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Ether Lipid Biosynthesis Promotes Lifespan Extension and Enables Diverse Prolongevity Paradigms

A dissertation presented

by

Lucydalila Cedillo

to

The Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Biological and Biomedical Sciences

Harvard University

Cambridge, Massachusetts

May 2022

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Dissertation Advisor: Dr. Alexander A. Soukas Lucydalila Cedillo

Ether Lipid Biosynthesis Promotes Lifespan Extension and Enables Diverse Prolongevity Paradigms

Abstract

Biguanides, including the world's most commonly prescribed drug for type 2 diabetes, metformin, not only lower blood sugar, but also promote longevity in preclinical models. Epidemiologic studies in humans parallel these findings, indicating favorable effects of metformin on longevity and on reducing the incidence and morbidity associated with aging-related diseases, such as cancer. In spite of these promising observations, the full spectrum of the molecular effectors responsible for these health benefits remains elusive. Through unbiased genetic screening in *C. elegans*, we uncovered a novel role for genes necessary for ether lipid biosynthesis in the favorable effects of biguanides. We demonstrate that biguanides govern lifespan extension via a complex effect on the ether lipid landscape requires enzymes responsible for both ether lipid biogenesis and polyunsaturated fatty acid synthesis. Remarkably, loss of the ether lipid biosynthetic machinery also mitigates lifespan extension attributable to dietary restriction, target of rapamycin (TOR) inhibition, and mitochondrial electron transport chain inhibition. Furthermore, overexpression of a single, key ether lipid biosynthetic enzyme, *fard-1*/FAR1, is sufficient to promote lifespan extension. These findings illuminate the ether lipid biosynthetic machinery as a novel therapeutic target to promote healthy aging.

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CHAPTER 1: INTRODUCTION

1.1 GENERAL ETHER LIPID BIOLOGY

1.1.1 General ether lipid structure

Canonical diacyl phospholipids are generally comprised of two fatty acid tails and a phosphate head group, all of which are bound to a glycerol backbone via ester linkages. The two, hydrophobic tails are attached to the *sn-1* and *sn-2* carbons of the glycerol backbone and the hydrophilic, phosphate head group is bound to the *sn-3* position. Ether lipids are structurally similar to diacyl phospholipids yet highly distinct due to the addition of a fatty alcohol, as opposed to a fatty acid, to the glycerol backbone at the *sn-1* position. The presence of an alkyl or alkenyl chain attached to the *sn-1* position of a glycerol backbone defines ether lipids as a unique, subclass of glycerophospholipids (Figure 1.1). The alcohol moiety bound to the phosphate head group in ether lipids is generally ethanolamine or choline, although minor species including glycerol, inositol, and serine have also been observed.

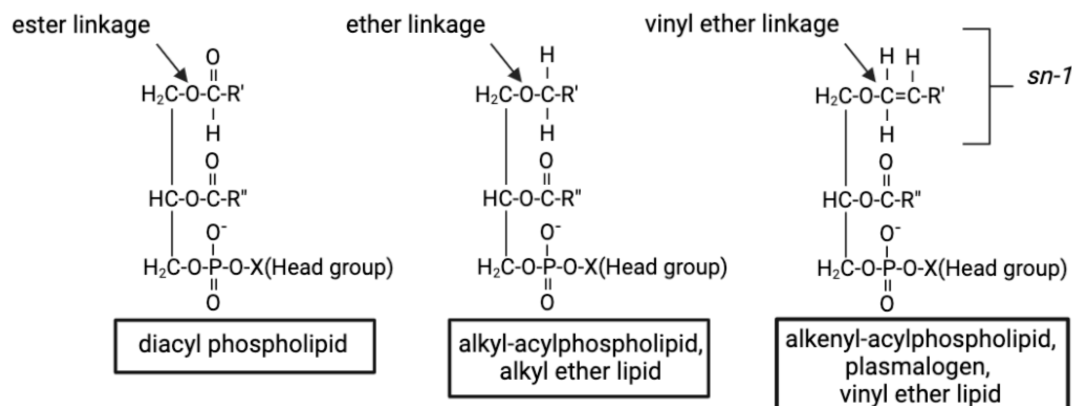


Figure 1.1. Chemical structures of diacyl phospholipids and ether lipids, adapted from (Dean and Lodhi, 2018). Diacyl phospholipids are comprised of two fatty acid tails attached to a glycerol backbone at the *sn-1* and *sn-2* position via ester linkages. These lipids also have a phosphate head group attached by an ester linkage to the *sn-3* position of this glycerol backbone. Ether-linked lipids are defined by the presence of an alkyl chain that is attached at the *sn-1* position via an ether bond. Similar to diacyl phospholipids, ether lipids generally have an ester-linked fatty acyl chain that is bound at the *sn-2* position of the glycerol backbone. Ether lipids can be broken into two smaller subclasses called: alkyl ether lipids and vinyl ether lipids. Alkyl ether lipids harbor an ether linkage at the *sn-1* position (C-O-C'), while vinyl ether lipids contain a *cis*-double bond adjacent to the ether linkage and are commonly referred to as plasmalogens. The most common radical groups bound to the phosphate head in ether lipids are ethanolamine or choline.

1.1.2 General ether lipid nomenclature

The alkyl ether linkage of ether lipids is represented by the "O-" prefix, for example PC(O-16:0/18:1(9Z)) indicates the presence of an ether linked, 16-carbon chain at the *sn-1* position and a delta-9 unsaturated, 18-carbon fatty acyl chain at the *sn-2* position. The alkenyl ether (also known as plasmalogen or vinyl ether, see Figure 1.1) species are represented by the "P-" prefix. *PE*(*P*-18:0/18:2(9Z,12Z)), for instance, denotes a *cis*-double bond adjacent to the ether linkage at the *sn-1* position (Figure 1.1). The PC and PE in the above examples represent the phosphatidylcholine (PC) and the phosphatidylethanolamine (PE) headgroups that are bound to the phosphate group at the *sn-3* position.

1.1.3 Ether lipid biosynthesis

Ether lipid precursors are first synthesized by enzymes localized in peroxisomes and further processing is completed in the endoplasmic reticulum (ER) (Ghosh and Hajra, 1986; Hajra et al., 1979; Hardeman and Van den Bosch, 1989; Kennedy, 1961; Singh et al., 1993; van den Bosch, 1974). In humans, the main enzymes involved in ether lipid biosynthesis *within* the peroxisomal matrix are, glyceronephosphate O-acyltransferase (GNPAT) and alkylglycerone phosphate synthase (AGPS). The first lipidic intermediate in ether lipid biosynthesis is formed within the lumen of the peroxisome where glycerol 3-phosphate (G3P) dehydrogenase converts G3P to

dihydroxyacetone phosphate (DHAP, see Figure 2.1 *A*). GNPAT then acylates DHAP at the *sn-1* position to generate 1-acyl-DHAP. Subsequently, AGPS exchanges the acyl chain for an alkyl group and forms an ether bond at the *sn-1* position to generate 1-alkyl-DHAP. The alkyl moiety used in previous step is synthesized outside of the peroxisome by the peroxisomal membrane-associated enzyme, fatty acyl-CoA reductase 1 (FAR1), and is transported into the peroxisomal lumen. Thus, FAR1 supplies the fatty alcohols used by AGPS to synthesize 1-alkyl-DHAP. Notably, the three key enzymes responsible for ether lipid biosynthesis are well conserved in *Caenorhabditis elegans* (*C. elegans*) as acyltransferase-like-7 (*acl-7/GNPAT)*, alkyl-dihydroxyacetonephosphate synthase-1 (*ads-1/AGPS)*, and fatty acyl-CoA reductase-1 (*fard-1/FAR1).*

The final peroxisomal step in ether lipid synthesis is carried out by acyl/alkyl-DHAP reductase, which reduces 1-alkyl-DHAP into 1-O-alkyl-G3P. This ether lipid precursor is then trafficked to the ER, with the aid of peroxisome- and ER-related tethering proteins, to undergo unique acyl chain remodeling and produce various ether lipid products (Dean and Lodhi, 2018; Hua et al., 2017; Singh et al., 1993). The *sn-1* alkyl moiety may also have a *cis*-double bond introduced at the *sn-1* position between the α-carbon immediately adjacent to the ether bond by the recently identified desaturase, *tmem-189* (Werner et al., 2020).

1.1.4 Ether lipids as cell membrane constituents

Ether lipids are key structural components of cell membranes. Ether lipids are involved in the maintenance of general membrane fluidity and the formation of lipid rafts within microdomains, which are important for promotion of membrane fusion and remote cellular signaling. The vinyl ether linkage of plasmalogens allows the proximal regions of the *sn-1* and *sn-2* chains to become parallel, resulting in close alignments and allows tighter packing of phospholipids in the membrane. The incorporation of plasmalogens in lipid raft microdomains, cholesterol-rich regions of membranes, therefore further enables an increase in viscosity that is essential for the organization of concentrated membrane-associated, cellular signaling proteins (Pike et al., 2002; Rodemer et al., 2003). In support of this notion, plasmalogen deficient GNPAT null mice exhibit disruptions in lipid raft microdomain formation and display abnormal cholesterol localization to a perinuclear compartment (Rodemer et al., 2003).

Using molecular dynamic simulations based on a coarse-grained lipid model, researchers went on to demonstrate that fundamental biological processes, such as vesicle fusion or budding, require the cell matrix to transition from a lamellar to a non-lamellar, inverted hexagonal state (Marrink and Mark, 2004). Within a lipid membrane, the *sn-2* acyl chains in diacyl phospholipids and plasmalogens are positioned in a manner that is perpendicular to the membrane surface. However, plasmalogens lack the carbonyl oxygen at the *sn-1* position and are therefore more lipophilic. The resulting stronger intermolecular hydrogen bonding between the head groups of plasmalogens

leads to a higher propensity of formation of an inverse hexagonal phase, which is essential for membrane fusion (Braverman and Moser, 2012; Marrink and Mark, 2004; Tresset, 2009)

The unique covalent structure created at the *sn-1* position in plasmenyl-ethanolamines, for example, has been shown to substantially impact the rate of membrane fusion. A comparison between small unilamellar vesicles (SUVs) containing plasmenylethanolamine or phosphatidylethanolamine species via stopped-flow spectrometry demonstrated that the *sn-1* vinyl ether linkage significantly increases calcium dependent membrane fusions by 3-6 fold. In other words, SUVs containing plasmalogens have a higher rate of membrane fusion than vesicles containing the ester-linked counterpart. This result can, in part, explain the predominance of plasmenylethanolamines in membrane compartments undergoing rapid membrane fusion (Glaser and Gross, 1994).

In addition to facilitating membrane fusion events, ether lipids are also structurally important for remote cellular signaling through the maintenance of intercellular junctions. During spermatogenesis in mice, one of the major roles of Sertoli cells (somatic cells present in seminiferous tubules) is to establish the blood-testis barrier between blood vessels and the seminiferous tubules. The blood-testis barrier assembles and disassembles to accommodate the translocation of prophase-stage sperm cells into the epithelium to complete meiosis. Ether lipid deficient (*Gnpat* -/-) mice exhibit downregulation and mistargeting of the transmembrane protein claudin-3 (CLDN3) and impaired blood-testis barrier disassembly, which ultimately results in

deficient spermatogenesis. This emphasizes the importance of ether lipids in the regulation of fundamental biological processes, such as the formation and maintenance of cell-cell junctions in mammalian germ cell development (Komljenovic et al., 2009).

1.1.5 Ether lipids have broad roles in cell differentiation, cellular signaling, and reduction of oxidative stress.

Ether lipids can serve as agonists to enable cell differentiation. For instance, the oxidation of low-density lipoprotein generates an ether lipid byproduct—hexadecyl azelaoyl phosphatidylcholine (azPC)—which contributes to the activation of peroxisome proliferator-activated receptor gamma (PPARγ). Using affinity-binding and PPAR response element (PPRE) luciferase reporter assays, azPC was shown to bind and activate PPARγ to similar degree as rosiglitazone, a well-established agonist for PPAR-induced transcription (Davies et al., 2001). PPARγ possesses a distinct selectivity for the *sn-1* ether linkage, since the diacyl homolog of azPC was not an effective ligand or agonist for PPARγ (Davies et al., 2001). PPARγ is a known transcription factor that controls metabolic and cellular differentiation genes that contain variations of the PPAR-response element (Hernandez-Quiles et al., 2021; Peymani et al., 2016; Wafer et al., 2017). Thus, ether lipids can play an active role in cell differentiation by directly acting as signaling molecules to activate transcription factors and stimulate signal transduction pathways.

Furthermore, ether lipid deficient *Gnpat (-/-)* mice have significantly fewer total *invariant natural killer T* (iNKT) thymocytes and a greater frequency of immature iNKT cells (Facciotti et al., 2012). Antigen responsiveness experiments to detect cytokine release from freshly isolated iNKT cells showed that the synthetic ether-bonded plasmalogen C16-lysophosphatidyl-ethanolamine stimulated the development and maturation of iNKT cells in the thymus. Together, this demonstrates that ether lipids can serve as iNKT cell antigens to stimulate thymic maturation of iNKT cells, and therefore, provides another means by which ether lipids can contribute to cellular differentiation, this time serving as antigens in the immune response (Facciotti et al., 2012).

