress the activated UPR^{ER} in mdt-15(RNAi) worms; moreover, supplementation with the PC precursor choline did not relieve the induced UPR^{ER} in mdt-15(RNAi) worms (Fig. 9). Together, these data show that the downregulation of polyunsaturated fatty acids indirectly affect PC and PE levels, but the reduced PC level does not contribute to the activated UPR^{ER} in mdt-15 worms.

3.2.2 Downregulation of C20:3 and C20:4 does not contribute to mdt-15 induced UPR^{ER}

The PC in mdt-15(RNAi) and SCD(RNAi) worms also showed a downregulation of C20:3 and C20:4 contents, suggesting that these downregulations could also contribute to the UPR^{ER} activation in these worms. However, these polyunsaturated fatty acids remained downregulated following dietary supplementation of mdt-15(RNAi) and SCD(RNAi) worms (Fig. 8B-C), yet the UPR^{ER} is completely restored in the supplemented SCD(RNAi) worms. Moreover, supplemented control(RNAi) worms also showed reduced C20:3 and C20:4 polyunsaturated fatty acids in PC (Fig. 8B). Thus, the reduced C20:3 and C20:4 levels in PC are unlikely to cause the residual UPR^{ER} in the mdt-15(RNAi) worms.
3.2.3 Substrate build-up of C18:0 does not contribute to mdt-15 induced UPR$^{ER}$

In addition to reduced levels of unsaturated fatty acids, the PC in mdt-15(RNAi) and SCD(RNAi) worms also shows an increase in saturated fatty acyl contents. Dietary fatty acid supplementation did not eliminate the upregulation of saturated fatty acyl contents (Fig. 8C). This is expected, as dietary rescue with downstream products should not suppress the upstream substrate build-up that occurs as a consequence of enzyme depletion. Nonetheless, in supplemented SCD(RNAi) worms, the UPR$^{ER}$ is reduced to WT level, suggesting that increased C18:0 alone is not sufficient to induce the UPR$^{ER}$ (note that the C18:0 content in PC is similar in supplemented mdt-15(RNAi) and SCD(RNAi) worms; Fig. 8B-C).

3.2.4 Additional mdt-15-mediated pathways for ER homeostasis may be both lipid-dependent and -independent

mdt-15(RNAi) worms, but not SCD(RNAi) worms, also display increased C16:0 in PC, likely due to mdt-15’s requirement for fat-5 expression (fat-5 catalyzes the conversion of C16:0 to C16:1 [122]). Exogenous C16:0 induces the UPR$^{ER}$ in various mammalian cell lines and in yeast, and thus might explain the residual UPR$^{ER}$ in mdt-15 worms. However, loss-of-function mutations in fat-5 do not result in severe build-up of C16:0 under normal conditions [122], perhaps due to compensatory mechanisms. In line with this lack of C16:0 accumulation, our preliminary data showed a lack of hsp-4p::gfp induction by fat-5 RNAi (data not shown). Such a negative results may relate to RNAi inefficiency; therefore, further experiments are required to better test the relationship between fat-5, C16:0 contents in PC, and UPR$^{ER}$ activation in C. elegans.
Alternatively, it is possible that not one individual fatty acid species, but rather the combination of fatty acid changes in supplemented \textit{mdt-15(RNAi)} worms underlies the residual UPR\textsubscript{ER} induction. In any case, as this remaining UPR\textsubscript{ER} is substantial, there likely exist other \textit{mdt-15-}mediated ER homeostatic pathways, perhaps including lipid-dependent and/or –independent processes.

\subsection{3.2.5 Additional \textit{mdt-15}–regulated pathways for ER homeostasis can be identified using unbiased genetic screen}

One method to identify \textit{mdt-15}–regulated pathways that contribute to ER homeostasis would be to perform an unbiased genetic screen. For this purpose, we designed a forward suppressor screen using the \textit{hsp-4p::gfp} as a readout and the \textit{mdt-15(tm2182)} as a sensitized background. As the \textit{mdt-15} mutation constitutively activates the UPR\textsubscript{ER} without disturbed proteostasis, mutations that suppress this UPR\textsubscript{ER} could reveal the cause(s) of the UPR\textsubscript{ER} activation in these worms.

