Inhibition of the mevalonate pathway in
C. elegans: Consequences and implications

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To my Family

Abstract

The mevalonate pathway in human is responsible for the synthesis of cholesterol and other important biomolecules such as coenzyme Q (a component of the electron transport chain in mitochondria), dolichols (important for N-linked glycosylation of proteins) and isoprenoids (important for the membrane association of small GTPases). This thesis concerns novel findings about the effect of statin on the mevalonate pathway using *C. elegans* as a model organism.

Statins are cholesterol-lowering drugs that inhibit HMG-CoA reductase, which is the rate-limiting enzyme of the mevalonate pathway, hence limiting the synthesis of cholesterol and other products from this pathway. *C. elegans* is a particularly powerful model to study the effect of statin on the non-cholesterol outputs of the mevalonate pathway because this pathway is well conserved in worms except for the key fact that the enzymes required for the synthesis of cholesterol are absent. We characterized a *hmgr-1(tm4368)* mutant, which lacks HMG-CoA reductase, and showed that its phenotypes recapitulate the effect of statin on *C. elegans* but in a more severe form. We also showed that inhibition of protein prenylation is a critical consequence of mevalonate pathway inhibition in *C. elegans*.

Since inhibition of the mevalonate pathway via statins or *hmgr-1* mutation, causes growth arrest and sterility, it is relatively easy to screen for resistant mutant. We screened ~150 000 mutagenized haploid genomes and isolated four statin-resistant mutants that carried gain-of-function mutations in *atfs-1*, a positive regulator of the mitochondrial-unfolded protein response (UPR^{mt}). Interestingly, preinduction of this response using ethidium bromide or paraquat in wild type worms or mammalian cells also conferred resistance to statin. Our observations suggest that statin resistance through maintenance of mitochondrial homeostasis is conserved among species, and that the lethal effects of statins in *C. elegans* are caused primarily through impaired protein prenylation leading to mitochondria dysfunction.

We also isolated an additional statin-resistant mutant that carried a partial loss-of-function mutation in *nduf-7,* which encodes a key component of the mitochondrial transport chain complex1 (ETC-1). This mutation also activates the UPR^{mt} and prolonged life span through production of ROS. Interestingly, the gene *ced-4* is required for lifespan extension in the *nduf-7(et19)* mutant but not for UPR^{mt} induction or resistance to statin.

Keywords: *C. elegans,* mevalonate, *atfs-1*, UPR^{mt}, prenylation, *nduf-7, ced-4.*

List of Papers

This thesis is based on the following publications, referred to by roman numerals in the text:

I. Loss of HMG-CoA reductase in *C. elegans* causes defects in protein prenylation and muscle mitochondria.

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II. The mitochondrial unfolded protein response activator ATFS-1 protects cells from inhibition of the mevalonate pathway.

Rauthan M, Ranji P, Aguilera Pradenas N, Pitot C, Pilon M.

Proc Natl Acad Sci USA. 2013 Apr 9; 110(15): 5981-6.

III. A mutation in *Caenorhabditis elegans* NDUF-7 Activates the Mitochondrial Stress Response and Prolongs Lifespan via ROS and CED-4.

Rauthan M, Ranji P, Abukar R, Pilon M.

G3(Bethesda). 2015 Jun 1; 5(8): 1639-48.

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Abbreviations

ATF6	Activating Transcription Factor 6
C. elegans	*Caenorhabditis elegans*
CAAX motif	Four-amino acid sequence at the carboxyl terminus of a protein
CoQ	Coenzyme Q
DHE	Dehydroergosterol
dsRNA	Double stranded RNA
EIF2alpha	α subunit of eukaryotic initiation factor 2
EMS	Ethyl methane sulfonate
ER	Endoplasmic reticulum
ETC	Electron transport chain system
FPP	Farnesyl-pyrophosphate
FTase	Farnesyl transferase
FTI	Farnesyl transferase inhibitor
FWD	Four wheel drive
GGPP	Geranylgeranylpyrophosphate
GGTase	Geranylgeranyl transferase
GOF	Gain-of-function
HMGR	3-hydroxy-3-methylglutaryl-CoenzymeA reductase
I6A	Isopentenyl adenosine
ICMT	Isoprenylcysteine carboxyl methyltransferase
IPP	Isopentenyl-pyrophosphate
IRE1	Inositol Requiring 1
KO	Knockout
LOF	Loss-of-function
Mevalonate	Mevalonic acid
mtROS	Mitochondrial ROS
MTS	Mitochondrial targeting signal
NAC	N-acetyl-cysteine
NLS	Nuclear localization signal
PERK	PKR-like ER kinase
PGC	Primordial germ cells
PI4K-beta	Phosphatidylinositol 4-kinase-beta
Rce1	Ras-converting enzyme1
REP	Rab escort protein
RNAi	RNA interference
ROS	Reactive oxygen species
UPR^{er}	Endoplasmic reticulum unfolded protein response
UPR^{mt}	Mitochondrial unfolded protein response
WGS	Whole genome sequencing
XBP1	X-box binding protein 1

Introduction

C. elegans as a model organism

Caenorhabditis elegans is a small (1 mm), free living soil nematode (roundworm) which was first introduced by Sydney Brenner in 1974 to study animal development and the nervous system (Brenner, 1974). In 1998, the entire genome of *C. elegans* was sequenced and revealed that the similarity between the genes of *C. elegans* and humans is quite significant. Indeed, about 40% of genes linked to human diseases have clear homologs in *C. elegans*. Since then, much effort leveraging both forward and reverse genetics approaches has been done to understand the function, regulation, interaction and expression of the *C. elegans* genes (Corsi, 2006).

C. elegans has a simple anatomy that includes, from anterior to posterior: mouth, pharynx, intestine, gonad, anus and tail (Fig.1). The organs are formed into two tubes: the outer tube includes muscles, nerve cells, the cuticle and the hypodermis, and the inner tube contains the pharynx and intestine. The gonad is in the pseudocoelomic cavity, which is fluid-filled and separates the outer and inner tubes, and consists of two U-shaped tubes that are connected to spermatheca on each side (Fig. 1) (Hubbard and Greenstein, 2005).
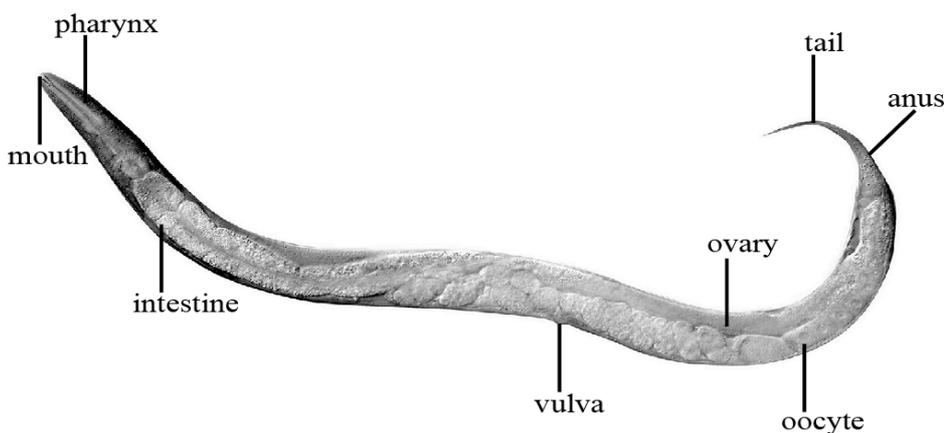
Figure 1. The main anatomical features of an adult hermaphrodite *C. elegans*

There are two sexes in *C. elegans*: hermaphrodites and males that consist of 959 and 1031 somatic cells respectively. The hermaphrodites are self-fertile, producing sperms first, which are stored in the spermatheca, and then oocytes (Schafer, 2005). Hermaphrodites can lay over 300 progeny during their lifetime. They can also be cross-fertilized by males.