Phenotypic characterization of mice with GNPAT deletions have also shown that ether lipids impact cell differentiation via their structural effects on membranes by physically impeding the steps needed for signal transduction. *Gnpat* (-/-) mice exhibit impaired spermatogenesis (Komljenovic et al., 2009) and defects in eye development (Rodemer et al., 2003). Ether lipids, as mentioned previously, are important for maintenance of cell-cell junctions in blood-testis barrier formation. A deficiency in ether lipid production therefore results in a structural disruption of sperm cell differentiation (Komljenovic et al., 2009). Similarly, with regards to eye development, detailed histological analysis of ether lipid deficient lens in mice suggests that plasmalogens play a structural role for the maintenance of cell-cell and cell-matrix interactions and anterior lens epithelial cell barrier function in order to maintain postnatal lens growth (Rodemer et al., 2003) Disruption of these cell-cell and cell-matrix interactions due to a lack of

ether lipids exemplifies how defects in maintenance of these structures can lead to defects in development, in this case impaired eye development.

Furthermore, Schwann cells of ether lipid deficient mice display impaired myelination (da Silva et al., 2014; Teigler et al., 2009). During the early postnatal phase of mouse development, a deficiency in plasmalogens impairs Schwann cell recognition of axons and prevents proper sorting of axons intended for myelination. Electron microscopy (EM) of ether lipid deficient Schwann cells show that these unsorted axons caused abnormal myelination and disrupted organization of Schwann cells (da Silva et al., 2014) Therefore, plasmalogens are necessary for correct assembly of myelin and organization of glial cells. These cells exhibited impaired protein kinase B (Akt) activation. Upstream regulators of Akt were not defective in the setting of plasmalogen deficiency, yet Akt was still present in membrane fractions of these mutant cells. Therefore, it is more probable that plasmalogens prevent proper Akt compartmentalization and subsequent activation in lipid raft microdomains (da Silva et al., 2014). The above examples highlight that ether lipids can not only impact cell differentiation and signaling by acting as signaling molecules themselves, but also by facilitating the structural organization of cell signaling proteins within membranes for efficient post-translational modification.

Ether lipids can also impact cellular signaling in a manner that is not directly related to cell development. In the context of eosinophil antiviral and antimicrobial defense mechanisms, eosinophil peroxidases produce reactive brominating species that attack the vinyl ether linkage of plasmalogens, which leads to production of lysophospholipids

and α-bromo fatty aldehydes. This is an example of cellular signaling because the resulting α-bromo fatty aldehydes were demonstrated to serve as phagocyte chemoattractants to signal an eosinophil inflammatory response (Albert et al., 2003). Similarly, activated neutrophils release reactive chlorinating species that target endothelial plasmalogens to generate α-chloro fatty aldehydes that serve as chemottractants to recruit additional neutrophils to sites of active inflammation (Thukkani et al., 2002). In both of these cases, ether lipids act as intermediates in signal transduction that enable the production of signaling molecules to execute a biological response.

With regards to plasmalogens serving as antioxidants, it is widely accepted that plasmalogen deficient cultured cells and *C. elegans* are more sensitive to oxidative damage as compared to their wild-type counterparts, and therefore plasmalogens may function as cellular antioxidants (Engelmann, 2004; Morand et al., 1988; Shi et al., 2016; Wallner and Schmitz, 2011; Zoeller et al., 1988). The first demonstrations of which were the rapid and preferential decomposition of plasmalogens during ultraviolet (UV) irradiation of Chinese hamster ovary (CHO) cells (Morand et al., 1988) and the demonstration that CHO cells deficient in plasmalogen biosynthesis are hypersensitive to photodynamic stress (Zoeller et al., 1988). These independent investigations into the effects of oxidative stress in the setting of plasmalogen deficiency, suggests that plasmalogens have an antioxidant role in membranes. This is further supported by recent demonstrations using biochemical methods and through examination of cell membrane models comparing the phenotypes of diacyl phospholipids and similarly structured ether lipids (Maeba et al., 2002; Reiss et al., 1997; Skaff et al., 2008). For

instance, stopped-flow analysis assessing the rate of reaction of reactive chlorinating and brominating species with plasmalogen models relative to phospholipid model compounds demonstrated that vinyl ether lipids are significantly more susceptible to oxidation relative to their diacyl analogs (Skaff et al., 2008). In addition, large unilamellar vesicles (LUVs) were used to compare effect of having ethanolamine plasmalogens in the bilayers of cholesterol-rich liposomal membranes relative to the impact of phosphatidylethanolamines as the diacyl counterpart control. Essentially, the vinyl ether bonds of ethanolamine plasmalogens were shown to be targeted more readily by reactive oxidative species (ROS) and thwarted the oxidation of nearby PUFAs, SFA, and MUFAs. This targeting of plasmalogens by ROS appears to be less detrimental to membrane integrity as demonstrated by EM, in which plasmalogen deficient bilayers of LUVs collapsed in response to oxidation treatment, whereas the membranes of LUVs that contained ethanolamine plasmalogens remained intact (Maeba et al., 2002). Altogether, this indicates that ether lipids, specifically plasmalogens, function directly as cellular antioxidants.

1.2 ETHER LIPID DEFICIENCY IN *C. ELEGANS* AND HUMANS

1.2.1 Ether lipid levels are significantly altered in common age-related diseases

There is a general understanding that ether lipid deficiency is associated with certain aging-related diseases. In the case of neurological disorders, there are studies comparing plasmalogen levels between control and post mortem brain samples from

Alzheimer's Disease (AD) disease patients and AD mouse models (Ginsberg et al., 1995; Han et al., 2001). AD patients had significantly lower levels of ethanolamine plasmalogens in the mid-temporal lobe cortex of the brain (Ginsberg et al., 1995). Although there was no assessment of a direct genetic or mechanistic cause, the demonstration that no such association was present in the brains of Parkinson's disease negative controls further supported that this phenotype is specific to AD patients (Ginsberg et al., 1995). Additionally, a comparison of plasmalogen content in the grey and white matter of AD human and mice patients via electrospray ionization mass spectrometry (ESI/MS) demonstrated that a deficiency in ethanolamine plasmalogens, specifically in grey matter, becomes more severe in patients as AD progresses (Han et al., 2001).

With regards to metabolic disorders, lipidome profiling of hypertensive male plasmalipidomes showed that hypertension is significantly and specifically associated with the deficiency of PC and PE-containing alkyl ether lipids. The most abundant acid moieties that these ether lipids specifically contained were arachidonic acid (20:4). Therefore, it is possible that deficiency of arachidonic acid-rich ether lipids may contribute significantly to hypertension pathogenesis (Graessler et al., 2009). Altogether, the above data support the notion that misregulation of ether lipid levels is significantly associated with age-related diseases.

1.2.2 Ether lipid levels are not causally linked to aging

Available evidence linking alterations in ether lipid levels to aging and longevity in humans is strictly correlative. An assessment of the plasma lipidome from middle-aged offspring of long-lived individuals revealed that this group collectively had higher levels of PC-ether lipids, specifically PC(O-34:3) and PC(O-36:3), than their spouses (control group) (Gonzalez-Covarrubias et al., 2013). Therefore, these PC-ether lipids are positively associated with familial longevity. While it is not possible to establish causality between alterations in ether lipid levels and aging or aging-related diseases based upon human studies to date, observations such as this imply that a deficiency in PC-ether lipids may be detrimental to health and associated with the aging process. Conversely, having more PC-ether lipids in plasma of humans is indicative of a more youthful lipidomic profile. As previously mentioned, this is concordant with AD patients having lower levels of PC ether lipids, if we classify having AD as a non-youthful state (Gonzalez-Covarrubias et al., 2013; Pradas et al., 2019).

Similarly, mass spectrometry based quantification of >100 ether lipids in healthy humans with exceptional lifespan (centenarians) to define a specific plasma phenotype of ether lipids demonstrates an increase in alkyl-PC ether lipids in centenarians (Pradas et al., 2019). It is worth noting that this study also revealed that a subset of PE-ether lipid species have a positive association with aging, meaning aged individuals displayed notable increases in alkyl-PE species while centenarians have decreased levels of these same ether lipids.

These studies are not able to establish cause versus effect, and further, may not be adequately controlled to justify conclusions drawn. For example, in order to group together aged and adult individuals and collectively compare their plasma ether lipid composition to that of centenarians, there is a strong presumption that the aged/adult subjects are not genetically or environmentally predisposed to being centenarians (Pradas et al., 2019). Thus, mechanistic studies require the ability to dietarily or genetically manipulate ether lipid levels. Given the detrimental effects of ether lipid depletion in humans (White et al., 2003), the use of model organisms, such as *C. elegans,* will be exceptionally important to investigate a potential causal link between ether lipid synthesis and aging.

1.2.3 *C. elegans* as potential models of ether lipid deficiency

Humans with inborn errors of metabolism deficient in the production of ether lipids present with rhizomelic chondrodysplasia punctata (RCDP), a rare autosomal recessive disorder. Children with RCDP are reported to not live past the age of six, due to a collection of severe abnormalities contributing to shorter lifespan, such as craniofacial abnormalities, feeding difficulties, and serious respiratory complications (White et al., 2003).

Loss-of-function mutations of any of these three ether lipid biosynthesis enzymes in worms results in the inability to produce ether-linked lipids, as in humans (Buchert et al., 2014; Drechsler et al., 2016; Honsho et al., 2013; Shi et al., 2016). However, in

contrast to humans, ether lipid deficient nematodes develop normally (see Figure 2.1*A*) (Shi et al., 2016). Although viable, there is evidence for fitness defects in ether lipid deficient *C. elegans*, including reduction in brood size and stress sensitivity (Drechsler et al., 2016; Shi et al., 2016), but the majority of these animals are still able to reproduce, permitting the maintenance of the strain over time. Although shortened lifespan has been previously reported for ether lipid *C. elegans* mutants, our laboratory conditions do not indicate a significant or reproducible impact on lifespan for mutants deficient in ether lipids. However, similar to previous work, these ether lipid deficient mutants still demonstrate a global loss of ether lipid production at the biochemical level via gas chromatography-mass spectrometry (GC-MS) (Shi et al., 2016). In the context of our laboratory conditions, we can move forward acknowledging that the *C. elegans* model for ether lipid deficiency is not extremely ill to the point that lifespan is severely compromised to a similar extent as in children. Therefore, *C. elegans* provide an opportunity to determine the biological roles of ether lipids in aging and longevity without developmental pleiotropies.

1.3 BIGUANIDES IMPACT LONGEVITY IN *C. ELEGANS* THROUGH VARIOUS MECHANISMS OF ACTION

1.3.1 Impact of the oral hypoglycemic metformin on lifespan

Metformin is the first line therapy for type 2 diabetes and the most frequently prescribed oral hypoglycemic medication worldwide (Inzucchi et al., 2012). Remarkably, metformin has been reported to have anti-aging benefits as demonstrated by

prospective studies where diabetic and cardiovascular disease patients treated with metformin exhibited increased rates of survival (Group, 1998; Scarpello, 2003). Furthermore, metformin also extends lifespan in invertebrate and vertebrate models and has been tested as a means of reducing aging-related diseases in humans (Barzilai et al., 2016). Early treatment of spontaneously hypertensive (SHR) female mice, C57BL/6, and B6C3F1 male mice with metformin led to an increase in lifespan when compared with vehicle-treated controls (Anisimov et al., 2011; Martin-Montalvo et al., 2013). Similarly, *C. elegans* treated with metformin also exhibit lifespan extension relative to those treated with vehicle (Cabreiro et al., 2013; Chen et al., 2017; Onken and Driscoll, 2010; Wu et al., 2016). Due to their relatively short lifespan (~2.5-3 weeks) (Zhang et al., 2020), *C. elegans* have been highly advantageous for longevity studies to provide mechanistic insight into metformin's prolongevity benefits.

1.3.2 Genetic effectors of metformin-mediated lifespan extension in nematodes

Although metformin treatment is a well-established form of lifespan extension in *C. elegans,* several cellular mechanisms have been proposed to elucidate how metformin exerts its anti-aging effects. For instance, metformin-mediated lifespan extension in *C. elegans* is suppressed when worms are grown on UV-killed *E. coli* (OP50) (Cabreiro et al., 2013). Pretreatment of *E. coli* with metformin transferred onto drug-free agar plates still results in worm lifespan extension, indicating that inherent changes in bacterial metabolism may impact host lifespan. Further investigation demonstrates that metformin increases lifespan through disruption of microbial folate

and methionine metabolism and subsequent methionine restriction in *C. elegans,* which suggests that metformin recapitulates a dietary restriction (DR)-like state (Cabreiro et al., 2013).

Other studies suggest that metformin is able to extend lifespan via a lysosomal pathway. Genetics knockdown of specific lysosome-related proteins and co-immunoprecipitation in mouse embryonic fibroblast (MEF) cells demonstrate that metformin-mediated activation of AMP-activated protein kinase (AMPK) requires formation of a v-ATPase-Ragulator-AXIN/LKB1-AMPK complex on the surface of lysosomes [vacuolar-ATPase (v-ATPase); axis inhibition protein (AXIN); liver kinase B1 (LKB1)] (Zhang et al., 2016). This study indicates that v-ATPase can act as an effector of metformin in the activation of AMPK. This proposed mechanism was recapitulated in a pure in vitro reconstitution system of purified lysosomes (Chen et al., 2017). Furthermore, genetic analyses in *C. elegans* demonstrated that the lysosome-based v-ATPase-Ragulator-AXIN/LKB1 pathway is necessary for metformin-mediated lifespan extension (Chen et al., 2017).

Furthermore, proteomics analysis of metformin treated *C. elegans* showed that metformin treatment resulted in a significant increase in respiration and metabolic heat production in wildtype worms, which resembles the mitohormetic pathway. Measurements of hydrogen peroxide levels in metformin treated worms revealed that metformin led to an increase in ROS production which contributes to its prolongevity effects. Additionally, peroxiredoxin-2 (PRDX-2), a factor involved in mitohormetic

regulation of lifespan, was discovered to be necessary in the downstream prolongevity response to oxidative stress (De Haes et al., 2014).