This screen will be carried out as illustrated in Fig. 17. Briefly, populations of nematodes can be sorted in the worm sorter (COPAS BIOSORT; similar to a FACS) based on their size and fluorescence. Analyzing wild-type \textit{hsp-4p::gfp} worms and mutant \textit{mdt-15(tm2182); hsp-4p::gfp} worm populations thus generates a distribution plot that captures the \textit{hsp-4} reporter GFP signal on the y-axis) and worm size on the x-axis (Fig. 17). As expected, wild-type \textit{hsp-4p::gfp} worms and \textit{mdt-15(tm2182); hsp-4p::gfp} mutants are distinguishable when analyzing the population distributions (see the “merged” window). To screen for suppressors whose \textit{hsp-4p::gfp} is restored to WT level in the \textit{mdt-15(tm2182)} background, we would select those individuals that
are below the \textit{mdt-15 hsp-4p::gfp} population and fall within the WT population (see “selected from Merged”).

Following initial candidate selection, irrelevant mutations would have to be counterscreened against. As the suppression of the \textit{hsp-4} reporter may be due to a defective \textit{ire-1/xbp-1 UPR}^{ER} (Fig. 6C and Fig. 13) or a defective transgenic reporter, we would thus perform a secondary screen to assess reporter functionality and inducibility. Specifically, candidates from the primary screen will be tested for their ability to induce the \textit{hsp-4p::gfp} reporter in response to tunicamycin; candidates unable to induce \textit{hsp-4p::gfp} would be discarded.

In short, this suppressor screen could efficiently identify additional \textit{mdt-15}-mediated ER homeostatic processes that are either lipid-dependent or independent. Identification and characterization of these factors/processes may unveil novel clients of the UPR^{ER}.
3.3 *mdt-15* induced UPR\textsuperscript{ER} occurs due to metabolic defects, not transcriptional defects

To date, MDT-15 and its orthologues have been exclusively described as co-activators [28-30, 32, 38-40, 123], but it is formally possible that MDT-15 might serve as a co-repressor for some UPR\textsuperscript{ER} genes such as *hsp-4*, which would explain why they are induced in *mdt-15* worms. However, this seems unlikely for two reasons. Firstly, unsaturated fatty acid supplementation partially suppresses *hsp-4* induction in *mdt-15* worms, and fully suppresses it in *SCD*(RNAi)

**Figure 17.** Outline for an unbiased genetic screen to identify additional *mdt-15*–mediated processes that are required for ER homeostasis.

The plots are screenshots of population distributions, as acquired with the COPAS worm bio-sorter. Red dots represent individual worms whose GFP signal is detected by the bio-sorter, and green dots represent the overlapping of any two red dots. The first window shows the GFP signal distribution of the *hsp-4p::gfp* reporter in WT worms. The second window represents the GFP signal distribution of *mdt-15(tm2182); hsp-4p::gfp* worms. The “merged” window is the overlap of these two plots. The last graph shows individuals from both the WT and the mutant population that fall within the selecting window.
worms, demonstrating that the UPR\textsuperscript{ER} activation in these worms is the result of metabolic defects.

Secondly, \textit{ire-1} or \textit{xbp-1} mutation prevents \textit{hsp-4} induction in \textit{mdt-15} worms (Fig. 6C and Fig. 13), showing that \textit{mdt-15} acts upstream of the canonical \textit{ire-1}/\textit{xbp-1} signalling.