Hermaphrodites have five pairs of autosomes (AA) and one pair of sex chromosomes (XX) whereas males have only one sex chromosome (XO). Self-fertilized hermaphrodites produce 0.01% male progeny through spontaneous X chromosome loss during meiosis but when a male fertilizes a hermaphrodite, 50% of the progeny are males since half of the male sperms contain no X chromosome (Nigon and Dougherty, 1949).

C. elegans mainly feed on bacteria as a food source and has a short life cycle of about three days at 20°C, which makes it an ideal laboratory animal. The life cycle of *C. elegans* consists of several stages and begins with a fertilized egg, which goes through four larval stages (L1-L4) then adulthood. Molting separates the different larval stages in *C. elegans*; stage-specific cuticle is made and the old one is shed at the end of each larval stage (Cassada and Russell, 1975).

In favorable laboratory conditions, the worms grow rapidly from embryo through four larval stages to adult with a normal life expectancy of 15-20 days. When food is scarce and population density is high, *C. elegans* larvae may opt for an alternative state after the L2 stage called the dauer diapause during which they can live for four months or even longer (Fielenbach and Antebi, 2008). When conditions for growth and reproduction improve, the dauer larvae undergo molting to enter the L4 stage then develop into normal adults (Fig. 2).
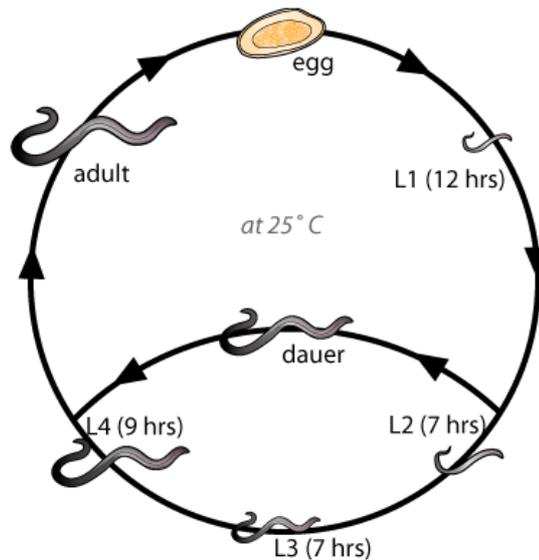
 Figure 2. The life cycle of *C. elegans* (Yuan, 2007)

There are a number of advantages and resources that make *C. elegans* a powerful tool for biological research. It has a transparent body, which allows us to observe all the cells in developing animals. In addition, the availability of knockout (KO) mutants and genetic methodologies such as mutagenesis, transgenesis, and RNA interference (RNAi) allows us to study animals at the molecular level (Leung et al., 2008). For example, the quickest way to determine gene function is to silence any of the ~20 000 genes by feeding dsRNA to the worms (Kamath et al., 2001). Another approach which leads to new genetic discoveries is to induce mutations with a chemical mutagen such as EMS (ethyl methane sulfonate) and screen for the phenotypes of interest (Jorgensen and Mango, 2002). The generation of transgenic worms is also a relatively easy technique which results in the formation of large extrachromosomal DNA arrays (Mello et al., 1991). *C. elegans* can also be frozen as glycerol stocks and stored at -80°C for long periods of time which is not possible with some other model organisms (Stiernagle, 2006).

The mevalonate pathway and its products

In the 1960s, Bloch and Lynen discovered the mevalonate pathway for cholesterol biosynthesis. The mevalonate biosynthetic pathway provides intermediates and products, which are necessary for cell survival and function (Fig. 3). The initial steps

of the mevalonate pathway provide substrate for the highly-branched sequence of reactions resulting in the production of cholesterol and a number of nonsterol isoprenoids (Grunler et al., 1994). These intermediates are required for post-translational modification of many proteins involved in important cellular processes including intracellular signaling, cell growth/differentiation, gene expression, protein glycosylation as well as cytoskeletal assembly (Buhaescu and Izzedine, 2007).

Figure 3. The mevalonate pathway (Bentinger et al., 2010)

The first reaction in the mevalonate pathway begins with the cytosolic enzyme acetoacetyl-CoA thiolase, which condenses two acetyl-CoA molecules to produce acetoacetyl-CoA. In the next step, which is catalyzed by HMG-CoA synthase, acetoacetyl-CoA is condensed with acetyl-CoA to produce HMG-CoA. HMG-CoA reductase, the rate-limiting enzyme of the mevalonate pathway, converts HMG-CoA to mevalonate in a two-step reaction, using two molecules of NADPH (Grunler et al., 1994; Kuzuyama, 2002). Mevalonic acid (mevalonate) is gradually converted into the 5-carbon molecule isopentenyl-pyrophosphate (IPP) via a multi-step phosphorylation and decarboxylation reactions then on to the 15-carbon molecule farnesyl-pyrophosphate (FPP) that is the substrate for the subsequent sterol and nonsterol branches of the pathway. Thus, synthesis of FPP plays an important role in the mevalonate pathway because FPP is the last common substrate for the production of all end products. Forming squalene from FPP requires squalene synthase, which catalyzes the head-to-head condensation of two FPP molecules, with reduction by NADPH. Then, subsequent cyclization steps results in sterol synthesis (Holstein and Hohl, 2004; Rauthan and Pilon, 2011).

One of the molecules synthesized via this pathway is cholesterol. Cholesterol is incorporated by lipoproteins in the cell membrane and regulates membrane fluidity. Cholesterol is also important for the production of steroid hormones and cell signaling (Edwards and Ericsson, 1999). Although insects have cholesterol as the main source of body sterol, they lack the enzymes required for de novo sterol biosynthesis; they must obtain sterols from their diet (Clark and Block, 1959; Jing et al., 2013). Nematodes are also cholesterol auxotrophs. In nature, the main sources of sterols for *C. elegans* are animal feces or yeast/plant debris whereas in laboratory condition *C. elegans* is maintained on agar plates containing cholesterol (Matyash et al., 2001). Cholesterol is an essential nutrient supplement affecting several development processes in *C. elegans* including molting, reproduction, dauer formation and metabolism (Shim et al., 2002). Feeding worms with dehydroergosterol (DHE), a fluorescent analog of cholesterol which mimics its property, revealed that DHE accumulates mainly in the pharynx, nerve ring, excretory gland cell, as well as gut during the L1–L3 stages. During adulthood, DHE molecules accumulate in oocytes and sperms, which is not surprising because these cells need high levels of membrane components for the development and growth of embryos (Matyash et al., 2001). Little

is known about the molecular mechanism of cholesterol transport and its distribution but it has been suggested that cholesterol uptake by *C. elegans* oocytes is mediated through an endocytotic pathway involving yolk proteins (Matyash et al., 2001). In the absence of yolk receptor RME-2, cholesterol forms in the body cavity rather than in the oocytes, which causes abnormal oocytes and low viability of embryos (Branicky et al., 2010).