Although metformin has been demonstrated to extend lifespan in conventionally used wildtype (N2) strains, the effects of genetic variation in the response to metformin's prolongevity effects has only recently been investigated. The *Caenorhabditis* Intervention Testing Program (CITP) is a collaborative effort conducted by multiple, independent institutions to identify compounds that confer reproducible longevity extension and health benefits in diverse *Caenorhabditis* strains and species. With regards to biguanides, the lifespan extension effect of metformin treatment in genetically diverse populations was assessed using three *Caenorhabditis* species and three strains within *C. elegans.* Metformin increases survival in three *C. elegans strains,* but has no effect or decreases lifespan in *C. briggsae* and *C. tropicalis* strains. Therefore, metformin's prolongevity benefits can extend to diverse genetic backgrounds, however genetic variation can profoundly influence whether metformin positively or negatively impacts lifespan. (Onken et al., 2022).

Furthermore, mid- and late-life metformin treatment in *C. elegans* revealed a negative impact on lifespan (Cabreiro et al., 2013; Espada et al., 2020). Metformin treatment in late-life exacerbated aging-related mitochondrial dysfunction and respiratory failure. *isp-1*(*qm150*) mutants have a mutation in complex III of the mitochondrial electron transport chain (ETC), which contributes to inherent lifespan extension. Metformin treatment of these *isp-1* mutant worms is detrimental for lifespan, indicating that metformin's toxicity is aggravated in the context of impaired mitochondrial

function (Espada et al., 2020). However, this late-life metformin toxicity pathway occurs in an AMPK-independent manner, unlike previously demonstrated forms of metformin-induced lifespan extension, which are dependent upon AMPK (Onken and Driscoll, 2010).

A recent targeted screen by our laboratory for suppressors of metformin effects in *C. elegans* implicated the nuclear pore complex and acyl-coA dehydrogenase family member 10 (ACAD10) as novel factors critical for growth inhibition and lifespan extension by metformin (Wu et al., 2016). The energy sensor AMPK, and the oxidative stress-responsive transcription factor *skn-1*/Nrf2 are not necessary for metformin-induced growth inhibition in *C. elegans*, but are both *required* for the drug's prolongevity effects (Cabreiro et al., 2013; Chen et al., 2017; Onken and Driscoll, 2010; Wu et al., 2016). Therefore, nematodes have previously served as a model to identify conserved targets of biguanides involved in metformin-mediated lifespan extension.

Altogether, these studies emphasize the variety of mechanisms by which metformin impacts longevity in *C. elegans*. As such the biological responses to metformin and the related oral biguanide phenformin (a potent biguanide no longer used in humans due to an increased likelihood of lactic acidosis) in vivo are complex, branching, and incompletely understood.

1.3.3 Prolongevity paradigms and corresponding genetic effectors in *C. elegans*

In addition to biguanide-mediated lifespan extension, there exist *C. elegans* models for various, distinct forms of prolongevity paradigms that emanate from disruption of: 1) mitochondrial function, 2) target of rapamycin complex I (TORC1)

signaling, 3) pharyngeal pumping/feeding, and 4) insulin/insulin-like growth factor 1 (IGF-1) signaling. As mentioned, metformin-induced lifespan extension is dependent upon *aak-2/AMPK* and *skn-1/Nrf-2* (Cabreiro et al., 2013; Chen et al., 2017; Onken and Driscoll, 2010; Wu et al., 2016), and acts independently of the forkhead family transcription factor, *daf-16/FoxO* (Onken and Driscoll, 2010).

With regards to mitochondrial function, various studies have demonstrated that reduced ETC activity results in lifespan extension in *C. elegans*. An example of which involves the gene, *isp-1,* which encodes for the Rieske iron-sulfur protein subunit of complex III of the mitochondrial ETC. Partial loss-of-function mutations, such as *isp-1(qm150),* impede electron transport and promote lifespan extension in *C. elegans* (Feng et al., 2001). Studies using *isp-1* and *aak-2* double mutants indicated that lifespan extension by *isp-1* is dependent upon *aak-2* (Curtis et al., 2006). In addition, genetic knockdown of *daf-16* significantly suppresses lifespan in *isp-1* mutants (Senchuk et al., 2018). *skn-1* has also been demonstrated to be necessary for lifespan extension in *isp-1* worms (Wei and Kenyon, 2016). Therefore, lifespan extension in *isp-1* mutant *C. elegans* requires the activity of *aak-2*, *daf-16*, and *skn-1*.

In the case of TORC1 signaling, TORC1 is a highly conserved serine/threonine kinase that modulates cell growth, proliferation, and survival in response to nutrient availability and growth factors. Pharmacologic or genetic inhibition of TORC1 has been shown to extend lifespan in *C. elegans (Robida-Stubbs et al., 2012; Vellai et al., 2003).* TORC1 impacts *C. elegans* longevity at least in part via the guanosine triphosphate hydrolases (GTPases), *raga-1* and *ragc-1.* For instance, *raga-1(ok701)* deletion mutants

lead to extension of lifespan in *C. elegans* and are dependent upon *skn-1* and *aak-2* (Schreiber et al., 2010; Zhang et al., 2019). Existing data suggests that lifespan extension via inhibition of TORC1 signaling may or may not be dependent upon *daf-16* (Robida-Stubbs et al., 2012; Vellai et al., 2003). With regards to *raga-1* specifically, TORC1-mediated longevity induced by *raga-1* RNAi appears to require *daf-16* (Robida-Stubbs et al., 2012). Therefore, lifespan extension in *raga-1* mutant *C. elegans* is dependent upon *aak-2*, *skn-1*, and possibly *daf-16,* although more evidence is required to establish this.

Dietary restriction (DR) is defined as the restriction of food intake without malnutrition (Masoro, 2005). *C. elegans* exhibit lifespan extension as a physiological response to DR (Greer and Brunet, 2009; Lakowski and Hekimi, 1998). Depending upon the manner in which DR is induced, the pathway by which lifespan extension occurs in *C. elegans* may have conflicting genetic dependencies (Greer and Brunet, 2009). In the context of this study, we focus on utilizing *eat-2(da465)* mutants, which have defects in pharyngeal pumping that lead to insufficient food intake (Lakowski and Hekimi, 1998). These *eat-2* mutants appear to act independently of *daf-16*, and extend lifespan through a mechanism that is distinct from *daf-2(e1370)* (Lakowski and Hekimi, 1998). RNAi knockdown of *skn-1* has been demonstrated to significantly suppress the lifespan of *eat-2(ad465)* mutants (Park et al., 2010). In addition, *eat-2(ad465); aak-2 (ok524)* double mutants still exhibit lifespan extension, similar to *eat-2(ad465)* mutants alone, thereby indicating that *eat-2* mutants (and by analogy, DR) may bypass aak-2 to extend lifespan (Curtis et al., 2006). Therefore, lifespan extension in *eat-2* mutant *C.*

elegans appears to act independently of *daf-16* and *aak-2*, and is likely to be dependent upon *skn-1*.

The insulin/IGF-1 pathway also plays an important role in aging. The *C. elegans* ortholog of the mammalian IGF-1 receptor is known as *daf-2,* which signals through the conserved phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) pathway to promote metabolism, cell survival, proliferation and growth in response to extracellular signals. Through this pathway, *daf-2* ultimately acts as an inhibitor of *daf-16* (Halaschek-Wiener et al., 2005; Lin et al., 1997; Ogg et al., 1997). Reduced insulin/IGF-1 signaling, e.g. *daf-2(e1370),* results in significant lifespan extension in *C. elegans.* This prolongevity paradigm is dependent upon *daf-16*, since reduced *daf-2* function leads to nuclear translocation and activation of DAF-16 to induce expression of genes that promote lifespan extension (Kenyon et al., 1993; Murphy, 2006; Senchuk et al., 2018; Tullet et al., 2008). *daf-2(e1370)* mediated lifespan extension is also dependent upon *aak-2* and *skn-1* (Blackwell et al., 2015; Grushko et al., 2021; Tullet, 2015). Therefore, lifespan extension in *daf-2* mutant *C. elegans* requires proper function of *daf-16*, *aak-2*, and *skn-1*.

Altogether, this illustrates that *C. elegans* can serve as a model for multiple prolongevity paradigms and the accompanying genetic effectors involved can provide insight into the mechanism by which novel paradigms of lifespan extension operate.

1.3.4 **A putative role for ether lipids in metformin's impact on longevity**

Through follow-up longevity studies from an original screen for suppressors of metformin activity (Wu et al., 2016), we identified three members of the ether lipid biosynthesis pathway in *C. elegans* as suppressors of metformin activity. Independent knockout mutations in *fard-1*, *acl-7*, and *ads-1* significantly suppress biguanide-induced lifespan extension, however, as elaborated on above, the importance of ether lipids to aging and longevity is not well established.

Here, we demonstrate that the ether lipid biosynthetic machinery underlies healthy aging in *C. elegans*. Ether lipids are necessary for lifespan extension stimulated by metformin or the related biguanide phenformin. Metabolomic analysis indicates that phenformin treatment leads to changes in multiple ether lipids, primarily those with elongated and desaturated fatty acids in the *sn-2* position. Interestingly, requirement for the ether lipid biosynthetic genes extends to multiple genetic paradigms used to study lifespan including defective mitochondrial electron transport function (*isp-1*), defective pharyngeal pumping/ dietary restriction (*eat-2*), and compromises in TORC1 activation (*raga-1*). Importantly, defects in ether lipid synthesis do not impact lifespan extension caused by a loss of function in the IGF-1 receptor homolog, *daf-2*, indicating that ether lipid deficiency does not simply forbid lifespan extension of any kind because of non-specific sickness. Finally, overexpressing *fard-1*, the enzyme that supplies all the fatty alcohols for ether lipid biogenesis in *C. elegans*, extends lifespan, suggesting that an increase in ether lipids alone is sufficient to promote healthy aging.

CHAPTER 2: ETHER LIPID BIOSYNTHESIS PROMOTES LIFESPAN EXTENSION AND ENABLES DIVERSE PROLONGEVITY PARADIGMS

2.1 SUMMARY

Metformin is the most widely used drug to treat patients with type 2 diabetes and extends lifespan in multiple species, yet the molecular targets responsible for these prolongevity benefits remain incompletely understood. To this end, we demonstrate that a subset of lipids, known as ether lipids, are necessary for biguanides' anti-aging effects and various other paradigms of lifespan extension. Strikingly, overexpression of FAR1, the enzyme responsible for fatty alcohol production in an initial step of ether lipid biosynthesis, is sufficient to stimulate lifespan extension and illuminates the prospect of leveraging these lipids as therapeutic targets to promote healthy aging.

2.2 RESULTS

2.2.1 Genes responsible for ether lipid biosynthesis are necessary for biguanide-induced lifespan extension.

From a previous RNAi screen of ~1000 metabolic genes for knockdowns that interfere with the growth-inhibitory properties of metformin in *C. elegans* (Wu et al., 2016), we identified several genes required for ether lipid biosynthesis. Ether lipids are distinguished from canonical phospholipids as the latter contain exclusively fatty acids conjugated to glycerol, whereas ether lipids contain a fatty alcohol conjugated to the glycerol backbone at the sn-1 position via an ether linkage (Figures 1.1 and 2.1 *A*). Confirming our screen results, RNAi knockdown of two genes involved in ether lipid biosynthesis, *fard-1* and *acl-7*, results in significant resistance to biguanide induced