Similar arguments hold for SAMS-1, which, by its virtue of synthesizing the universal methyl-group donor SAM, could in principle affect gene expression through processes such as histone methylation; because the UPR\textsuperscript{ER} activation due to \textit{sams-1} depletion is fully rescued by dietary choline supplementation, it must originate from changes in PC synthesis, not chromatin methylation defects due to loss of \textit{sams-1} driven methyl-group production. Thus, our data suggest that the UPR\textsuperscript{ER} (as well as UPR\textsuperscript{mito}) is induced entirely because of compromised PC synthesis, as all the phenotypes investigated in this study.

3.4 UPR\textsuperscript{ER} activation and ER proteostasis

Numerous studies have shown that the UPR\textsuperscript{ER} can be triggered by ER membrane disequilibrium, such as increased membrane phospholipid saturation, inositol depletion, altered PC/PE ratio, and ER membrane cholesterol accumulation [42, 96, 110, 112, 114]. In these studies, the changes in lipid composition apparently activate the UPR\textsuperscript{ER} indirectly through protein misfolding. For example, improving proteostasis with pharmacological chaperones alleviates UPR\textsuperscript{ER} activation in the obese mice with altered liver PC/PE ratios, as well as in yeast with severely reduced oleic acid levels [114, 124].

Likewise, and in contrast to the genetic interactions we identified in \textit{mdt-15}(RNAi), \textit{SCD}(RNAi), and \textit{sams-1}(RNAi) worms, yeast mutants with extremely low PC levels exhibit synthetic lethality
in combination with *ire-1* mutations [124]. Our investigation thus changes the current view on how lipid disequilibrium induces UPR\textsuperscript{ER}, as we observe an activated UPR\textsuperscript{ER} without evidence of misfolded proteins in *mdt-15* worms or worms defective for membrane phospholipid biosynthesis. This clearly contrasted worms depleted of factors that are directly (e.g. ER chaperone ENPL-1) or indirectly (e.g. ER Ca\textsuperscript{2+} homeostasis, SCA-1) involved in ER protein quality control.

### 3.4.1 Proteostatic disturbance may be dependent upon the severity of membrane lipid aberrancy

Why do the genetic alterations observed here not result in protein misfolding, yet activate the UPR\textsuperscript{ER}? After all, the activation of the UPR\textsuperscript{ER} in leptin deficient *Lep\textsuperscript{ob}* mice or in yeast defective for PC synthesis can be rescued by chemical chaperones [78, 125], implicating misfolded proteins as the stimulus of the UPR\textsuperscript{ER}. Perhaps the lipid disturbances in the worm models used in our study are less severe than those in grossly obese *Lep\textsuperscript{ob}* mice or in yeast strains completely or substantially devoid of PC: even the *sams-1* worms, the model with the most substantially altered membranes studied here, undergo a relatively mild change in PC/PE ratio compared to the changes observed using the *cho2* or *opi3* mutants in yeast [107, 124, 126]. This may reflect a threshold effect, whereby comparably mild alterations of lipid profiles activate the UPR\textsuperscript{ER} but do not cause accumulation of misfolded proteins, whereas drastic changes in lipid distribution or saturation not only induce the UPR\textsuperscript{ER} but also impair proteostasis. Further experiments will be required to address this hypothesis.
3.4.2 Protein degradation may not be activated in our mutant contexts

In principle, our observations that mdt-15, SCD, or sam-s-1 depletion does not result in misfolded protein aggregations could reflect a constitutively active UPR\textsuperscript{ER} and/or ERAD, which might counteract protein the accumulation of misfolded proteins. We consider this unlikely as our gene expression profiling showed that, although the overlap between the inducible UPR\textsuperscript{ER} genes and those activated by mdt-15 depletion is significant, a large fraction of classical UPR\textsuperscript{ER} genes are not induced in mdt-15(RNAi) worms (Fig. 10A). As such, these worms do not undergo a full UPR\textsuperscript{ER} akin to that in response to tunicamycin.