Dolichol is also synthesized by this pathway and is formed through several condensation reactions beginning from FPP and IPP molecules. Dolichol is found in all tissues as well as most organelles membranes of eukaryotic cells and is required for N-linked glycosylation. The phosphorylated form of dolichol, dolichol phosphate, acts as a lipid carrier for the formation of the oligosaccharides Glc3-Man9-GlcNAc2 (where Glc is Glucose, Man is Mannose, and GlcNAc is N-acetylglucosamine) (Cantagrel and Lefeber, 2011) which is then transferred onto certain asparagine residues of nascent polypeptides. It has been reported that dolichol is metabolized very slowly and accumulates in tissues during the aging process (Carroll et al., 1992).

Isopentenyl adenosine, one of the other products of the mevalonate pathway, is made from isopentenyl diphosphate (IPP). Diphosphomevalonate decarboxylase catalyzes the synthesis of IPP, of which the isopentenyl group is transferred by tRNA-isopentenyltransferase-1 to adenosine in position 37 of tRNA producing isopentenyl adenosine (i6A) (Persson et al., 1994). The presence of isopentenyl adenosine in tRNA improves the interaction between A:U pairing thus increasing the fidelity during translation (Persson et al., 1994). It has been reported that loss of function of *tit1*, which encodes i6A-transferase in *S. pombe* and *MiaA* in Salmonella and *E.coli* results in decreased translational efficiency of mRNAs (Lamichhane et al., 2013). Moreover, mutation in this gene, tRNA-*IPT* (i6A-transferase), influences several biological functions. For example, in *C. elegans, gro-1* encodes i6A-transferase and mutation in this gene causes a slower developmental rate and increased lifespan (Lamichhane et al., 2013).

Coenzyme Q (CoQ), also called ubiquinone, is an important cofactor in the mitochondrial electron transport chain (ETC) and is synthesized via the mevalonate pathway although it can also be obtained from the diet.

CoQ includes a benzoquinone ring, which can be reversibly reduced (ubiquinol, $CoQH_2$) or oxidized (CoQ), and a tail with a different number of isoprenyl units, which is species specific. For example, *Saccharomyces cerevisiae* has six isoprene units (CoQ_6), *C. elegans* contains nine isoprenoid units (CoQ_9), and mammalian species contains different proportions of CoQ_9 and CoQ_{10} (Navas et al., 2007).

CoQ is present in mitochondria and is required for oxidative phosphorylation and ATP production. In this context, CoQ acts as a mobile electron carrier, which transports electrons from complex I (NADH coenzyme Q reductase) and II (succinate dehydrogenase) to complex III (cytochrome bc_1 complex) in the mitochondrial respiratory chain. In a reduced form of its own, $CoQH_2$ acts as an antioxidant protecting biological membranes against oxidation by reactive oxygen species (ROS), as well as preventing the peroxidation of lipoprotein lipids present in the circulation. Therefore, dietary supplementation of coenzyme Q_{10} may be beneficial since the level of $CoQ_{10}H_2$ is increased within the circulating lipoproteins, thus preventing LDL peroxidation (Mohr et al., 1992; Molyneux et al., 2008). In *C. elegans,* exogenous CoQ_{10} supplementation also partially rescues important gene expression alteration and growth retardation in CoQ deficient mutant *clk-1* (Fischer et al., 2014).

CoQ_{10} deficiency often alters mitochondrial energy production and produces more reactive oxygen species (ROS) as a result of reduced scavenging capacity which can induce various diseases such as cardiovascular disease, diabetes and cancer (Molyneux et al., 2008). Hernandez and colleagues reported that expression of autophagic genes both at the transcriptional and translational level increased in CoQ deficient fibroblasts due to the production of excess ROS. This affects mitochondrial structure, which in turn determines their self-elimination (Rodriguez-Hernandez et al., 2009).

Finally, two isoprenoid moieties, namely farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP) are synthesized via the mevalonate pathway and are used in the isoprenylation of proteins. Protein prenylation is a posttranslational modification in which isoprenoid lipids bind to conserved cysteine residues at or close to the C termini of certain proteins and promote their attachment to the cell membranes (Palsuledesai and Distefano, 2015). Isoprenylation of proteins was first discovered in fungi in 1987, and almost 10 years later, the first prenylated

protein, lamin B, was identified (Wolda and Glomset, 1988). Since then, this modification has been investigated extensively because of its essential role in the activity of proteins. In particular, prenylation of small GTPses is achieved in a multi-step process that involves farnesyltransferase (FTase) or geranylgeranyltransferase type 1 (GGTase-I) which catalyzes the attachment of a single farnesyl (15 carbon) or geranylgeranyl (20 carbon) isoprenoid group, respectively, to a cysteine residue in a sequence called "CaaX motif". In this motif, "C" is cysteine, "a" is an aliphatic amino acid, and the "X" residue is usually methionine, glutamine, serine, alanine, or cysteine (Yokoyama et al., 1992). X usually determines if small GTPases should undergo farnesylation or geranylgeranylation (Leung et al., 2007). When X is a methionine, serine, glutamine, or alanine, the substrate is farnesylated, but if it is leucine or phenylalanine, the substrate is geranylgeranylated. However, Rab geranylgeranyltransferase (known as RGGT, GGTase-II or geranylgeranyltransferase type II) can form a different class of protein prenyltransferases. It has been suggested that this enzyme functions together with Rab escort protein (REP) where REP binds to RGGT and the complex associates with unprenylated Rabs. In the next step, the RGGT enzyme catalyzes the transfer of two geranylgeranyl groups to two cysteine residues in sequences such as CXC or CCXX close to the C-terminus of Rab proteins (Casey and Seabra, 1996; Glomset et al., 1990). Alternatively, Rab attaches to REP proteins, which are then presented to RGGT (Leung et al., 2007).

In a second step, Ras-converting enzyme1 (Rce1) removes the last three amino acids (-aaX) from the isoprenylated cysteine residue by endoproteolysis. Finally, isoprenylcysteine carboxyl methyltransferase (ICMT) adds a methyl group to the cysteine residue (Zhang and Casey, 1996). The small GTPases can then become-membrane bound, hence activatable. Proteins that undergo prenylation include Ras, nuclear lamins and Ras-related small GTP-binding proteins, such as Rho, Rab, Rac, the γ subunit of the trimeric G proteins, and others (Vicent et al., 2000). These prenylated proteins are involved in many essential processes such as cell growth, differentiation, cytoskeletal function and vesicle trafficking (McTaggart, 2006). Isoprenylation of proteins occurs shortly after translation and it is irreversible during the lifetime of the protein. The vital importance of protein prenylation was demonstrated using knockout mice lacking *Rce1* or *Icmt*, which are embryonically lethal (Leung et al., 2007; Perez-Sala, 2007).

It is evident from the above overview that blocking the mevalonate pathway using HMG-CoA reductase inhibitors such as statins will result in low levels of mevalonate and its downstream products, and that this may have significant effects on many critical cellular functions (Caraglia et al., 2005).

The structure of HMG-CoA reductase

As already mentioned, 3-hydroxy-3-methylglutaryl-CoenzymeA reductase (HMGR) is the rate-limiting enzyme of the mevalonate pathway and catalyzes the production of mevalonate from HMG-CoA molecule (Luskey, 1986). Once mevalonate is synthesized, it can be converted to different molecules. HMGR is localized to the endoplasmic reticulum (ER) via an N-terminal domain that consists of eight transmembrane domains (339 amino acids) and a linker sequence, which connects N-terminal domain to the globular C-terminal domain (548 amino acids) located in the cytosol (Fig. 4). The N-terminal domain is required for correct localization of this enzyme in the ER and for sensing the sterol concentration, whereas the C-terminal domain is necessary for the catalytic activity of HMGR (Basson et al., 1988; Chen et al., 2012; Liscum et al., 1985).