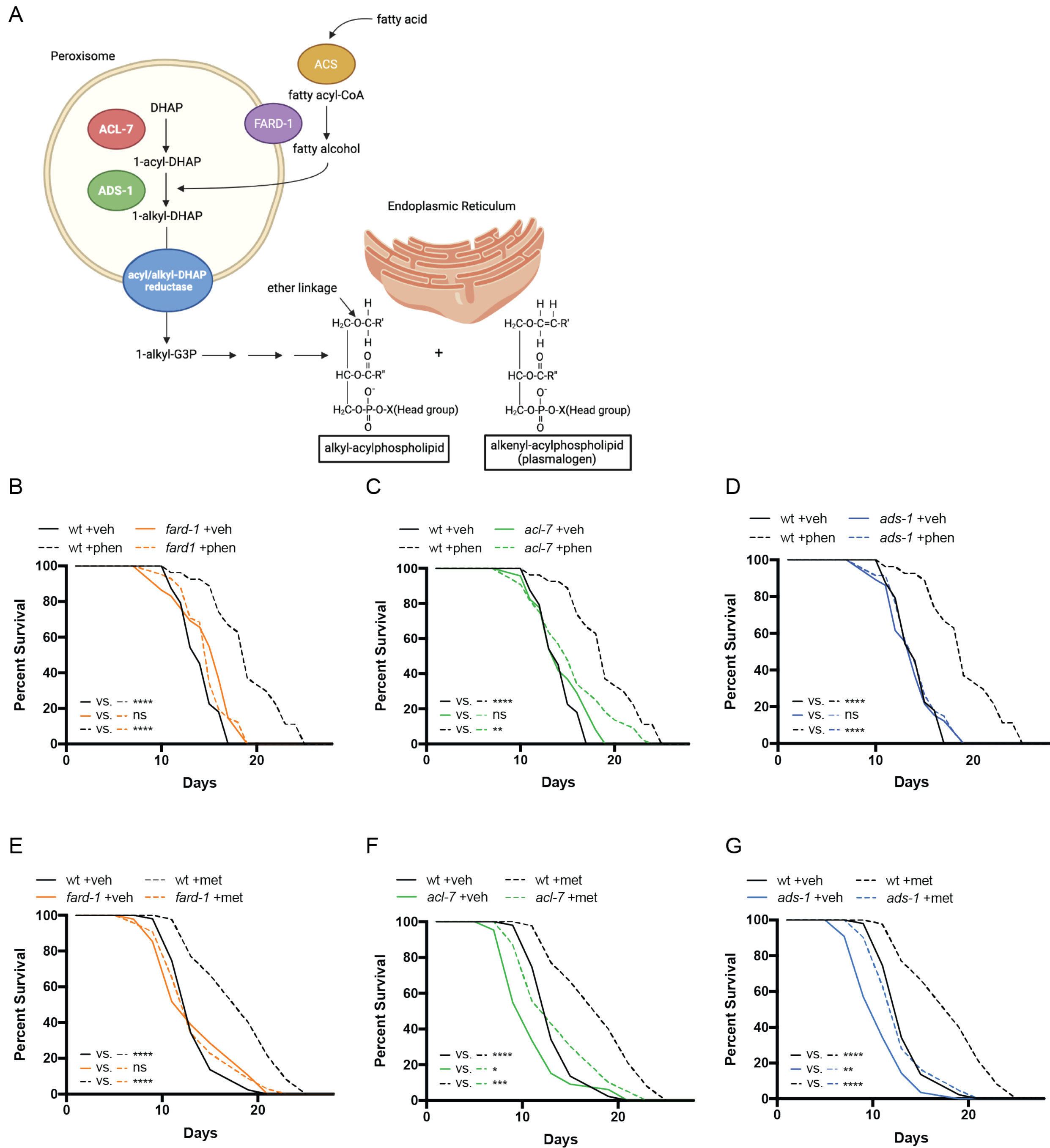
growth inhibition (Figure 2.2 *A*). Our lab has previously demonstrated that biguanide effects on growth in *C. elegans* share significant overlap mechanistically with the machinery by which metformin extends lifespan in the worm (Wu et al., 2016). Indeed, loss-of-function mutations in any of three genes encoding enzymes required for ether lipid biosynthesis, *fard-1*, *acl-7* or *ads-1*, significantly suppresses lifespan extension induced by both metformin and the related biguanide phenformin (Figure 2.1 *B*-*G*, Dataset 2.1 for tabular survival statistics and biological replicates). Confirming that these mutations confer resistance to metformin by compromising ether lipid synthetic capacity, RNAi knockdowns of *fard-1* and *acl-7* also partially impair lifespan extension promoted by phenformin (Figure 2.2 *B* and *C*). Studies from this point forward are presented predominantly with phenformin because 1) phenformin is known to be more readily absorbed without need for a specific transporter, unlike metformin, (Segal et al., 2011; Sogame et al., 2009; Wu et al., 2016) and 2) our experience indicates more consistent lifespan extension with phenformin in *C. elegans*.

Because ether lipids are a major structural component of cell membranes, one possibility is that deficiencies in ether lipid synthesis compromises drug action by reducing biguanide bioavailability in the worm. To test this, we compared the relative levels of biguanides present in vehicle- and biguanide-treated wild type to the three ether lipid synthesis mutants by liquid chromatography-tandem mass spectrometry (LC-MS/MS). A comparison of normalized concentrations of phenformin across all four strains shows that phenformin abundance is significantly higher in phenformin-treated samples relative to the corresponding vehicle-treated controls, and that phenformin levels are similar across

wild type and all three ether lipid mutant strains (Figure 2.1*H* and Figure 2.2*D*). Similar results were obtained when comparing levels of metformin wild type and ether lipid mutant animals (Figure 2.1*I* and Figure 2.2*E*). Thus, deficiency in ether lipid synthesis does not significantly impact uptake or clearance of biguanides in *C. elegans.*

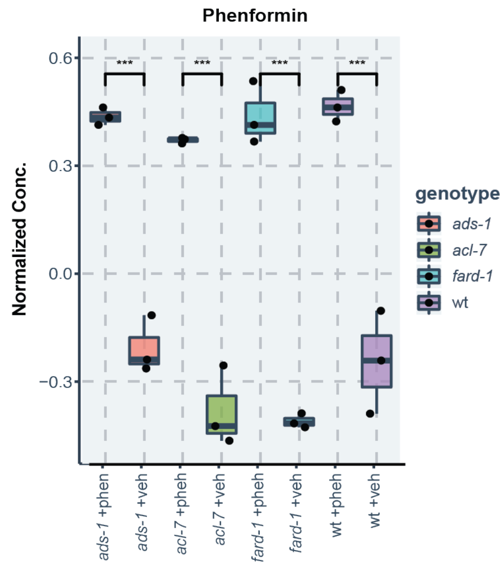
Figure 2.1. Genes responsible for ether lipid biosynthesis are necessary for biguanide-induced lifespan extension. Ether lipid synthesis is catalyzed by three enzymes: fatty acyl reductase FARD-1, acyltransferase ACL-7 and alkylglycerone phosphate synthase ADS-1 (*A*). The latter two are localized to the peroxisomal lumen. *Missense* mutations in *fard-1* (*B*), acl-7 (*C*), and *ads-1* (*D*) in *C. elegans* suppress phenformin-induced lifespan extension. A deficiency of ether lipid synthesis in *fard-1* (*E*), *acl-7* (*F*), and *ads-1* (*G*) worm mutants blunts metformin-induced lifespan extension. Results are representative of 3 biological replicates. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ by log-rank analysis. See also Figure 2.2 and refer to Dataset 2.1 for tabular survival data and biological replicates. Normalized concentrations of phenformin (*H*) and metformin (*I*) in vehicle, 4.5mM phenformin, or 50 mM metformin treated wild type *C. elegans* versus *fard-1*, *acl-7*, and *ads-1* mutants. n= 3 biological replicates; ***, $P < 0.001$ by two-tailed students *t*-test. Box represents $75^{th}/25^{th}$ percentiles, while whisker represents higher/lower hinge +/- [1.5 * interquartile range (IQR)].

(Figure 2.1. continued)

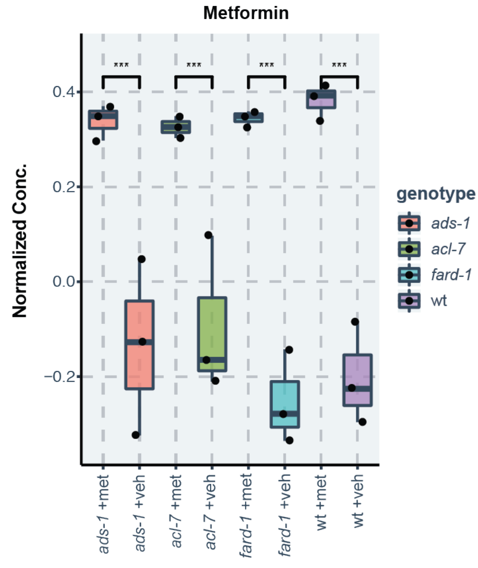


(Figure 2.1. continued)

H



I



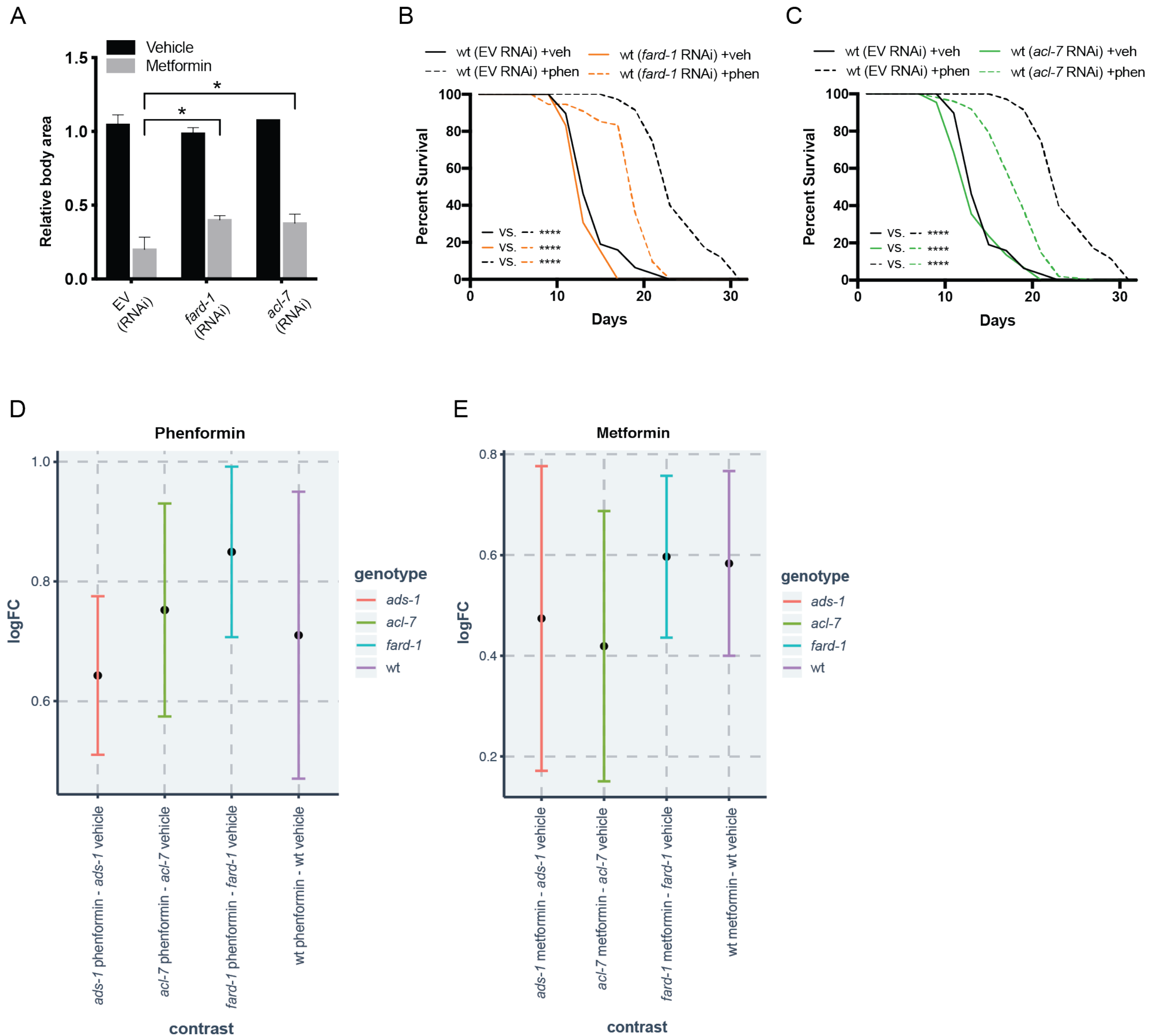


Figure 2.2. RNAi knockdown-mediated reduced function of genes responsible for ether lipid biosynthesis partially suppresses biguanide effects of growth and lifespan without affecting biguanide levels. RNAi to *fard-1* and *acl-7* induce *C. elegans* resistance to growth inhibition by 160 mM metformin treatment (*A*). *p < 0.05, by two-way ANOVA, 2 biological replicates. RNAi knockdown of *fard-1* (*B*) and *acl-7* (*C*) in *C. elegans* partially suppresses phenformin's effect on lifespan extension. For *B* and *C,* results are representative of 3 biological replicates. ****, $P < 0.0001$ by log-rank analysis; for tabular survival data and biological replicates see also Dataset 2.1. Log

(Figure 2.2 continued)

fold change (LogFC) of phenformin abundance in samples treated with 4.5 mM phenformin versus vehicle reveals that the increase in phenformin levels in wild type and 3 ether lipid deficient mutants is similar (*D*). LogFC of metformin abundance in samples treated with 50 mM metformin versus vehicle show that metformin increases are similar across all 4 strains (*E*). Bars represent mean and 95% confidence intervals.

2.2.2 Phenformin induces changes in ether lipid metabolite levels

We reasoned that if biguanides require ether lipid biosynthesis to promote lifespan extension that phenformin may promote changes in abundance of one or several ether lipids. To investigate the impact of biguanides on ether lipids at a high level, we first utilized GC-MS analysis. GC-MS recapitulates the observation that *fard-1* mutants show absence of 18-carbon containing plasmalogens and an accumulation of stearic acid (18:0) relative to wild type controls (Figure 2.3 *A* and *B*). We then asked if phenformin impacts the levels of stearic acid and 18-carbon alkenyl ether lipids in wild type animals and if those corresponding changes are absent in *fard-1* mutants. Strikingly, phenformin-treated wildtype worms display a significant increase in 18-carbon plasmalogens versus vehicle, whereas no such increase is evident in drug treated *fard-1* worms (Figure 2.3 *C*). In addition, relative proportions of stearic acid levels within the total fatty acid pool are significantly increased in *fard-1* mutants treated with phenformin versus vehicle treated *fard-1* controls. In comparison, the relative proportion of stearic acid does not rise in phenformin treated wild type animals,

suggesting that stearate is being utilized for ether lipid production (Figure 2.3 *D*). We conclude that phenformin treatment leads to an overall increase of alkenyl-type ether lipid levels in *C. elegans*.

To investigate changes in relative abundance across the entire landscape of alkyl and alkenyl ether lipids in response to phenformin, we utilized LC-MS/MS lipidomics. This method detected 270 alkyl and alkenyl ether lipids, from those reported previously to be abundant versus rare (Figure 2.3 *E*, and Dataset 2.2) (Drechsler et al., 2016). After filtering for ether lipids significantly altered in abundance in response to phenformin, we implemented a spectral clustering model to visualize the difference of the average normalized value in wild type as compared to the three ether lipid mutants *fard-1*, *acl-7*, and *ads-1*. Two clusters of ether lipids have a significant change in metabolite concentrations in wild type worms but not *fard-1*, *acl-7*, and *ads-1* mutants treated with phenformin compared to vehicle (Figure 2.3 *E* and Table 2.1, clusters 1 and 3). As an orthogonal strategy, we utilized a linear model to identify ether lipids with a significant change in abundance between vehicle and biguanide treatment in wild type animals, without a corresponding change in the ether lipid deficient mutants (Figure 2.3*F*). These two independent models 1) had strong overlap among ether lipids increased in response to phenformin (of 14 ether lipids from cluster 1 spectral clustering (Figure 2.3*E*) and 13 increased from the linear model (Figure 2.3*F*), 12 are overlapping, Table 2.1) and 2) indicate an ether lipid metabolite signature of phenformin treatment that is prevented in ether lipid biosynthetic mutants.