In addition to the microarrays, quantification of selected ERAD genes suggests that this pathway is not transcriptionally activated in mdt-15(RNAi) worms. Moreover, misfolded protein aggregates were not absent in mdt-15(RNAi), SCD(RNAi), or sam-s-1(RNAi) worms, when compared to negative control (Hou et al, 2014 submitted). Therefore, the UPR\textsuperscript{ER} likely employs different downstream responses dependent on the molecular nature of the insults and a different upstream sensing mechanism. It will be important to identify and dissect the regulatory events that distinguish misfolded proteins from membrane lipid sensing to comprehensively understand the complexity of the UPR\textsuperscript{ER}.

3.4.3 Activation of the UPR\textsuperscript{ER} in mdt-15 worms is not due to general sickness

Lastly, the elevated UPR\textsuperscript{ER} in mdt-15 worms might be a part of a general stress response akin to the activation of cytoprotective pathways upon depletion of essential genes in C. elegans [127]. However, this does not appear to be the case. For one, mdt-15 inactivation does not upregulate the UPR\textsuperscript{mito}, which is induced by the inactivation of many essential genes [127]. Moreover, hsp-4
induction is not a general response to the inactivation of any essential gene; rather, it indicates the loss of an activity critical for ER function, such as protein trafficking, phospholipid synthesis, or Ca\(^{2+}\) homeostasis. Thus, induction of the UPR\(^{ER}\) implies disturbed ER function; therefore, the induction of the UPR\(^{ER}\) in \textit{mdt-15} worms most likely reflects ER disequilibrium, but not general sickness.

3.5 Activation of the UPR\(^{ER}\) does not correlate to stress resistance or longevity

It seems curious that the \textit{mdt-15} or \textit{SCD} deficient worms have highly activated UPR\(^{ER}\) but yet they are not resistant to proteostastic stresses. On the other hand, \textit{sams-1} inactivation constitutively activates the UPR\(^{ER}\) (Fig. 13 and [127]), and leads to tunicamycin resistance in the adult survival assay (Fig. 12A). On the contrary, the long-lived insulin-deficient mutant \textit{daf-2} worms is ER stress resistant, yet the UPR\(^{ER}\) is apparently set at a lower threshold. This means that in the \textit{daf-2} mutant, higher dosage or larger magnitude of proteostatic stresses is required to induce the UPR\(^{ER}\) as strongly as the WT. Although \textit{sams-1} deficient worms have an activated UPR\(^{ER}\), as opposite to that in the insulin-deficient mutants, \textit{sams-1} worms are also long lived [128]. Apparently, the UPR\(^{ER}\) can be set at high or at low levels, with no one strict requirement for longevity or stress resistance.

3.6 Defects in PC production trigger the UPR\(^{mito}\)

Different from mdt-15 or SCD deficiency, \textit{sams-1} depletions also strongly induces the UPR\(^{mito}\). Therefore, defective unsaturated fatty acid synthesis only triggered the UPR\(^{ER}\), but not the, indicating a specific role for PC polyunsaturation in ER homeostasis. Apparently, the ER is more
sensitive to the unsaturation of membrane lipids than the mitochondria, likely due to its greater demand for membrane fluidity and its functions in lipid and protein biosynthesis. In contrast, disruption of PC synthesis compromised the homeostasis of both the ER and the mitochondria. This perhaps reflects a greater and a more general role of PC in organelle membrane integrity.

3.7 The fatty acid composition of cardiolipin is distinctive from that of PC and PE

The lack of UPR\textsuperscript{mito} activation in \textit{mdt-15}(RNAi) worms prompted us to characterize the fatty acid composition of cardiolipin, a lipid unique to the inner mitochondrial membrane [97]. Although others have previously quantified overall cardiolipin levels in worms [129], we provide to our knowledge the first analysis of cardiolipin fatty acid profiles in \textit{C. elegans}. We found that the overall levels and the fatty acid composition of cardiolipin were not significantly different between control(RNAi) and \textit{mdt-15}(RNAi) or \textit{SCD}(RNAi) worms, which aligns with the lack of UPR\textsuperscript{mito} activation in these worms.