Figure 4. The structure of HMGR (Chen et al., 2012)

HMGR is perhaps the most highly regulated enzymes in animals. In the presence of sterols, HMGR binds to the ER proteins Insig-1 and Insig-2 that are linked to gp78, a ubiquitin-ligating enzyme which transfers ubiquitin from Ubc7, a ubiquitin-conjugating enzyme to a pair of lysine residues (lysines 89 and 248) in the membrane domain of HMGR. Once HMGR becomes ubiquitinated, it is extracted and delivered to the 26S proteasome for degradation. This mechanism is poorly understood but it is likely that gp78-bound ATPase VCP/p97 and its associated cofactors are involved in the degradation process. Accumulation of geranylgeraniol, a 20-carbon isoprenoid also contributes to the degradation through an undefined mechanism (Fig. 5)

(DeBose-Boyd, 2008; Sever et al., 2003). The expression of a truncated enzyme consisting of only the C-terminal domain is not influenced by the level of sterols even though a stable enzyme is formed (DeBose-Boyd, 2008).
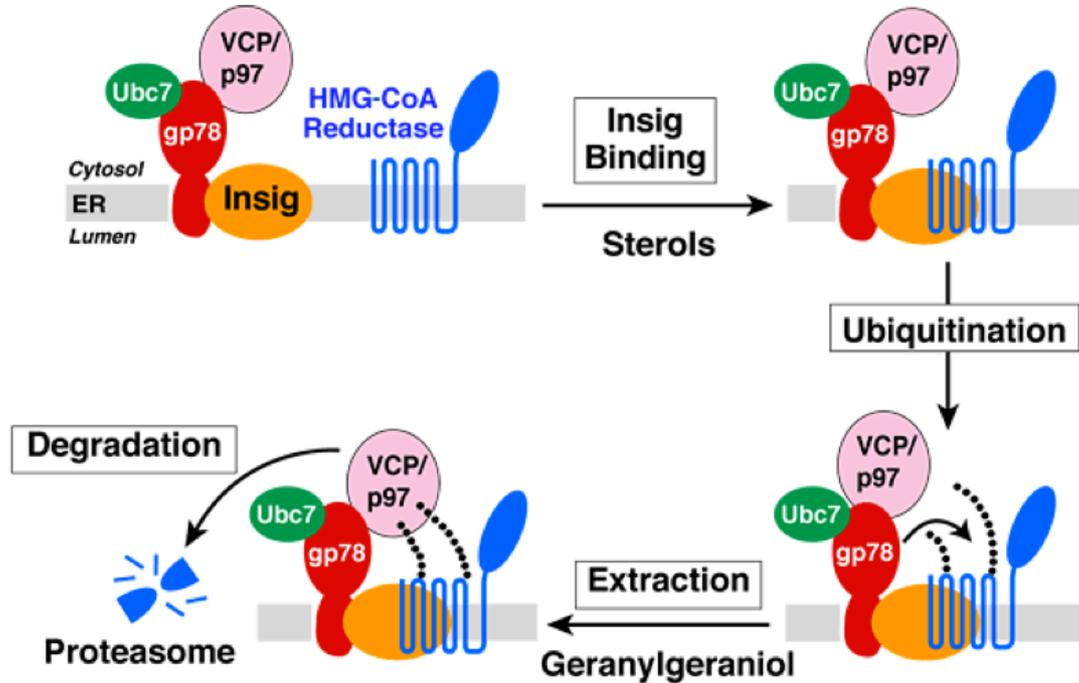
Figure 5. Pathway for the degradation of HMG-CoA reductase in the presence of sterols or geranylgeraniol (DeBose-Boyd, 2008)

HMGR is a tetrameric molecule, which consists of two functional dimers and each dimer has binding sites for HMG-CoA. Since each dimer has two active sites, it is therefore possible for HMGR to either exist as a dimer or tetramer. At each active site, the HMG moiety of HMG-CoA is brought into the proximity of NADPH molecule whose binding pocket is placed in the neighboring monomer. Therefore, the interaction between the two substrates occurs at the interface of the two monomers of a dimer. Interestingly, the tetrameric form of HMGR does not seem to be involved in the substrate binding (Istvan et al., 2000).

Site of statin binding to HMG-CoA reductase

In a clinical setting, the HMGR enzyme is targeted by statin drugs, which are prescribed to lower serum cholesterol levels. The mechanism by which statins bind to

HMGR and inhibit its activity was examined by 3-dimensional X-ray crystallography. All different types of statins share common structural features that resemble the HMG portion of HMG-CoA in either the open chain (pravastatin and atorvastatin) or closed-ring lactone (lovastatin, simvastatin, fluvastatin and cerivastatin) form. The hydrophobic ring structure permits tight binding to the reductase enzyme and a side group on the ring determines the solubility and pharmacokinetic properties of statins (Brosseau and Schaefer, 2002).

Statins are very effective competitive inhibitors as they bind to HMGR approximately 1000-fold more tightly than the natural substrate. Once statins bind to HMGR via their HMG-like moiety, the enzyme undergoes conformational changes which prevents the possibility of being replaced by the endogenous substrate (Istvan, 2003).

The unfolded protein response

In the course of this thesis, specific cellular responses to mevalonate inhibition were studied. Specifically, these are the unfolded protein responses of the endoplasmic reticulum and of the mitochondria, termed UPR^{er} and UPR^{mt}, respectively. They will now be briefly described here.

The UPR^{er} is initiated by three transmembrane proteins in response to accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (ER). These transmembrane proteins are: Inositol Requiring 1 (IRE1), PKR-like ER kinase (PERK), and Activating Transcription Factor 6 (ATF6). In the absence of stress, The ER chaperone (BiP) binds to these receptors and keeps them inactive. In *C. elegans, hsp-4* encodes the homolog of the mammalian BiP (Sasagawa et al., 2007). Upon ER stress, BiP dissociates from its receptors, which leads to homodimerisation and activation of these receptors. IRE1 dimerizes and autophosphorylates itself once it becomes activated. The activated cytosolic domain of IRE1 splices the X-box binding protein 1 (XBP1) mRNA, facilitating its translation to act as a transcription factor, i.e. the XBP1 protein. XBP1 together with the activated form of ATF6 traffic to the nucleus to upregulate the ER chaperones. In addition, the activated cytosolic domain

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of PERK phosphorylates the α subunit of eukaryotic initiation factor 2 (eIF2alpha), which leads to inhibition of translation and cell cycle arrest (Oslowski and Urano, 2011).

A similar pathway, which regulates mitochondrial homeostasis, is UPR[mt]. This response is activated when the unfolded protein load in the mitochondria exceeds the capacity of the mitochondrial chaperones (Pellegrino and Haynes, 2015). Upon mitochondrial stress, the ClpXp protease chops off the unfolded proteins to small peptides, which are then transported out via the ABC-transporter HAF-1. The presence of small peptides in the cytosol leads to activation of the bZip transcription factor ATFS-1 (also known as ZC376.7) through an unknown mechanism. Once ATFS-1 becomes activated, it traffics to the nucleus and interacts with homeobox proteins, namely DVE-1 and UBL-5. This complex then binds to the promoters of mitochondrial chaperone genes and activates mitochondrial chaperones including *hsp-6* and *hsp-60* in *C. elegans* that are necessary for relieving stress and re-establishing homeostasis (Haynes and Ron, 2010).