Three important features are evident from analysis of the lipidomics data. First, specific ether lipids containing non-oxidized fatty alcohols that significantly increase and decrease in response to phenformin treatment, indicating that biguanides do not simply increase abundance of all alkyl or alkenyl ether lipid species (Figure 2.3*F* and Dataset 2.2). Second, 11 of the 15 ether lipids increased in abundance from both analyses are alkenyl-type (plasmalogen) ether lipids, indicating that the predominant governance of ether lipid abundance by biguanides may be on those lipids containing an ether bond to an alkenyl group in the *sn-1* position. This data agrees with our GC/MS data (Figure 2.3*C*) indicating an increase in plasmalogen-type ether lipids in response to phenformin. Third, examination of the molecular identities of the ether lipids increased in response to phenformin indicates that 9 of 15 harbor elongated, polyunsaturated fatty acids in the *sn-2* position, suggesting a potential role for the enzymes implicated in the biogenesis of these fatty acids in metformin action.

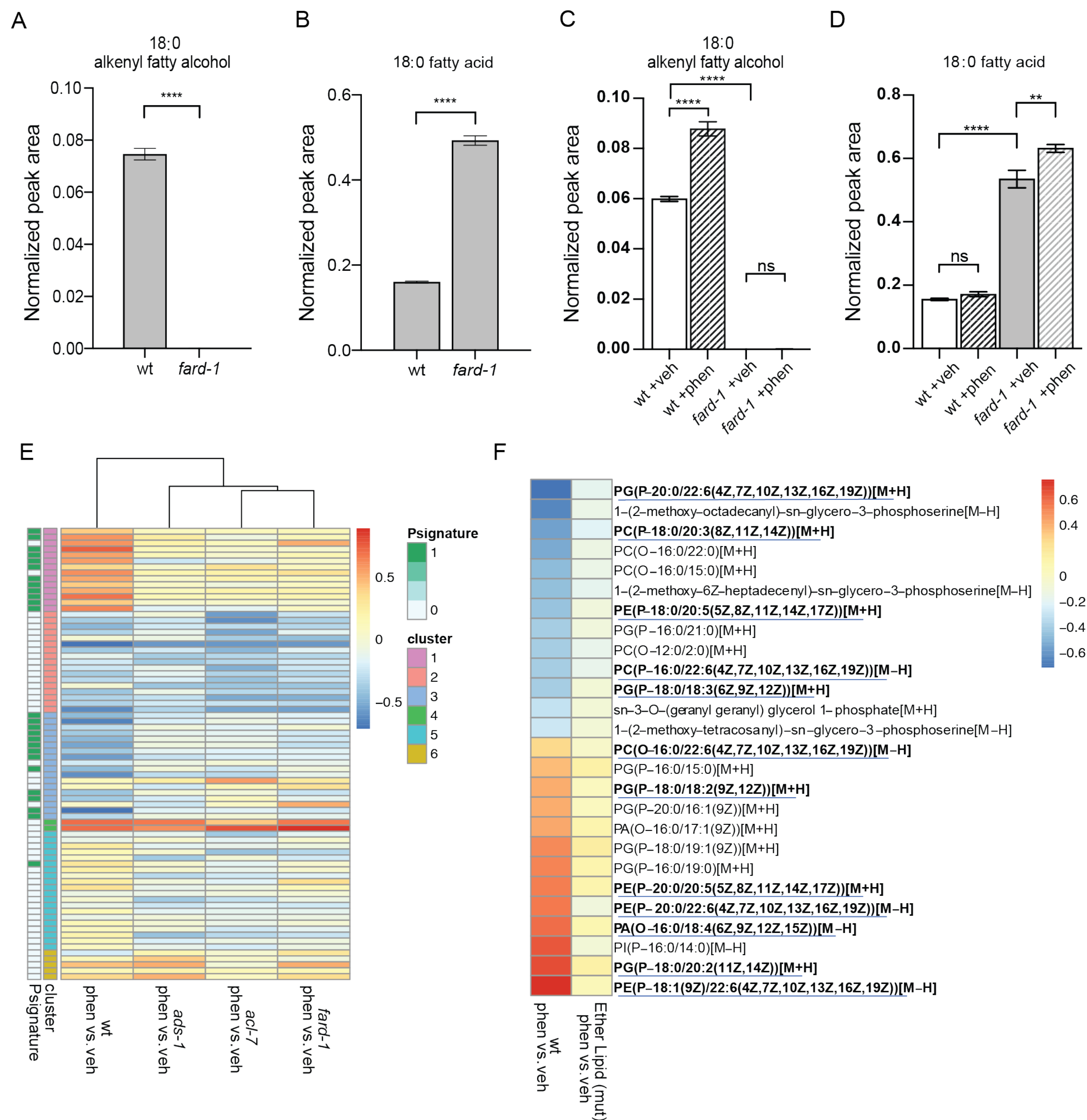


Figure 2.3. Phenformin induces an ether lipid metabolite signature that is blunted in ether lipid biosynthesis mutants. Loss-of-function *fard-1* mutants have significant reduction in 18:0 fatty alcohol derivatized from 18-carbon containing alkenyl ether lipids (also known as plasmalogens) by GC/MS (*A*) and corresponding accumulation of the fatty acid stearate (18:0, *B*). Wild type worms treated with 4.5 mM phenformin display a

(Figure 2.3 continued)

significant increase in 18:0 fatty alcohol derivatized from alkenyl ether lipids relative to vehicle control, with levels remaining essentially undetectable in *fard-1* mutants on vehicle or drug (*C*). Phenformin 4.5mM treatment does not impact stearate levels in wild type worms, however it does result in a greater accumulation of stearate in *fard-1* mutants (*D*). For *A-D*, **, $P < 0.01$; ****, $P < 0.0001$, by two-way ANOVA, n = 3 biological replicates. Cluster analysis of ether lipids significantly altered in response to phenformin (*E*). Cluster 1 denotes a set of ether lipids that increased significantly in phenformin treated wild type worms but are unaffected in ether lipid mutants *fard-1*, *acl-7* and *ads-1*. Cluster 3 represents a set of ether lipids that decreased significantly in phenformin treated wild type worms but unaffected in ether lipid mutants. n=3 biological replicates; adj. *P*-value < 0.05. See Dataset 2.2 and Table 2.1 for raw mass spectrometry data and identity of metabolites in clusters, respectively. Heat map of 26 ether lipids identified by linear modeling with significant fold change in abundance between vehicle and 4.5mM phenformin treatment in the wildtype background that do not show significant differences between vehicle and drug treated ether lipid deficient mutant (*F*). 10 of 13 ether lipids increased in abundance are alkenyl-type (plasmalogen, indicated by “P” in their name prior to the fatty alcohol designation) ether lipids, versus alkyl-type (indicated by “O” in their name prior to fatty alcohol designation). Twelve ether lipids (bold and underlined) harbor very long chain polyunsaturated fatty acids at the *sn-2* position. n=3 biological replicates; adj. $P<0.05$. P PG, phosphatidyl glycerol; PE phosphatidyl ethanolamine; PC phosphatidyl choline; PI, phosphatidyl inositol; PA, phosphatidic acid.

Table 2.1. Molecular identity and spectral cluster number of ether lipid metabolites significantly altered in abundance in response to phenformin versus vehicle in wild type animals and ether lipid mutants.

Compound Molecular Identity	Cluster
PA(O-16:0/17:1(9Z))[M+H]	1
PA(O-16:0/18:4(6Z,9Z,12Z,15Z))[M-H]	1
PA(O-18:0/18:4(6Z,9Z,12Z,15Z))[M-H]	1
PE(P-18:1(9Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))[M-H]	1
PE(P-20:0/20:5(5Z,8Z,11Z,14Z,17Z))[M+H]	1
PE(P-20:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))[M-H]	1
PG(P-16:0/15:0)[M+H]	1
PG(P-16:0/18:4(6Z,9Z,12Z,15Z))[M+H]	1
PG(P-16:0/19:0)[M+H]	1
PG(P-18:0/18:2(9Z,12Z))[M+H]	1
PG(P-18:0/19:1(9Z))[M+H]	1
PG(P-18:0/20:2(11Z,14Z))[M+H]	1
PG(P-20:0/16:1(9Z))[M+H]	1
PI(P-16:0/14:0)[M-H]	1
1-(2-methoxy-13-methyl-6Z-tetradecenyl)-sn-glycero-3-phosphoethanolamine[M+H]	2
1-(2-methoxy-6Z-pentadecenyl)-sn-glycero-3-phosphoethanolamine[M+H]	2
1-(2-methoxy-6Z-tetradecenyl)-sn-glycero-3-phosphoethanolamine[M+H]	2
1-(2-methoxy-pentadecanyl)-sn-glycero-3-phosphoserine[M-H]	2
1-O-(2-methoxy-4Z-hexadecenyl)-sn-glycero-3-phosphocholine[M+H]	2
Int_Std_Glutamic_Acid[M-H]	2
PA(O-20:0/22:1(11Z))[M+H]	2
PC(O-15:0/2:0)[M-H]	2
PG(O-18:0/17:2(9Z,12Z))[M-H][M-H]	2
PG(O-20:0/21:0)[M+H]	2
PG(P-18:0/20:3(8Z,11Z,14Z))[M+H]	2
PG(P-18:0/20:5(5Z,8Z,11Z,14Z,17Z))[M+H]	2
PG(P-18:0/21:0)[M-H]	2
PG(P-20:0/18:2(9Z,12Z))[M+H]	2
PG(P-20:0/18:3(6Z,9Z,12Z))[M+H]	2
PI(P-16:0/0:0)[M-H]	2
PS(P-18:0/18:4(6Z,9Z,12Z,15Z))[M-H]	2
1-(2-methoxy-6Z-heptadecenyl)-sn-glycero-3-phosphoserine[M-H]	3
1-(2-methoxy-octadecanyl)-sn-glycero-3-phosphoserine[M-H]	3
1-(2-methoxy-tetracosanyl)-sn-glycero-3-phosphoserine[M-H]	3
PC(O-12:0/2:0)[M+H]	3

(Table 2.1 continued)

Compound Molecular Identity	Cluster
PC(O-16:0/15:0)[M+H]	3
PC(O-16:0/22:0)[M+H]	3
PC(P-16:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))[M-H]	3
PC(P-18:0/20:3(8Z,11Z,14Z))[M+H]	3
PC(P-20:0/20:5(5Z,8Z,11Z,14Z,17Z))[M-H]	3
PE(P-18:0/20:5(5Z,8Z,11Z,14Z,17Z))[M+H]	3
PE(P-20:0/20:3(8Z,11Z,14Z))[M-H]	3
PG(O-16:0/21:0)[M+H]	3
PG(P-16:0/16:0)[M+H]	3
PG(P-16:0/21:0)[M+H]	3
PG(P-18:0/18:3(6Z,9Z,12Z))[M+H]	3
PG(P-18:0/22:4(7Z,10Z,13Z,16Z))[M+H]	3
PG(P-20:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))[M+H]	3
sn-3-O-(geranylgeranyl)glycerol 1-phosphate[M+H]	3
PC(P-18:0/20:3(8Z,11Z,14Z))[M-H]	4
Phenformin[M+H]	4
1-(2-methoxy-5Z-hexadecenyl)-sn-glycero-3-phosphoethanolamine[M+H]	5
1-(2-methoxy-tetradecanyl)-sn-glycero-3-phosphoserine[M+H]	5
Metformin[M+H]	5
PA(O-16:0/17:2(9Z,12Z))[M+H]	5
PC(O-14:0/22:0)[M+H]	5
PC(O-16:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))[M-H]	5
PC(O-17:0/2:0)[M+H]	5
PC(P-18:0/20:5(5Z,8Z,11Z,14Z,17Z))[M+H]	5
PC(P-18:0/20:5(5Z,8Z,11Z,14Z,17Z))[M-H]	5
PE(P-16:0/19:1(9Z))[M+H]	5
PG(O-16:0/18:3(9Z,12Z,15Z))[M-H][M-H]	5
PG(O-16:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))[M+H]	5
PG(O-20:0/20:4(5Z,8Z,11Z,14Z))[M+H]	5
PG(P-16:0/22:0)[M+H]	5
PG(P-16:0/22:1(11Z))[M+H]	5
PG(P-16:0/22:4(7Z,10Z,13Z,16Z))[M+H]	5
PG(P-18:0/20:5(5Z,8Z,11Z,14Z,17Z))[M-H]	5
PG(P-20:0/14:1(9Z))[M+H]	5
PG(P-20:0/17:1(9Z))[M+H]	5
PS(P-18:0/22:0)[M-H]	5
1-O-(2R-hydroxy-pentadecyl)-sn-glycerol[M+H]	6
PE(P-18:0/18:1(9Z))[M+H]	6
PG(P-16:0/19:1(9Z))[M+H]	6
PG(P-16:0/22:2(13Z,16Z))[M+H]	6
PS(O-20:0/20:0)[M-H][M-H]	6

2.2.3 Phenformin stimulates peroxisomal localization of FARD-1.

In order to begin to understand the governance of ether lipid biosynthesis by biguanides, we generated *C. elegans* expressing a FARD-1::RFP translational reporter from an extrachromosomal array under its own promoter (Figure 2.4 *A*). Imaging of these worms reveals that FARD-1 is expressed in intestine and appears to localize nearby structures resembling lipid droplets (Figure 2.4 *B*). Given that ether lipid biogenesis is reported to occur in peroxisomes, we crossed the FARD-1::RFP reporter to an animal bearing a GFP reporter that illuminates peroxisomes in intestine (GFP fused to a C-terminal peroxisomal targeting sequence 1(PTS1)). FARD-1::RFP fluorescence partially overlaps with peroxisomally-targeted GFP in intestine (Figure 2.4 *C*). Compellingly, treatment with phenformin leads to an increase in overlap between FARD-1::RFP and GFP::PTS1 (Pearson's R value=0.84) relative to vehicle treated controls (Pearson's R value=0.73) (Figure 2.4 *C*). The same trend is present in another biological replicate where the Pearson's correlation value for the overlap between GFP and RFP in the phenformin treated transgenic is R= 0.57 versus R= 0.39 in vehicle. These results suggest that biguanides may govern ether lipid biosynthesis by recruiting greater amounts of FARD-1 to peroxisomes.