Interestingly, we found that the fatty acid composition of \textit{C. elegans} cardiolipin differs from that in mammals: greater than 65% of its acyl chains consist of C20:3\textit{n}-6 and C20:4\textit{n}-6, whereas most eukaryotic cardiolipins are composed almost exclusively of 18-carbon fatty acids, primarily C18:2 [130]. Moreover, our analysis also showed that the fatty acid composition of cardiolipin is quite distinct from that of other phospholipid species. For instance, compared to PC and PE, cardiolipin is deficient in the polyunsaturated fatty acids C20:4\textit{n}-6 and C20:5 (Fig. 16A to 5E-F [130]). This suggests that the different fatty acid compositions of the mitochondrial and ER membranes underlie the distinct cellular functions of these organelles.
3.8 PC production, UPR\textsuperscript{mito}, and longevity

In \textit{C. elegans}, activating the UPR\textsuperscript{mito} can promote longevity in a cell non-autonomous fashion [131]. Is this why \textit{sams-1} worms are long-lived? As the UPR\textsuperscript{mito} activation in \textit{sams-1}(RNAi) worms can be fully suppressed by choline supplementation, it is possible that choline rescue would reverse the longevity of \textit{sams-1} worms. Moreover, these data suggest that PC biosynthesis may influence lifespan, potentially through modulating the UPR\textsuperscript{mito}. Therefore, it would be very interesting to test whether suppressing the UPR\textsuperscript{mito} induction alone suppresses the extended lifespan resulted from \textit{sams-1} inactivation.

Recently, Walker \textit{et al.} showed that defective PC production leads to activation and upregulation of the SREBP-mediated lipogenesis [107]. As such, \textit{sams-1} worms overexpress \textit{SCD} and exhibit increased triglyceride storage. This implicates \textit{mdt-15} in the extended longevity upon PC reduction, as MDT-15 co-activates \textit{SCD} with SBP-1 [29, 30]. Lastly, lipogenesis has been linked to longevity, though the correlation remains controversial [132]. Perhaps in the context of \textit{sams-1} loss-of-function or PC reduction in general, one could explore the role of \textit{mdt-15} in longevity, and to further dissect the relationship between lipid metabolism, stress responses, and longevity in general.
3.9 Conclusions

In summary, we concluded the following from the current study (also see Fig. 18):

- *mdt-15* and *SCD* are required for normal lipid unsaturation, especially in the dominant membrane phospholipid PC.

- *mdt-15* maintains ER homeostasis by influencing PC unsaturation whereas it does not strongly affect the relative level of membrane lipids.

- There are additional, unidentified *mdt-15*-mediated pathways that contribute to ER homeostasis.

- Membrane lipid disequilibrium, specifically decreased PC desaturation or production, activates the UPR\textsuperscript{ER} without disturbed proteostasis in *C. elegans*.

- Membrane lipid unsaturation predominantly impacts the ER than mitochondria whereas normal PC synthesis is essential for both organelles.

- FA composition of the mitochondrial membrane-specific cardiolipin is distinctive from those of the dominant phospholipids PC and PE.

Our data imply that the UPR\textsuperscript{ER} employs distinctive mechanisms to sense membrane lipid disequilibrium and proteostatic disturbances. Signalling outcomes downstream of stimuli-specific UPR\textsuperscript{ER} activation differ from each other. How does the UPR\textsuperscript{ER} undertake these tasks? Perhaps through revealing new *mdt-15*-mediated processes, future studies will shed light on the stimuli-specific downstream regulators of this remarkable ER homeostatic response.
Figure 18. Working model proposed by this study.

mdt-15 is specifically required for the polyunsaturation of membrane lipids, particularly the dominant phospholipid PC; in contrast, mdt-15 does not strongly affect relative lipid levels. Membrane lipid unsaturation (mediated by mdt-15 and SCD), PC biosynthesis (mediated by sams-1) and additional unidentified mdt-15-regulated processes are essential for maintaining ER homeostasis. Defects in any of these pathways constitutively activate the UPRER, but without disturbed proteostasis. Thus, dependent upon the initial stimuli, the UPRER employs distinct upstream sensing mechanisms to activate specific downstream regulatory events, leading to different signaling outcomes.
Chapter 4: Materials and Methods