Why we study the mevalonate pathway particularly in *C. elegans*

The cholesterol synthesis sub-branch of the mevalonate pathway has been studied extensively due to the suspected role of cholesterol in the development of atherosclerosis. In the USA, atherosclerosis is a major cause of mortality, affecting approximately 13-14 million adults. However, in large-scale clinical trials for patients receiving statins the risk of stroke and overall mortality are reduced by 29% and 22%, respectively (Istvan et al., 2000). While most of the effects of statins are linked to reducing cholesterol levels, it is likely that they also block the non-sterol branches of the mevalonate pathway, thus reducing the formation of isoprenoids and affecting protein prenylation with potentially important biological consequences. This could explain some of the side effects of statin use such as muscle pain and immunosuppression. Given that millions of patients take statins on a daily basis, this subject merits careful investigation.

Statins are also interesting as potential anti-cancer drugs. For instance, oncogenic Ras is activated in many human tumors, and inhibiting prenylation of constitutively active

forms of Ras could be a target for treatment (Ripple et al., 2000). Some support for this strategy has come from in vivo models: targeting the farnesyl-transferase of activated Ras rescued the multivulva phenotype of the *let-60(n1046)* gain-of-function allele in *C. elegans.* Similarly, the growth of lung tumors in mice with a K-ras mutations and treated with farnesyl transferase inhibitors, such as FTI-276 or tipifarnib, was reduced significantly compared to control mice (Appels et al., 2005; Morck et al., 2009).

Indeed, focus on the prenylation branch of the mevalonate pathway is increasing from a clinical perspective because of our interesting appreciation that prenylated molecules are crucial for cell growth and differentiation and thus may turn out to be interesting therapeutic targets in the treatment of cancers, autoimmune disorders, atherosclerosis, and Alzheimer disease (Buhaescu and Izzedine, 2007).

Besides their beneficial effects, statins may also have adverse side effects including hepatotoxicity, myotoxicity, neuropathy and so on. The actual mechanisms by which these and other effects are obtained are poorly understood (Wang et al., 2008). As previously stated, we use *C. elegans* as a model organism to specifically study the non-cholesterol effects of statins because much of the mevalonate pathway is conserved between human and *C. elegans* except for the cholesterol branch, which is absent in *C. elegans*.

Earlier studies showed that inhibition of HMG-CoA reductase using RNAi or statins causes severe phenotypes such as growth arrest and sterility in *C. elegans* (Morck et al., 2009). Thus, screening for mutants that are resistant to statins may help us to better understand the effects, and hence the clinical side-effects, of statin on the non-cholesterol branches in higher organisms. This then was the starting point.

During our studies we wished to answer questions of the following type:

1) What are the consequences of inhibition of HMG-CoA reductase?
2) Are the phenotypes caused by statins reversible?
3) How can statin-resistant mutants cope with the effects of statin?

Results and Discussion

In paper I, the *hmgr-1(tm4368)* mutant which lacks the HMG-CoA reductase gene was characterized. This mutant has phenotypes that recapitulate the effects of statins, though in a more severe form. Another finding from this study was that different concentrations of mevalonate are required for different physiological processes, with reproduction requiring the highest amount of mevalonate. Moreover, we showed that the mevalonate pathway is required for the activation of the mitochondrial unfolded protein response (UPR^{mt}): the *hmgr-1(tm4368)* mutant fails to activate this response even when exposed to inducers such as paraquat.

Papers II-III describe the results from a screen for novel mutants resistant to statin-induced lethality. Four such mutants carried gain-of-function mutations in the *atfs-1* gene, which is the key regulator of the mitochondrial unfolded protein response (UPR^{mt}), and a fifth mutant had a partial loss-of-function mutation in *nduf-7*, which encodes a key component of the mitochondrial electron transport chain complex 1 (ETC-1).

Paper I: Loss of HMG-CoA reductase in *C. elegans* causes defects in protein prenylation and muscle mitochondria.

In order to study the effects of mevalonate pathway inhibition in *C. elegans*, we characterized the *C. elegans* mutant *hmgr-1(tm4368)* that lacks a functional HMG-CoA reductase gene.

The *hmgr-1(tm4368)* mutant strain is probably a null mutant due to a 620-bp deletion extending around the first three exons. Mutation in this gene is lethal since the homozygous *hmgr-1(tm4368)* mutants segregating from a heterozygote arrest at the L1 stage (Shi and Ruvkun, 2012). However, if mevalonate is added to the growth media, the mutant worms survive. We tested the effect of different concentrations of mevalonate and we concluded that low concentration of mevalonate (2 mM) is sufficient to rescue the growth of L1s into adults after 96 hrs, 10 mM is required to restore a normal life span, and 20 mM is necessary to make the worms fertile. These observations suggest that different physiological processes are dependent on the

availability of different levels of mevalonate, with reproduction requiring the highest amounts.

We also examined the consequences of mevalonate withdrawal on the *hmgr-1(tm4368)* mutant background and found that this recapitulates many of the pharmacological effects of statins but in a more severe form. We previously showed that inhibition of *hmgr-1* using statin leads to activation of endoplasmic reticulum unfolded protein response (UPR^{er}) using *Phsp-4::GFP as* a reporter (Morck et al., 2009). This response is usually triggered by environmental and genetic factors that disrupt endoplasmic reticulum (ER) homeostasis through activation of a signaling network to reduce the stress, promoting cell survival and adaptation (Oslowski and Urano, 2011). The *Phsp-4::GFP* reporter is also strongly activated in the *hmgr1(tm4368)* mutant grown without mevalonate for 24 hrs, and is completely silenced after 72 hrs when 20 mM mevalonate is again provided. This result shows that ER stress is reversible. Dead oocytes or embryos produced on either side of the spermatheca in the young adult of *hmgr-1(tm4368)* mutant grown in the absence of mevalonate also showed strong UPR^{er} activation. Expression of UPR^{mt} reporters was also examined. In the absence of stress, the expression of GFP from the *hsp-60* promoter is at a basal level, but increases significantly when mitochondrial stress is induced using 0.5 mM paraquat (Haynes and Ron, 2010; Ranji et al., 2014).

Phsp-60::GFP was not activated in either statin-treated worms or *hmgr-1(tm4368)* mutant background grown in the absence of mevalonate unless small amounts of mevalonate were provided. This suggests that the mevalonate pathway is required for UPR^{mt} activation (Liu et al., 2014; Ranji et al., 2014). We propose that depletion of isoprenoids on one or a few small GTPases due to statin-mediated inhibition of HMG-CoA reductase may cause both the activation of the UPR^{er} and the inhibition of UPR^{mt} activation. Several molecular candidates are known to impact both the UPR^{er} and UPR^{mt}. For example, Runkel and colleagues proposed that downregulation of *pifk-1* using RNAi affects both expression of *Phsp-4::GFP* and *Phsp-60::GFP* reporters (Runkel et al., 2013) *pifk-1* is an ortholog of the kinase *Four wheel drive (Fwd)* in *Drosophila* and of phosphatidylinositol 4-kinase-beta (PI4K-beta) in humans. The *Drosophila Fwd* is a key regulator of the small G-protein RAB11, which functions in membrane trafficking during cytokinesis and is thus an example of a GTPase regulator that can influence both the UPR^{er} and UPR^{mt} when downregulated.