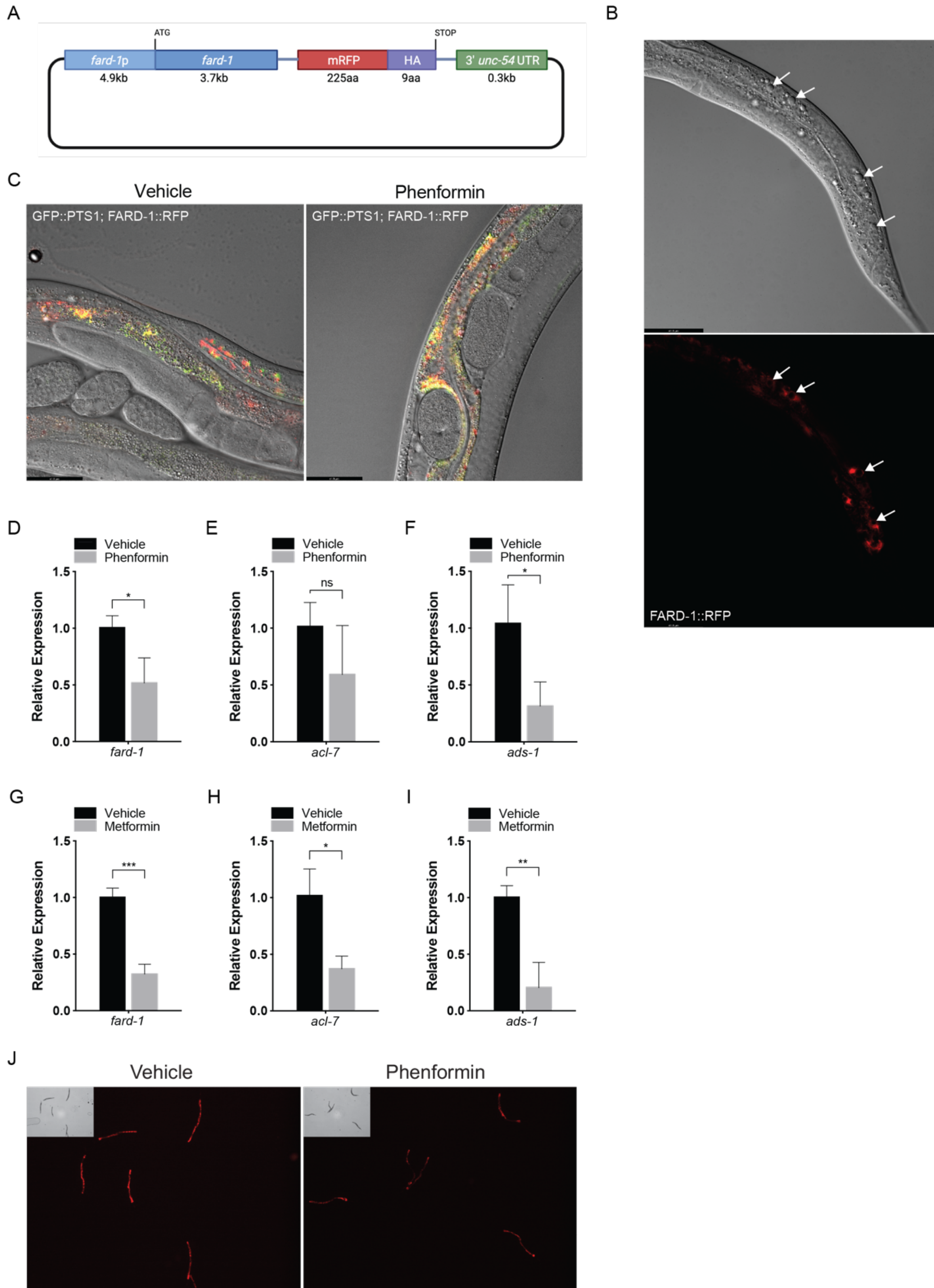
Curiously, expression of mRNAs encoding FARD-1, ACL-7, and ADS-1 are all decreased or unchanged in abundance upon treatment with biguanide via quantitative real time RT-PCR (Figure 2.4 *D-I*). A parallel decrease in overall levels of the FARD-1::RFP transgene was seen with phenformin treatment (Figure 2.4*J*). These seemingly

paradoxical data are consistent with the possibility that biguanides increase activity of ether lipid biosynthetic pathways post-translationally given that ether lipids have been previously reported to exert negative feedback on protein levels of FARD-1 (Honsho et al., 2010).

We surmised that if ether lipid synthesis is occurring principally in the peroxisome and that peroxisomal localization of FARD-1 was required for the prolongevity response to biguanides, that disruption of peroxisomal protein targeting should also impair phenformin-stimulated lifespan extension. Indeed, either *prx-5* or *prx-19* RNAi impair lifespan extension prompted by phenformin fully or partially, respectively (Figure 2.5*A* and *B*). PRX-5 is involved in protein import into the peroxisome matrix and PRX-19 functions as a chaperone and import receptor for peroxisomal membrane proteins; both of which are critical for peroxisomal biogenesis and function. Thus, either disruption of ether lipid biosynthetic machinery or of a principal site of ether lipid biosynthesis impairs phenformin's prolongevity benefit.

Figure 2.4. Phenformin impacts the spatial expression of intestinally expressed FARD-1. Schematic representation of the *C. elegans* FARD-1::RFP overexpression construct and translational reporter (*A*). FARD-1::RFP exhibits intestinal expression in *C. elegans* (*B*). FARD-1 displays a cytoplasmic distribution and an association with structures resembling lipid droplets (*B, arrows*). Co-expression of FARD-1::RFP and peroxisomally targeted GFP::PTS1 in transgenic animals indicates partial colocalization of FARD-1 with peroxisomes in intestine (*C*). Treatment of a FARD-1::RFP and GFP::PTS1 transgenic with 4.5mM phenformin leads to an increase in colocalization of RFP and GFP (Pearson's R value=0.84) in the intestine relative to vehicle (Pearson's R value=0.73) (*C*), representative of two biological replicates. Levels of *fard-1*, *acl-7*, and *ads-1* mRNA decrease in *C. elegans* treated with 4.5 mM phenformin versus vehicle. n = 3 biological replicates; ns, not significant; *, $P < 0.05$ by unpaired *t*-test (*D-F*). Levels of *fard-1*, *acl-7,* and *ads-1* mRNA decrease in *C. elegans* treated with 50 mM metformin versus vehicle. n = 3 biological replicates; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by unpaired *t*-test (*G-I*). Phenformin (4.5 mM) results in decreased expression of the FARD-1::RFP translational reporter. n = 3 biological replicates; total assessed: N = 1200 worms per condition (*J*).

(Figure 2.4. continued)



2.2.4 Functional genomics implicates fatty acid elongases and desaturases in biguanide-mediated lifespan extension.

Analysis of specific ether lipids significantly altered in abundance in response to phenformin (Figure 2.3*F*) indicated 13 ether lipids with long and/or polyunsaturated fatty acids at the *sn-2* position. These types of fatty acids are largely synthesized endogenously in *C. elegans* by fatty acid desaturases and fatty acid elongases (Perez and Van Gilst, 2008; Perez and Watts, 2021) (Figure 2.5 *C*). We reasoned that these desaturases and elongases may also contribute mechanistically to biguanide mediated lifespan extension. Indeed, RNAi knockdown of three fatty acid desaturases and two fatty acid elongases in phenformin-treated *C. elegans* blunted phenformin-stimulated lifespan extension relative to empty vector controls (Figure 2.5 *D*-*H*). Notably, these five genes all contribute to the production of fatty acids 18-20 carbons in length with three or more double bonds. Although knockdown of fatty acid desaturases and elongases in *C. elegans* results in inherent lifespan extension on vehicle relative to wild type controls on empty vector RNAi as has been previously reported (Horikawa et al., 2008; Shmookler Reis et al., 2011) these RNAi knockdown completely mitigate phenformin-driven lifespan extension (Figure 2.5 *D*-*H*). These results suggest that fatty acid desaturases and elongases promote biguanide-mediated lifespan extension through contribution of long and polyunsaturated fatty acids to the synthesis of ether lipids.

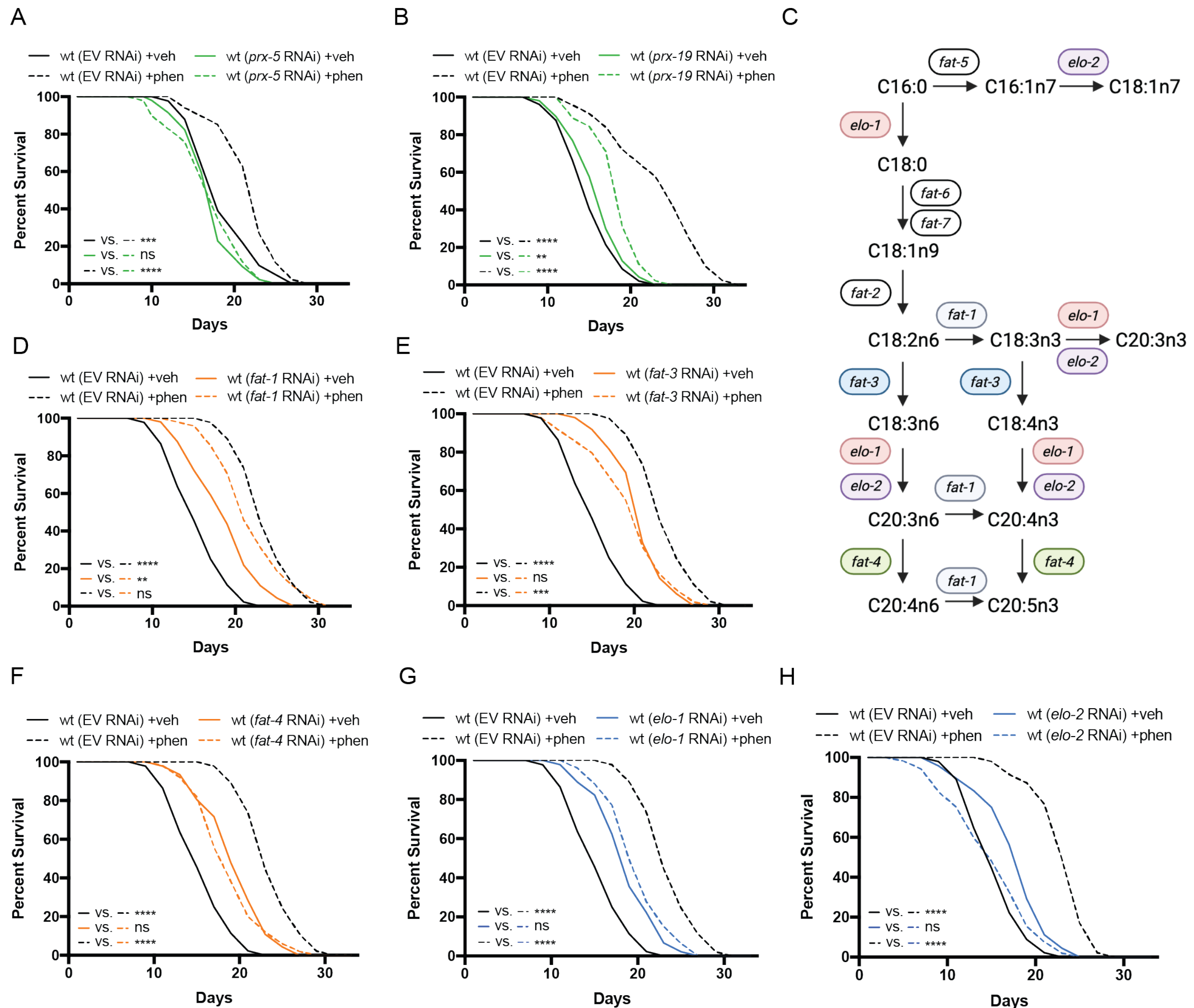


Figure 2.5. Peroxisomal biogenesis factors, fatty acid elongases, and fatty acid desaturases are required for the prolongevity effects of biguanides. Knockdown of *prx-5* (*A*) and *prx-19* (*B*) by RNAi significantly suppresses phenformin-mediated lifespan extension. Schematic representation of the polyunsaturated fatty acid (PUFA) synthesis pathway in *C. elegans* (*C*), adapted from (Watts, 2016). RNAi of three fatty acid desaturases (*D-F*) and two fatty acid elongases (*G* and *H*) involved in the synthesis of 18- and 20-carbon polyunsaturated fatty acids blunt phenformin-mediated lifespan extension in wild type worms. For *A, B* and *D-H,* results are representative of 2-3 biological

(Figure 2.5 continued)

replicates. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ by log-rank analysis; see also Dataset 2.1 for tabular survival data and biological replicates.