4.1 Worm strains

We used the following worm strains: N2 wild-type [133], NL2099 rrf-3(pk1426) II [134], SJ 30 ire-1(zc14) II; zcIs4 [hsp-4p::GFP] V [119], SJ17 xbp-1(zc12) III; zcIs4 [hsp-4p::GFP] V [56], mdt-15(tm2182) III [31], mdt-15(tm2182) III; zcIs4 [hsp-4p::gfp] V (this study), mdt-15(tm2182) III; zcIs13 [hsp-4p::gfp] V (this study), SJ4005 zcIs4[hsp-4::GFP] V [119], SJ4100 zcIs13 [hsp-6p::gfp] V [135], SJ4058 zcIs9 [hsp-60::GFP] V [135]. To construct the vha-6p::SRP-2^{H302R}::GFP strain, the 1.2-kb vha-6 promoter, full-length srp-2, and gfp were amplified by PCR with the following primers: TH1811 vha6_P-for+Xba1 (aatctagagcatgtacctttatagg), TH1812 vha6_P-rev+Xma1 (aacccgggtaggttttagtcgccctg), TH1861 srp-2_for +Kpn1 (aaggtaccatgtcgtgagctgccctg), and TH1862 srp-2_rev + Kpn1 (aaggtaccagcatgtaaccccaaggaac). The resulting PCR products were cloned into the expression vector pBS already containing the unc-54 3’UTR. vha-6p::SRP-2^{H302R}::GFP was then synthesized by site directed mutagenesis using the Quikchange Lightning kit (Agilent technologies #210515) according to the manufacturer’s instructions and with the primers TH 1920 SRP-2_H302R (catctcgtcagggatccgcaaagcaatcattgaagt) and TH1921 SRP-2_H302R_antisense (acttcaatgattgctttgcggatccctgacgagatg). Transgenic worms were generated by microparticle bombardment of unc-119(ed4) mutants with 10 µg of linearized DNA constructs using a Biolistic PDS-1000/HE (Bio-Rad) gene gun, as described [136].
4.2 Worm growth conditions

We cultured *C. elegans* strains using standard techniques [133] and *E. coli* OP50 as food source, except for RNAi. As *mdt-15(tm2182)* and *ire-1(zc14)* mutants display developmental delay, they were allowed to grow an extra four and one hours, respectively, to achieve developmental synchronicity with WT worms. For GC-MS, RT-qPCR and immunoblot experiments, all worms were collected after 40-43 hours of incubation at 20°C since the L1 larvae were placed on food. For the SRP-2 aggregatation experiment, worms were allowed to grow for 72 hours to reach day-1 adulthood since L1 larvae. For developmental assays, L1 larvae were fed on RNAi bacteria and larval stages were scored after 48 hours development at 20°C. For the *ire-1(zc14)* synthetic interaction experiment, 8 L4 larvae were placed on corresponding RNAi plates and allowed to develop and lay progeny at 25°C for 2-3 days, then their live progeny were counted under light microscope.

4.3 Feeding RNA interference

Feeding RNA interference (RNAi) was performed using nematode growth medium (NGM)-RNAi plates containing 25 μg/mL carbenicillin, 1 mM IP triglyceride, and 12.5 μg/mL tetracycline, and seeded with the appropriate HT115 RNAi bacteria. The *mdt-15, fat-6 (=SCD), sams-1, ire-1, enpl-1, sca-1, and atf-6* RNAi plasmids were from the Ahringer library [137] and were sequenced prior to use. We refer to *fat-6* as *SCD* because *fat-6* RNAi depletes both *C. elegans* SCDs (*fat-6* and *fat-7*) due to sequence similarity [122]. For all RNAi experiments, we acquired DIC micrographs to document phenotypes. Tunicamycin (654380, EMD Millipore), thapsigargin (BML-PE180-00055, Enzo Life Sciences), antimycin A (A8674, Sigma), and fatty acid sodium salts (C18:1n-9, S-1120; C18:2, S-1127; C20:5; S-1144; Nu-Chek Prep) were added.
into the cooled RNAi media prior pouring, with a final concentrations of: C18:1n-9: 300 μM, C18:2: 150 μM and C20:5: 150 μM.