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Further research will be required to examine the role of PIFK-1 in these two processes (Runkel et al., 2013). Another small GTPase identified in RNAi screen for suppressors of the UPR^mt in *C. elegans* was RHEB-1. RHEB-1 is involved in the mTOR pathway, which has important roles in maintaining mitochondrial homeostasis. However, The exact function of RHEB-1 in UPR^mt is still not clear (Groenewoud and Zwartkruis, 2013).

We used a GFP-based prenylation reporter to confirm that inhibition of the mevalonate pathway results in reduced protein prenylation in the *hmgr-1* mutant. In this reporter, the CAAX C-terminal motif of a small GTPase RAS-2 is added to 3' the GFP coding sequence such that the GFP becomes membrane-enriched when it gets prenylated (Morck et al., 2009). As with statin-treated worms, the *hmgr-1(tm4368)* mutant exhibits a diffused GFP pattern in the absence of mevalonate. However, this phenotype is reversed when 20 mM mevalonate is provided.

In a separate study (Paper II), we showed that gain-of-function (*gof*) mutations in the UPR^mt activator ATFS-1 could protect worms against mevalonate pathway inhibition using statins (Rauthan et al., 2013). ATFS-1 is a leucine zipper transcription factor that contains a mitochondrial targeting signal (MTS) at its N terminus and a nuclear localization signal (NLS) at its C-terminus. In the presence of mitochondrial stress, ATFS-1 is not targeted to mitochondria and instead accumulates in the nucleus to activate UPR^mt response (Nargund et al., 2012; Rauthan et al., 2013). We examined whether *atfs-1* could suppress the *hmgr-1(tm4368)* phenotypes. For this purpose, a double mutant carrying the gain-of-function allele *atfs-1(et15)* and the *hmgr-1(tm4368)* null allele was generated. This double mutant was not viable in the absence of mevalonate, but interestingly showed a much improved benefit from as little as 1 mM mevalonate in terms of growth and protein prenylation. Our results therefore show that UPR^mt activation allows *C. elegans* to survive and reproduce even when output from mevalonate pathway is drastically reduced. However, it is important to point out that we still do not know what aspect of the UPR^mt is critical in this context.

Accumulating evidence suggests that depletion of isoprenoids particularly GGPP via statins leads to myopathy and in rare cases rhabdomyolysis (Cao et al., 2009; Sakamoto et al., 2011). To determine if inhibition of HMG-CoA reductase can induce

muscle damage in *C. elegans*, we used the PD4251 strain that carries the transgene *ccIs4251,* which expresses GFP in nuclei and mitochondria of all body wall muscles. We found that inhibition of HMG-CoA reductase causes disorganization of the muscle mitochondria in *C. elegans*, and that this effect could be completely prevented by inclusion of 20 mM mevalonate in the culture medium or partially rescued by *atfs-1(gof)* mutation. In connection with this result, Sakamoto and colleagues reported that in the presence statins, rat myofibers gets vacuolated due to depletion of RAB-1A. RAB-1 is an important molecule for the maintenance of intracellular tubular network structure and it is more susceptible to GGPP depletion among small GTPases (Sakamoto et al., 2011). RAB-1 is also involved in the ER-to-Golgi trafficking. Incubation of rat myofibers with brefeldin A, which inhibits ER-to-Golgi trafficking, resulted in the same phenotype as statin-treated myofibers. Perhaps, one or more small GTPases, which depend on the mevalonate pathway for their membrane association via prenylation, regulate muscle skeletal regeneration.

We next wanted to examine the expression pattern of *hmgr-1*. To this end, we created a transcriptional reporter, *Phmgr-1::GFP*, which contained 3.06 kb of 5' UTR fused to the coding sequence of GFP, and a translational reporter, *Phmgr-1::HMGR-1::GFP,* which contained 3.06 kb of 5' UTR and the complete *hmgr-1* gene fused to the coding sequence of GFP. The transcriptional reporter is expressed in several tissues, but predominantly in spermatheca, excretory canal cell, vulva muscles, the pharyngeal muscles pm3 and pm8, the anal depressor and, more weakly, in the intestine. The translational reporter showed strong expression in the spermatheca, excretory canal cell and pharyngeal muscles, and is also expressed in the gonad sheath cell and the ventral nerve cord. Since the translational reporter could rescue the *hmgr-1(tm4368)* mutant, we concluded that these reporters provided an accurate view of *hmgr-1* expression.

Knowing that reproduction requires the highest amount of mevalonate to rescue the *hmgr-1(tm4368)* mutant and that the HMGR-1 protein is expressed at remarkably high levels in the spermatheca leads us to think that the mevalonate pathway may regulate germline development. This is consistent with studies by Van Doren and colleagues who found that HMGR is highly expressed in the *Drosophila* somatic gonad and is required for the migration of germ cells to the gonad. Primordial germ

cells (PGC) migrate actively through the gut epithelium, reaching the midgut surface, and then move into the mesoderm where they associate with their somatic cells to form the gonad (Starz-Gaiano and Lehmann, 2001). In the absence of *hmgcr* (*hmgcr*[clb]), PGCs fail to migrate from endoderm to mesoderm. Sometimes, the PGCs migrate to the mesoderm but they then lose their connection with the gonadal mesoderm, and hence become scattered in the embryo (Van Doren et al., 1998). Interestingly, misexpression of *hmgcr* as well as farnesyl-diphosphate synthetase (*fpps*) (which synthesizes FPP), geranylgeranyl diphosphate synthetase (*qm*) (which in turn converts FPP to GGPP), and geranylgeranyl transferase type I (*b-ggt1*) guide PGCs to tissues other than the gonadal mesoderm (Santos and Lehmann, 2004). Related studies were also performed by Thorpe and colleagues in zebrafish. They showed that embryos treated with geranylgeranyl transferase inhibitors but not farnesyl transferase inhibitors had abnormal germ cell migration very similar to that observed in statin-treated embryos (Thorpe et al., 2004). It is still not clear how *hmgcr* attracts germ cells, but at least two models have been proposed. In the indirect model, geranylgeranylated proteins such as Ras, Rac and Rab would enhance the expression and secretion of an attractant molecule whereas in the direct model, a geranylgeranyl-PP protein produced in the mesoderm is transported out via an ABC transporter and is recognized by germ cells, guiding their migration (Santos and Lehmann, 2004). This mechanism has been studied in yeast, where ABC transporters Ste6 secretes farnesylated pheromones required for cell mating (Cesari, 2009). Further studies need to be performed to examine whether *hmgcr* plays a role in germ cell migration or development in *C. elegans*.

In conclusion, we now believe that inhibiting HMG-CoA reductase via statins decreases the lipid moieties required for prenylation of small GTPases. Small GTPases are involved in many cellular processes and it is still unknown which critical GTPases and vesicular trafficking pathways are most affected by statins.

Paper II: The mitochondrial unfolded protein response activator ATFS-1 protects cells from inhibition of the mevalonate pathway.

In this study, we mutagenized wild type worms with ethyl methane sulfonate (EMS) and looked for mutants resistant to fluvastatin-induced lethality. Chemical

mutagenesis is the quickest and most straightforward way to induce mutations in the germ line at high frequency (Kutscher and Shaham, 2014). In total, we screened ~150 000 mutagenized haploid genomes and isolated five mutants that can grow in the presence of fluvastatin (0.5-1mM), which is lethal for normal worms. Incubation of wild type L1 larvae on fluvastatin plates leads to developmental arrest at concentrations of 0.125 mM or higher (Morck et al., 2009).