2.2.5 Genes involved in ether lipid biosynthesis are required in multiple longevity paradigms.

Given the critical role of ether lipids in the response to biguanides, we next endeavored to determine whether these molecules also play a broader role in diverse longevity paradigms involving metabolic or nutrient-sensing pathways. Given mechanistic parallels between biguanide-mediated lifespan extension and several other longevity paradigms, we chose to analyze *C. elegans* mutant strains that exhibit lifespan extension in response to 1) reduced mitochondrial function (*isp-1*), 2) disrupted mTORC1 signaling (*raga-1*), 3) abnormal pharyngeal pumping (*eat-2*), or 4) inhibition of insulin/insulin-like growth factor-1 signaling (*daf-2*) (Apfeld et al., 2004; Curtis et al., 2006; Schreiber et al., 2010; Senchuk et al., 2018). To determine whether requirement for the ether lipid biosynthetic machinery in aging generalizes to other lifespan extension paradigms, we knocked down each of the three ether lipid biosynthetic enzymes by RNAi in wild type *C. elegans* and four long-lived genetic mutants: *raga-1*, *isp-1*, *eat-2*, and *daf-2*. Knockdown of *fard-1*, *acl-7*, and *ads-1* by RNAi results in suppression of lifespan extension in *isp-1*, *raga-1*, and *eat-2* mutants (Figure 2.6 *A*-*C*). However, knockdown of ether lipid synthesis genes by RNAi did not impact lifespan

extension in *daf-2* mutants (Figure 2.6 *D*). Thus, the ether lipid biosynthetic machinery plays a broad role in lifespan extension. Importantly, *daf-2* mutants still promote lifespan extension in the setting ether lipid deficiency, which indicates that the absence of ether lipid production is not simply a cause of non-selective sickness that renders worms invincible to any form of lifespan extension.

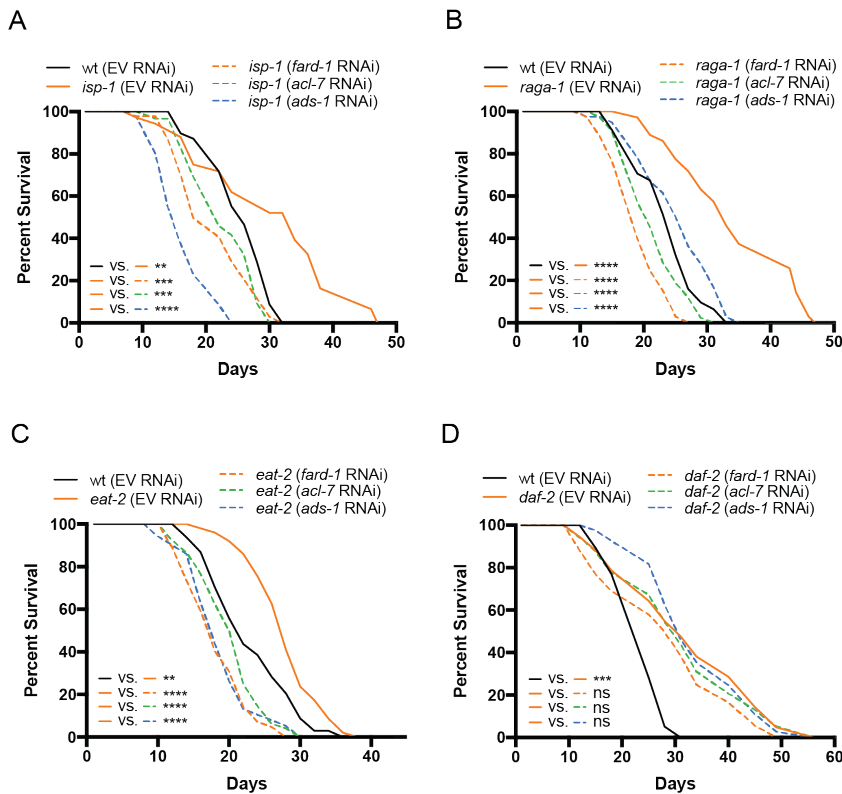


Figure 2.6. Genes involved in ether lipid biosynthesis are required in multiple longevity paradigms, but are not necessary for *daf-2*-dependent lifespan extension. *isp-1*, *raga-1*, and *eat-2* mutants display extended lifespan relative to wild type animals that is suppressed by knockdown of any of the three members of the ether

(Figure 2.6 continued)

lipid biosynthetic pathway (*A*-*C*). *daf-2* mutants display extended lifespan relative to wildtype animals (*D*). RNAi knockdown of *fard-1*, *acl-7*, and *ads-1* does not impact lifespan extension in these mutants. For *A*-*D,* results are representative of 2-3 biological replicates. ns, not significant; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ by log-rank analysis. For tabular survival data and biological replicates see also Dataset 2.1.

2.2.6 Overexpression of FARD-1 is sufficient to promote lifespan extension

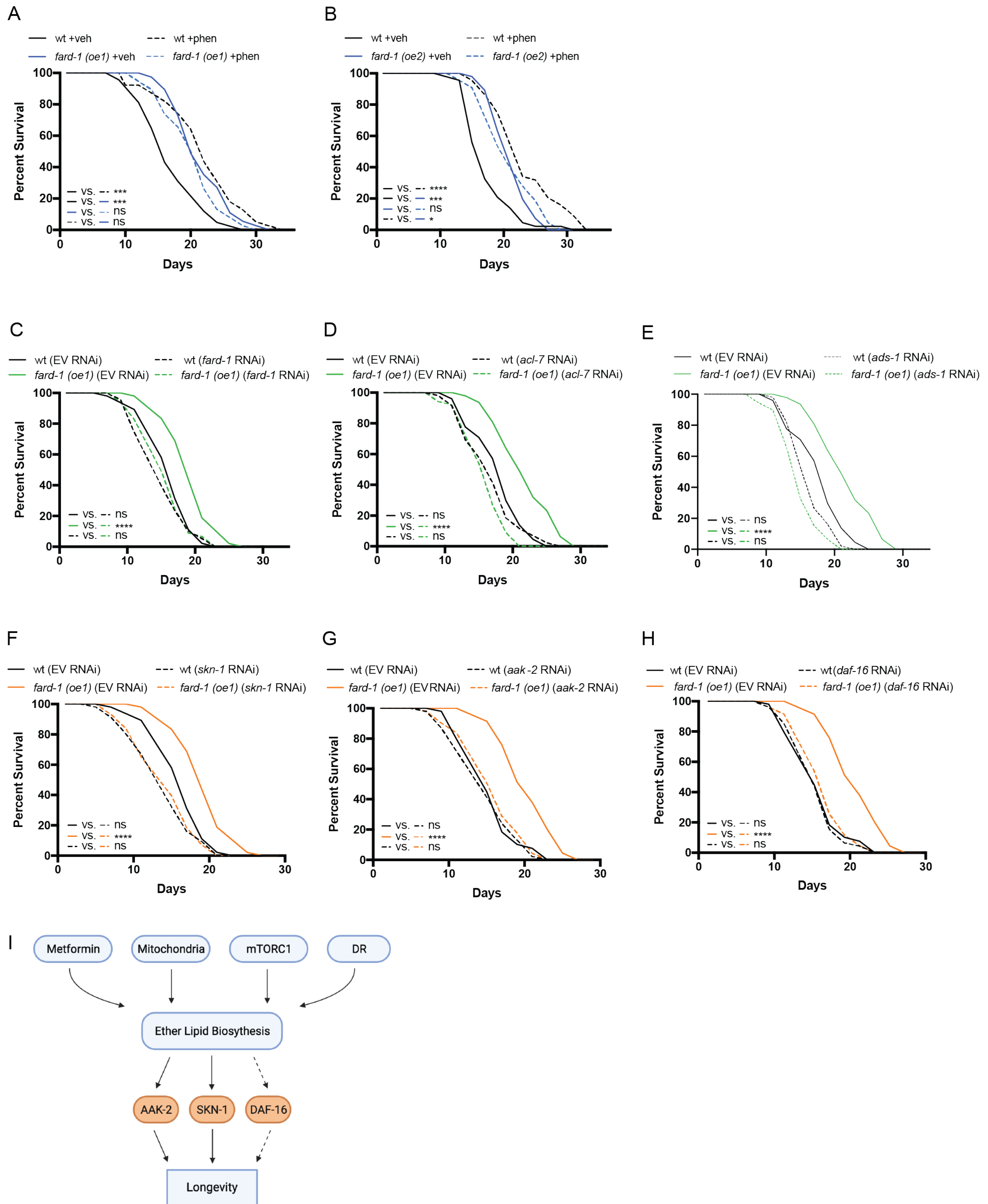
To determine whether stimulation of ether lipid biosynthesis is sufficient to prompt lifespan extension, we tested the effect of *fard-1* overexpression (*oe*) on *C. elegans* lifespan. Strikingly, *fard-1*(*oe*) alone results in significant lifespan extension (Figure 2.7 *A*). This result is similar between two independent *fard-1*(*oe*) transgenic lines (Figure 2.7 *B*). To confirm that *fard-1*(*oe*) lifespan extension is dependent upon ether lipid biosynthesis, we knocked down *fard-1*, *acl-7*, and *ads-1* by RNAi in the *fard-1*(*oe*). As predicted, knockdown of three ether lipid biosynthetic enzymes leads to significant suppression of *fard-1*(*oe*) lifespan extension (Figure 2.7*C*-*E*).

To determine the lifespan extension attributable to *fard-1* overexpression shares any genetic dependencies of biguanide-mediated longevity, we independently knocked down *skn-1*/*Nrf2*, *aak-2/*AMPK and *daf-16*/*FoxO* by RNAi in a *fard-1*(*oe*) background. While *skn-1*/*Nrf2* and *aak-2/*AMPK have previously been demonstrated to be necessary for metformin-stimulated lifespan extension, *daf-16/FoxO* has not (Kenyon et al., 1993;

Onken and Driscoll, 2010). Lifespan extension attributable to *fard-1*(*oe*) is suppressed by these three gene knockdowns (Figure 2.7*F*-*H*), indicating that it is mechanistically similar but not identical to biguanide-mediated lifespan extension (Cabreiro et al., 2013; Onken and Driscoll, 2010). In aggregate, these results support the notion that ether lipids are an important requirement in multiple, diverse longevity paradigms, and further that *fard-1*(*oe*) is a genetically distinct lifespan extension paradigm in *C. elegans.*

Figure 2.7. FARD-1 overexpression is sufficient to extend lifespan and is mechanistically similar, but not identical to biguanide-mediated lifespan extension. Two independently-generated *fard-1* overexpression (oe) transgenic strains exhibit lifespan extension that is not further extended by concomitant phenformin treatment (*A*-*B*). RNAi knockdown of *fard-1* (*C*), *acl-7* (*D*), and *ads-1* (*E)* suppresses *fard-1* overexpression (oe) lifespan extension, indicating that the *fard-1* (oe)-mediated lifespan extension is dependent upon ether lipid synthesis. RNAi of *skn-1* (*F*), *aak-2* (*G*), and *daf-16* (*H*) suppress *fard-1* (oe)-mediated lifespan extension. For *A*-*H*, results are representative of 2-3 biological replicates. ns, not significant; *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$ by log-rank analysis. See also Dataset 2.1 for tabular survival data and biological replicates. Model for the role of the ether lipid biosynthetic machinery in biguanide-mediated lifespan extension (*I*).

(Figure 2.7. continued)



2.3 EXPERIMENTAL PROCEDURES

2.3.1 *C. elegans* genetics. Strains were maintained at 20°C. The following strains were used in this study: N2 (wild-type strain), BX275 *fard-1*(*wa28*), BX259 *acl-7*(*wa20*), BX10 *ads-1*(*wa3*), CB1370 *daf-2*(*e1370*), MQ989 *isp-1*(*qm150*), VC533 *raga-1*(*ok701*), DA465 *eat-2*(*da465*), MGH48 *mgIs43*[*ges-1p*::GFP::PTS1]. For *fard-1* overexpression, the following strains were generated: MGH471 *alxEx122*[*fard-1p*::FARD-1::mRFP::HA *myo-2p*::GFP] (*fard-1 oe1*), MGH472 *alxEx124*[*fard-1p*::FARD-1::mRFP::HA *myo-2p*::GFP] (*fard-1 oe2*).

2.3.2 Statistical analysis of metabolomics data. We performed t-tests with multiple comparison corrections using the Benjamin-Hochberg method to test the difference between vehicle and biguanide for each strain. To test the hypothesis that drug uptake was similar in wildtype (N2) and ether lipid mutants, we used a linear model to estimate the drug levels (with corresponding confidence intervals) for each strain as follows,

$$\text{compound_concentration} = \beta_0 + \beta_1 * \text{sample_group} + \varepsilon$$

where:

β_0 =intercept

β_1 =group effect

ε =noise

Mass integration for 270 ether lipids was extracted from scan mode LC-MS/MS. Non-variant lipids were excluded from subsequent analyses. Next, we identified phenformin signatures that were induced by the drug and prevented in the lipid mutants using spectral clustering, which is a machine-learning algorithm to identify groups of compounds with similar concentration profiles. After selecting for 270 ether lipids, spectral clustering was performed using the *SNFtool* package (version 2.3.0) with k = 6 based on the difference between average log-normalized values of phenformin treated samples and the vehicle. We further estimated the effect size and significance of the ether lipids in the phenformin treated wild type and mutant samples compared to the vehicle using the *limma* package (version 3.44.3).