4.4 Thin layer chromatography and gas chromatography-mass spectrometry

For thin layer chromatography (TLC) and gas chromatography-mass spectrometry (GC-MS) analysis, mid-L4 larvae were harvested in distilled water, settled by gravity at room temperature and washed with water three to four times until bacteria were no longer apparent. Then, worm pellets were snap-frozen in liquid N$_2$ and stored at -80°C. Lipids were extracted, separated and quantified using TLC and GC-MS as described [138], with the addition of cardiolipin standards.

4.5 RNA isolation and real-time quantitative PCR

Developmentally synchronized L4 larvae were collected as described for GC-MC analysis; RNA isolation, purification, and real-time quantitative PCR (qPCR) was performed as described [30-32]. Primer sequences are listed in Table 4. For tunicamycin induction assays, synchronized early L4 larvae were transferred onto NGM plates containing 5 or 10 μg/ml tunicamycin and incubated for five hours.

4.6 DIC, fluorescence microscopy and confocal microscopy

For DIC and fluorescence microscopic experiments, worms were picked and mounted on 2% agarose pads containing NaN$_3$ as described [31]. We captured images on a CoolSnap HQ camera (Photometrics) attached to a Zeiss Axioplan 2 compound microscope, and used MetaMorph Imaging Software with Autoquant 3D digital deconvolution for image acquisition.
To monitor SRP-2$^{H302R}$::GFP aggregates, synchronized vha-6p::SRP-2$^{H302R}$::GFP expressing L1 larvae were fed for 72 hours with the indicated RNAi bacteria. After immobilizing the worms on agarose pads with NaN$_3$, SRP-2$^{H302R}$ aggregates of a defined region in the intestine were counted immediately using an Axioimager (Zeiss) with a 100x magnification. We also acquired images with the Texas Red filter set to identify and eliminate autofluorescence of the gut granules. Each image illustrates the first two pairs of the anterior intestinal cells of a day-one adult worms.

To monitor SRP-2$^{H302R}$::GFP aggregates using confocal microscopy, 10-15 day-1 adult worms were directly placed onto a glass slide with 10 μl of M9 buffer containing NaN$_3$ (without agarose pads). To avoid dehydration of samples, we used nail polish to seal the edge of the cover slips. We used a Leica SP5 confocal microscope and Leica AF system software to capture and count SRP-2 aggregates. The confocal imaging settings were optimized as follows: 100x objective magnification; laser: argon and HeNe543 with 15% power; channels: GFP (excitation: 488 nm, emission: 490-510 nm), DAPI (for monitoring autofluorescence; excitation: 405 nm, emission: 410-480 nm); and DIC. All signals were captured by Leica HyD™ detectors. We obtained the final signal by overlapping the GFP and DAPI channels and scanning through the z-axis, and only scoring the GFP signal as aggregates.

4.7 Protein extraction and immunoblots

Whole-worm proteins were extracted by sonication in denaturing SDS buffer with or without cOmplete Protease Inhibitor Cocktail (#4693116001, Roche). Protein concentrations were determined using the RC DC Protein Assay kit (#500-0121, Bio-Rad), and SDS-PAGE analysis and immunoblotting were performed as described [30] with antibodies against Ser51-Phospho-eIF2α (#9721, Cell Signalling Technologies), pan-actin (#8456, Cell Signalling Technologies),
and anti-rabbit HRP conjugated (#7074, NEB). Detection was done using ECL (#32109, Pierce). Quantification of relative signal intensity was done using ImageJ. The arbitrary number was obtained by dividing the percentage intensity of the P-eIF2α band with that of the actin band, or the YFP band with that of the tubulin band.