In paper I, we further characterized four of these mutants *(et15-et18)*. These mutants were resistant to two types of statins (fluvastatin and rosuvastatin) as well as to ibandronate, which inhibits farnesyl diphosphate synthase. The statin-resistant mutants could also suppress the growth defect phenotype of the *hmgr-1(tm4368)* null mutant but only when small doses of mevalonate were provided (1mM). In other words, the *et15-et18* mutants can not survive if the mevalonate pathway is completely non-functional, as in the *hmgr-1(tm4368)* mutant. We therefore conclude that some residual but important HMG-CoA reductase activity remains in statin-treated worms and that this limited output is sufficient for survival and reproduction in the *et15-et18* mutant backgrounds.

Using a gene identification strategy based on outcrossing and whole-genome sequencing (Zuryn et al., 2010), we discovered that all four statin-resistant mutants had substitution mutations at amino acid positions 4 or 6 in the mitochondrial targeting signal (MTS) of the protein ATFS-1. ATFS-1 (also known as ZC376.7) is a leucine zipper transcription factor that activates the UPR[mt]. ATFS-1 contains a MTS at its N terminus and a nuclear localization signal (NLS) at its C terminus. ATFS-1 shuffles between mitochondria and nucleus depending on the condition. In the absence of mitochondrial stress, the MTS part is dominant and ATFS-1 is targeted to the mitochondrial matrix where it gets degraded by the Lon protease. During mitochondrial stress, ATFS-1 accumulates in the nucleus. ATFS-1 interacts with transcription factors, namely DVE-1 and UBL-5, to activate target genes, including the mitochondrial chaperones HSP-6 and HSP-60 (Schulz and Haynes, 2015).

To further confirm that the four isolated *atfs-1* mutant alleles (*et15-et18*) are responsible for the statin resistance in these mutants, we performed RNAi against the

atfs-1 gene: this caused the loss of statin resistance, hence confirming that ATFS-1 is key to the resistance in the mutant.

Several other lines of evidence confirm that *atfs-1* is critical for worms to survive in the presence of statin: 1) The null *atfs-1(gk3094)* allele is hypersensitive to statin; 2) The UPR^mt reporters *hsp-60::GFP* and *hsp-6::GFP* (but not the UPR^er reporter *hsp-4::GFP*) are constitutively expressed in the mutants; and 3) *atfs-1(et15)* heterozygous worms are resistant to statin. We conclude that constitutive activation of the UPR^mt is the most likely mechanism by which the novel *atfs-1* alleles confer resistance to statins. Surprisingly, the UPR^mt reporter *Phsp-60::GFP* was not activated in wild type worms cultivated in the presence of fluvastatin, suggesting that worms treated with statins are unable to activate the one protective response that would allow them to survive statin treatment.

We believe that mitochondrial targeting is impaired by the mutations (amino acid substitution) in the MTS region of the *atfs-1(et15-18)* alleles, which allows for increased nuclear localization of this protein and hence activation of the UPR^mt. This hypothesis was tested by injecting an *atfs-1* transgene lacking the MTS into *atfs-1(gk3094)* null mutants then testing the resulting transgenic worms for statin resistance. Consistent with the hypothesis, the MTS-deficient *atfs-1* transgene confers statin resistance, while a similar transgene lacking both region (MTS and the NLS) showed no resistance at all.

We next tried to examine if pre-activation of the UPR^mt could lead to statin resistance in wild type worms, yeasts or even mammalian cells. Pre-activation of the UPR^mt was done using EtBr (an inhibitor of mitochondrial DNA replication), or paraquat (an inducer of oxidative stress). We found that preinduction of UPR^mt could protect *C. elegans*, yeast *Schizosaccharomyces pombe* and mammalian fibroblast line NIH 3T3 from the effects of statins. We concluded that statin impairs mitochondria homeostasis even in organisms where the main output of the pathway is sterols.

As our results suggested that inhibition of the mevalonate pathway could impair mitochondrial homeostasis, we next examined the possible effects of statin treatment

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on the CoQ levels. CoQ is an important factor in mitochondrial respiration and a number of studies in animals have reported that CoQ levels are depleted both in tissues and blood after statin therapy (Di Stasi et al., 2010; Schulz and Haynes, 2015).

Our work suggests that the effects of statins in *C. elegans* are not caused by reduction in CoQ levels. We showed that the *atfs-1(et15)* mutant respires normally even in the presence of fluvastatin, and that CoQ supplementation in statin plates did not protect *C. elegans* from the toxic effect of statins (CoQ_{10} was tested at 50 μg/mL and concentrations of CoQ_9 ranging from 10 to 80 μg/mL were tested). CoQ is dietarily available to *C. elegans* that are fed *Escherichia coli*. In addition, the *atfs-1(et15)* mutant did not show resistance to rotenone, antimycin A, or sodium azide, which are known as inhibitors of the mitochondrial respiratory chain.

Knowing that the effects of statins on mitochondria are not related to inhibition of CoQ synthesis, we next tested the prenylation branch of the mevalonate pathway using gliotoxin as an inhibitor of farnesyl transferase and our prenylation reporter (*pGLO-1P::GFP-CAAX*). The gain-of-function *atfs-1(et15)* allele showed partial resistance to gliotoxin, whereas the loss-of-function *atfs-1(gk3094)* allele conferred hypersensitivity. Moreover, monitoring of a prenylation reporter revealed that GFP remains partially enriched on the intestinal cells in *atfs-1(et15)* and *atfs-1(et18)* mutants even when these are treated with gliotoxin. In connection with our result, Cao et al also showed that prenylation is the most likely branch affected by statins. They showed that blocking prenylation via statins or geranylgeranyl inhibitors impaired the structure of muscle cells in zebrafish, mice and mammalian culture. Moreover, the addition of geranylgeraniol (permeable form of geranylgeranyl pyrophosphate)(Crick et al., 1997) but not farnesol could rescue the effect of statin on myotubes (Cao et al., 2009).

In summary, we believe that inhibition of the prenylation branch of the mevalonate pathway is the critical cause of the statin effects in *C. elegans*, and that activation of the UPR^{mt} machinery maintains mitochondria homeostasis even in the presence of statin. Activation of this response likely allows the cells to better use the residual output from the mevalonate pathway and hence maintains prenylation of essential GTPases.

Paper III: A mutation in *C. elegans* NDUF-7 activates the mitochondrial stress response, and prolongs lifespan via ROS and CED-4

In this study, we isolated a new mutant allele *(et19)* in a forward genetic screen for mutants that could grow in the presence of 0.5 mM fluvastatin, a concentration that is lethal for wild type worms. The *et19* mutant worms also confer resistant to another class of statin (rosuvastatin), indicating that they have a generic resistance to statins rather than to one particular subtype. Interestingly, mevalonate supplementation had no beneficial effect on the growth of the *et19* mutant allele, just as it did not benefit mutants carrying the *atfs-1* gof alleles described in Paper II.

Based on the prior knowledge that constitutive activation of UPR^mt allows the mutants *atfs-1* (*et15-et18*) to survive in the presence of statin, we hypothesized that the *et19* mutant might also display constitutive activation of the mitochondrial stress response. Consistent with this hypothesis, *et19* mutant worms showed strong expression of *hsp-60::GFP,* a known marker of UPR^mt, even when grown on normal plates.