4.8 Pharyngeal pumping assay

We monitored the pharyngeal pumping rate of late L4 larvae using an Olympus SZX7 light microscope equipped with 20X eye pieces. We counted pumping rates of randomly selected L4 larvae by counting the total number of pumps per one minute. At least 5 animals were counted for every genotype and data points were collected from 4 independent experiments.

4.9 Glutathione assay

L4 larvae were collected and fast-freeze with ethanol-dry ice cold bath, and stored at -80°C. We extracted glutathione (using buffer provided by the enzymatic kit) and protein contents using sonication as described in section 4.7. We measured total glutathione levels using the ApoGSH™ Glutathione colorimetric detection kit (#K261-100, Biovision) and detected absorbance using a POLARstar Omega plate reader (wavelength: 405 nm; BMG labtech). Glutathione level was normalized to total protein level measured as described in section 4.7.

4.10 Statistical analysis

For RT-qPCR, GC-MS analysis, larval survival on tunicamycin and the ire-1(zc14) synthetic interaction assay, we performed F-tests on at least three independent biological replicates per experiment; we then performed two-tailed student t-tests with equal (F-test <0.05) or unequal
variance (F-test>0.05) to determine p-values. We used GraphPad Prism 5 to generate survival curves and calculated statistical significance using the Log-rank (Mantel-Cox) test. We used GraphPad Prism 5 to generate the dot-plots for the protein aggregation assay and the pharyngeal pumping assay; for both assays, we pooled all data points from 3-4 independent experiments together and performed the Mann Whitney test for statistic analysis.
References


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Appendices

Table 1. p-values representing the significance of the changes in lipid profiles of unsupplemented and supplemented control(RNAi) and mdt-15(RNAi) worms.
The table shows the p-values representing the significance of the change in relative FA abundance (first table), FA abundance per total lipids (second table), and relative lipid levels (third table). The p-values are for the comparison of unsupplemented control (RNAi), unsupplemented mdt-15(RNAi), or FA-supplemented mdt-15(RNAi) worms, respectively. N=3, statistical analysis by two-tailed student t-test.

<table>
<thead>
<tr>
<th>Relative FA abundance</th>
<th>PC</th>
<th>PE</th>
<th>CL</th>
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</thead>
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<td>0.82</td>
<td>0.42</td>
<td>0.75</td>
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<td>mdt-15 RNAi</td>
<td>0.20</td>
<td>0.60</td>
<td>0.75</td>
</tr>
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<td>mdt-15 RNAi+UFAs</td>
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Table 2. p-values representing the significance of the changes in lipid profiles between wild-type (WT) and mdt-15(tm2182) worms.
The table shows the p-values representing the significance of the change in relative FA abundance (first table), FA abundance per total lipids (second table), and relative lipid levels (third table). The p-values are for the comparison of WT worms with mdt-15(tm2182) mutant worms. N=3, statistical analysis by two-tailed student t-test.

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<th>PE</th>
<th>triglyceride</th>
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<td>0.01</td>
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Table 3. p-values representing the significance of the changes in lipid profiles of unsupplemented and supplemented control(RNAi) and SCD(RNAi) worms.
The table shows the p-values representing the significance of the change in relative FA abundance (first table), FA abundance per total lipids (second table), and relative lipid levels (third table). The p-values are for the comparison of unsupplemented control(RNAi) worms with FA-supplemented control (RNAi) worms, unsupplemented SCD(RNAi) worms, or FA-supplemented SCD(RNAi) worms, respectively. N=3, statistical analysis by two-tailed student t-test.

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<th>Relative FA abundance</th>
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<td>SCD RNAi</td>
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<td>PS</td>
<td>PI</td>
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**Table 4. Primer sequences used for RT-qPCR.**

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