Based on the outcrossing and whole genome sequencing (WGS) method, we found that *et19* mutant has a single missense mutation in the *nduf-7* gene*,* which encodes one of components of the complex I in the mitochondrial electron transport chain system (ETC). This mutation introduces a premature STOP codon that results in a protein five amino acids smaller than the wild type version. We believe that this mutation is a partial loss-of-function allele since the null *nduf-7(tm1436)* mutant allele lacking the second exon and part of the third exon is lethal while *nduf-7(et19)* is viable.

To further confirm that the *nduf-7* mutation is responsible for the statin resistance, we used RNAi to knockdown the *nduf-7* gene both in wild-type and *et19* mutant worms. Knockdown of the *nduf-7* gene in wild-type worms induced the UPR^mt whereas the same RNAi treatment in *et19* mutant worms resulted in larval arrest.

This suggests that *nduf-7(et19)* is partially functional in this mutant but that further inhibition using RNAi brings the activity below an essential threshold. Another proof that *et19* is a partial loss of function allele of *nduf-7* came from the introduction of a wild-type copy of this gene in the *et19* mutant: the wild type copy of *nduf-7* could

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suppress the expression of *hsp-60::GFP* and rescue the growth defect in *et19* mutant on normal plates. The same transgene could also rescue the lethality of the *nduf-7(tm1436)* deletion mutant.

Knowing that the *nduf-7(et19)* mutation and RNAi against *nduf-7* causes activation of UPR^mt reporter, we next examined whether this response is dependent on ATFS-1. Activation of the UPR^mt in the *nduf-7(et19)* mutants does not occur when the *atfs-1* gene is inhibited by RNAi, suggesting that UPR^mt activation in the *nduf-7(et19)* mutant occurs via ATFS-1. However, RNAi against *nduf-7(et19)* did not activate the *hsp-4::GFP* reporter, a known marker of UPR^er, suggesting that the stress response is specific for mitochondria. We also performed a suppressor screen of *atfs-1(et15);zcIs9(Phsp60::GFP)* in an effort to identify essential components between *atfs-1* and its target *hsp-60* promoter. This screen resulted in the isolation of 14 intragenic loss-of-function alleles of *atfs-1* itself, and nothing else. This suggests that ATFS-1 may act directly on the *hsp-60::GFP* promoter, or that eventual partners or downstream effectors of ATFS-1 are not easily be identified using forward genetics. Note that all the novel *atfs*-1 loss-of-function *(lof)* alleles also lost their resistance to statin.

Several studies reported that mutations in the mitochondrial electron transport chain (ETC) components are linked to longevity in various species including yeast, *C. elegans*, *Drosophila*, and mice (Hwang et al., 2012). In addition to genomic mutations, which affect mitochondrial proteins, it has been shown that RNAi against the genes encoding subunits of mitochondrial complexes in *C. elegans* such as *isp-1* and *nuo-6* also extends lifespan, which is mediated through decreased electron transport and elevation of production of reactive oxygen species (ROS) by mitochondria (Yang and Hekimi, 2010). Mitochondrial ROS (mtROS) acts as signaling molecules communicating between mitochondria and different cells under physiological condition. Therefore, it is really curcial for the cells to maintain ROS at a certain level. A slight increase in the level of mtROS is sufficient for adaptation to stress due to activation of several signaling pathways while higher levels of mtROS is toxic for the cells and leads to cell death (Chandel, 2014).

We examined whether the *nduf-7(et19)* mutant allele functions similarly as other long-lived mutants such as *nuo-6* and *isp-1* mutants that bear mutations in subunits of complex I and III respectively. We found that the *nduf-7(et19)* mutant has a low respiration rate, indicative of a compromised ETC function, as well as an extended lifespan. We next examined whether the impaired mitochondrial function in *nduf-7(et19)* leads to elevation of ROS level. There are several fluorescent indicator dyes that detect different types of ROS produced in the cell. For example, MitoSox is a dye that detects only superoxide whereas the H_2DCFDA dye is sensitive to a variety of ROS but not superoxide (Yang and Hekimi, 2010). For our experiments we chose to use the antioxidant N-acetyl-cysteine (NAC) rather than a fluorescent dye because the results would be functional rather than descriptive (Yang and Hekimi, 2010). Interestingly, NAC could reduce the expression of UPR^{mt} reporter as well as the extended lifespan of the *nduf-7(et19)* mutant. These results suggest that the UPR^{mt} activation and lifespan extension are both dependent on elevated ROS level in *nduf-7(et19)* mutant. It is possible that elevated ROS is an activating signal for ATFS-1.

Hekimi and colleagues have suggested that one candidate signaling pathway that could sense mtROS is the intrinsic apoptosis pathway consisting of CED-9/Bcl2, CED-4/Apaf1, and CED-3/Casp9, and activated by CED-13, an alternative BH3-only protein (Yee et al., 2014). Based on their model, the individual proteins or pairs of interacting proteins of the apoptotic signaling pathway seem to have a function independent of apoptosis which may affect longevity and promote survival under stressful condition in *C. elegans*. They showed that having a mutation in *ced* genes of the apoptotic pathway could suppress the longevity of long-lived mutants such as *isp-1* and *nuo-6*, but have no effect on lifespan by themselves (Yee et al., 2014).

We made a double mutant *nduf-7(et19);ced-4(n1162)* and examined their phenotypes in terms of longevity as well as resistance to statin. We found that lifespan extension is suppressed in the double mutants but not its resistance to statin. This suggests that *ced-4* is required for the longevity of the *nduf-7(et19)* mutant but is not required for the constitutive activation of UPR^{mt}.

In summary, we believe that the mutation in *nduf-7(et19)* may disrupt the assembly of this subunit and its interaction with other subunits in complex I (Mimaki et al., 2012) which leads to elevation of ROS and eventually activation of UPR[mt].

Activation of the UPR[mt] probably results in adaptation of cells to clear up subsequent ROS insults and improve mitochondrial function by turning on antioxidant genes including superoxide dismutase and glutathione metabolism as a feedback mechanism (Barbour and Turner, 2014; Schulz and Haynes, 2015). Additionally, ROS increases mitochondrial biogenesis and mitochondrial DNA content to improve mitochondrial capacity under stress. While low level of ROS increases the expression of mitochondrial chaperones as well as antioxidant genes to improve mitochondrial function, irreparable mitochondria which are damaged by an excess of ROS are degraded rapidly by mitophagy (Schulz and Haynes, 2015).

Understanding the mechanism of how cells react to mitochondrial oxidative stress will require more investigation that may be relevant in understanding the pathogenesis of complex diseases.

Future Prospects

During the course of this thesis work, we found that inhibition of the mevalonate pathway via statins interferes with the prenylation of small GTPases important for mitochondrial homeostasis. However, this treatment does not activate UPR^{mt}, which is a remarkable observation. Our understanding of small GTPases and their effector proteins, which might be affected by statins, remains primitive and a future goal is to identify which small GTPases are responsible for the statin effects. In particular, it will be interesting to investigate which small GTPases, when silenced, lead to activation of UPR^{er}, activation of UPR^{mt} and suppression of UPR^{mt}. We have a list of GTPases (43 GTPses present in a genome-wide RNAi library; Rauthan and Pilon) that can undergo either farnesylation or geranylgeranylation (type 1 and type 2). We are planning to screen those GTPases by RNAi since it is the quickest method to identify which ones are involved in these three processes.

This thesis produced several results that may have clinical relevance. For example, it would be interesting to examine whether the regulation of UPR^{mt} varies among individuals receiving statin treatment and whether such variation contributes to the varied susceptibility of patients to the side effects of statins.

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