All plots were generated using the *ggplot2* package in R. All analyses were conducted using the R statistical Software (4.1.0).

2.3.3 Generation of *fard-1 C. elegans* transgenic lines. For FARD-1 expression, the entire genomic sequence of the *fard-1* locus (3659 bp), including introns and exons, plus 4910 bp of promoter were amplified and cloned into a modified Fire vector driving *fard-1* fused to mRFP and a HA epitope tag at the C-terminus. The following cloning primers were used:

forward: 5’- TGCATGCCTGCAGGTCGACTTTGACAAAAGTTCTGTTGCCG -3’

and

reverse:

5’- TTTGGGTCCTTTGGCCAATCGCTTTTTTGAAGATACCGAGAATAATCC -3’.

The FARD-1 overexpression construct was injected at 10 ng/uL (alxEx122) and 18 ng/uL (alxEx124) into the gonad of wild type adult animals with salmon sperm DNA as a carrier and 1.5 ng/uL *myo-2p*::GFP as a co-injection marker.

2.3.4 Longevity assays. Lifespan analysis was conducted at 20°C, as previously described (Soukas et al., 2009). Briefly, synchronized L1 animals were seeded onto NGM (for mutant treatment) or RNAi plates (for RNAi) and allowed to grow until the L4-young adult (YA) stage. On day 0 of adulthood as indexed in the figure legend, ~50-60 L4/YA worms per plate (unless otherwise noted) were transferred onto fresh NGM or RNAi plates. These NGM and RNAi plates were supplemented with 30 µM and 100 µM 5-fluorodeoxyuridine (FUdR) to suppress progeny production, respectively. For biguanide treatment, about ~55-60 synchronized L1 animals (unless otherwise noted) were seeded onto plates containing 50 mM metformin or 4.5 mM phenformin. At the L4/YA stage, these worms were transferred to plates containing biguanide treatment and FUdR for the remainder of their life. Dead worms were counted every other day. Statistical analysis was performed with online OASIS2 resources (Han et al., 2016).

2.3.5 RNA interference (RNAi) assays. RNAi clones were isolated from a genome-wide *E. coli* RNAi library, sequence verified, and fed to animals as described (Kamath and Ahringer, 2003). RNAi feeding plates (6 cm) were prepared using a standard NGM recipe with 5 mM isopropyl-B–D-thiogalactopyranoside and 200 µg/ml carbenicillin. RNAi clones were grown for 15 hours in Luria Broth (LB) containing 100 µg/ml carbenicillin with shaking at 37°C. The stationary phase culture was then collected, concentrated through centrifugation, the supernatant was discarded, and the pellet was resuspended in LB to 20% of the original culture volume; 250 µl of each RNAi clone concentrate was added to RNAi plates and allowed to dry at least 24 hours prior to adding biguanide. Drug treatment was added to seeded RNAi plates and allowed to dry at least 3 hours before adding worms.

2.3.6 Quantification and statistical analysis. Statistical analyses were performed using Prism (GraphPad Software). The statistical differences between control and experimental groups were determined by two-tailed students *t*-test (two groups), one-way ANOVA (more than two groups), or two-way ANOVA (two independent experimental variables), with corrected P values < 0.05 considered significant. The log rank test was used to determine significance in lifespan analyses using online OASIS2.

2.3.7 GC/MS lipidomics. Lipid extraction and GC/MS of extracted, acid-methanol-derivatized lipids was performed as described previously (Pino and Soukas, 2020; Pino et al., 2013). Briefly, 5000 synchronous mid-L4 animals were sonicated with a probe sonicator on high intensity in a microfuge tube in 100-250 microliters total volume.

Following sonication, lipids are extracted in 3:1 methanol: methylene chloride following the addition of acetyl chloride in sealed borosilicate glass tubes, which were then incubated in a 75°C water bath for 1 hour. Derivatized fatty acids and fatty alcohols were neutralized with 7% potassium carbonate, extracted with hexane, and washed with acetonitrile prior to evaporation under nitrogen. Lipids were resuspended in 200 microliters of hexane and analyzed on an Agilent GC/MS equipped with a Supelcowax-10 column as previously described (Pino and Soukas, 2020). Fatty acids and alcohols were indicated as the normalized peak area of the total of derivatized fatty acids and alcohols detected in the sample.

2.3.8 LC/MS-MS lipidomics. Wildtype, *fard-1*, *acl-7*, and *ads-1* worm mutants were collected using conditions that enabled our reported longevity phenotypes. Briefly, collection for LC/MS-MS processing comprised of 3 replicates of these 4 strains that were independently treated with vehicle (ddH$_2$O), 50mM metformin, and 4.5mM phenformin on 10cm NGM plates. A set of untreated worms were included for optimization of LC-MS/MS processing, for a total of 39 samples. A total of ~6,000 animals (2 x 10cmM plates, 3,000 worms per plate) were utilized per sample. These worms were washed with M9 (4x), concentrated into 200uL of M9, and then flash frozen with liquid nitrogen in 1.5mL Eppendorf microcentrifuge tubes. Worm pellets were transferred to 2 mL impact resistant homogenization tubes containing 300 mg of 1 mm zirconium beads and 1 mL of 90:10 ethanol:water. Using a Precellys 24 tissue homogenizer, samples were homogenized in three 10 second cycles at 6400 Hz followed by 2 minutes of sonication. Samples were then placed at -20 °C for one hour to facilitate protein precipitation. Samples were

transferred to 1.5 mL microfuge tubes and centrifuged at 14,000 g for 10 minutes at 4 °C. After centrifugation, 120 μL of supernatant was dried *in vacuo* and resuspended in 120 μL of 80:20 methanol:water containing internal standards 1 ng/μL CUDA and 1 ng/μL MAPCHO-12-d38. Lipidomic data was acquired by injecting 20 μL of sample onto a Phenomenex Kinetex F5 2.6 μm (2.1 x 100 mm) column at 40 °C and flowing at 0.35 mL/min. Metabolites were eluted using (A) water containing 0.1% formic acid and (B) acetonitrile:isopropanol (50:50) containing 0.1% formic acid using the following gradient: 0% B from 0-1 min, 0-50% B from 1-6 mins, 50-100% B from 6 to 17 minutes and 100% B hold from 17- 20 mins. Compounds were detected using a Thermo Scientific QExactive Orbitrap mass spectrometer equipped with a heated electrospray ionization (HESI) source operating in positive and negative ion mode with the following source parameters: sheath gas flow of 40 units, aux gas flow of 15 units, sweep gas flow of 2 units, spray voltage of +/-3.5 kV, capillary temperature of 265°C, aux gas temp of 350°C, S-lens RF at 45. Data was collected using an MS1 scan event followed by 4 DDA scan events using an isolation window of 1.0 m/z and a normalized collision energy of 30 arbitrary units. For MS1 scan events, scan range of m/z 100-1500, mass resolution of 17.5k, AGC of $1e^6$ and inject time of 50 ms was used. For tandem MS acquisition, mass resolution of 17.5 k, AGC $5e^5$ and inject time of 80 ms was used. Data was collected using Thermo Xcalibur software (version 4.1.31.9) and analyzed using Thermo QualBrowser (version 4.1.31.9) as well as MZmine 2.36.

CHAPTER 3: DISCUSSION

3.1 DISCUSSION

In an unbiased RNAi screen of ~1000 metabolic genes, we identified ether lipid biosynthesis as critical to the longevity-promoting effects of metformin in *C. elegans*. Our results show that the biguanides metformin and phenformin promote lifespan extension through complex effects on the landscape of ether lipids, an effect that may be mediated by governance of peroxisomal localization of the sole enzyme capable of synthesizing fatty alcohols, FARD-1. Two observations demonstrate the broad importance of these findings: 1) the ether lipid biosynthetic machinery is also required for multiple, diverse paradigms of lifespan extension, and 2) *fard-1* overexpression alone is sufficient to promote lifespan extension. Thus, ether lipids form a heretofore unappreciated dimension of lifespan modulation, and are sufficient to support healthy aging through multiple, central longevity effectors.

Differences in ether lipid abundance and composition are correlated with diseases of aging. The uniform lethality associated with human genetic ether lipid deficiency, as in the case of patients diagnosed with RCDP and Zellweger syndrome, has made it difficult to study the role of ether lipids in aging and aging-associated diseases (Braverman et al., 1997; Itzkovitz et al., 2012; Motley et al., 1997; Purdue et al., 1997). Nonetheless, observational studies suggest decreases in certain plasmalogen species in Alzheimer's Disease, suggesting a link between ether lipids and aging-related pathologies (Goodenowe et al., 2007; Grimm et al., 2011; Han et al., 2001). Ether lipids have conflicting roles in cancer; while loss of the ether lipid biosynthetic machinery profits renal and ovarian cancer cell survival by enhancing resistance to ferroptosis (Zou et al., 2020),

in other contexts, ether lipid deficiency results in impaired pathogenicity in breast and ovarian cancer cells (Benjamin et al., 2013). Cancer cells generally have higher levels of ether lipids compared to normal cells, leading others to suggest that ether lipids confer pro-survival benefit (Albert and Anderson, 1977; Benjamin et al., 2013; Snyder and Wood, 1969). However, certain ether lipid species have also been reported to have anti-tumor properties (Arthur and Bittman, 2014; Jaffrès et al., 2016). Thus, akin to the results we present here, it is critical to understand ether lipids in context, and the impact of specific ether lipid species rather than the whole class *en masse* to understand which may play a beneficial versus detrimental role in health.

Studies in long-lived animal models suggest there is an association between ether lipid content and animal longevity, such as in the naked mole-rat (*Heterocephalus glaber*) (Mitchell et al., 2007) and the mud clam *Arctica islandica* (Munro and Blier, 2012). Higher plasmalogen levels in naked mole-rat tissues versus mice, are speculated to contribute to protection of cellular membranes via a reduction of oxidative stress (Mitchell et al., 2007). Similarly, exceptionally long-lived humans harbor higher levels of phosphatidylcholine-derived, short chained alkyl ether lipids and a lower levels of phosphatidylethanolamine-derived longer chained plasmalogens (Pradas et al., 2019), but these associations are of unclear functional significance. Although it is clear that ether lipid deficiency in *C. elegans* both prevents normal lifespan (Shi et al., 2016) and longevity downstream of mitochondrial dysfunction, mTOR deficiency, caloric restriction, and biguanides alike, the precise molecular species conferring this activity remains unclear. Each of these longevity paradigms have features of nutrient deficiency or the response

to nutrient deficiency, so it is possible that ether lipids are at least part of the common effector arm conferring benefit in aging to various forms of metabolic stress.

Our results suggest that ether lipids containing long and/or desaturated fatty acids at the sn-2 position may be essential to the health promoting effects of biguanides. Although we see major shifts in abundance of alkenyl ether lipids, genetic evidence of necessity of ether lipids, and requirement for the synthesis of mono- and poly-unsaturated fatty acids in biguanide-induced longevity, determination of the specific lipids necessary for promoting healthy aging awaits the ability to modulate the level of specific ether lipid species. Further, disruption of ether lipid biosynthesis has been shown to increase the proportion of stearate (18:0) and other saturated fatty acids (Shi et al., 2016), and our own data show that some ether lipids increase in abundance while others decrease in response to phenformin. Thus, at this time, we cannot rule out the possibility that biguanide-stimulated alterations in ether lipid biosynthesis serves to divert accumulation of more “toxic” lipid species that are detrimental to lifespan. This will require a deeper understanding of the regulation of specific steps dictating the synthesis and modification of ether lipids of different fatty alcohol and fatty acid composition.

Our data are the first to suggest that peroxisomal trafficking of the fatty alcohol reductase FARD-1 could be a mechanism for regulation of favorable ether lipids in aging. Indeed, our observation that FARD-1 mRNA and protein levels are decreased by biguanides is consistent with the suggestion that 1) biguanides promote ether lipid biogenesis by promoting post-translational FARD-1 activation and 2) previous work demonstrating that higher levels of ether lipids promotes proteasomal degradation of

peroxisomal Far1 protein (Honsho et al., 2010). Modulation of the peroxisomal localization of FARD-1 may provide the first clue to identify the precise ether lipids necessary to promote longevity.

In aggregate, data presented here indicate that ether lipid biosynthesis plays a broader role in aging than previously described. The necessity of the ether lipid machinery in metformin- and phenformin-stimulated lifespan extension and in multiple longevity paradigms indicates that ether lipids serve as a lynchpin through which lifespan is modulated (Figure 2.7*I*). Our demonstration that overexpression of FARD-1 alone results in lifespan extension provides an exciting opportunity to identify ether lipids that promote health and the effector mechanisms through which they act. Finally, these results support the exciting possibility that modulation of ether lipids pharmacologically or even dietarily may provide a new potential therapeutic target in aging and aging-related diseases.

Appendix – Supplementary Materials

Dataset 2.1 (separate file). Tabular and survival data including three biological replicates (unless otherwise noted) are shown for lifespan experiments related to Figures 2.1, 2.2, 2.5, 2.6, and 2.7. Data present a summary of the conditions tested which, if applicable, include: (1) drug treatment with vehicle control and 4.5 mM phenformin or 50 mM metformin and/or (2) RNAi treatment to knockdown expression of the specific denoted gene. The *C. elegans* strain, number of subjects, restricted mean (days), standard error, 95% confidence interval (C.I.), 95% median C.I., and *P*-values for relevant comparisons are noted amongst all conditions. *, $P < 0.05$, **; $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ by log-rank analysis.

Dataset 2.2 (separate file). Raw mass spectrometry data for ether lipids detected by LC-MS/MS. Data from three biological replicates are shown for molecules indicated for vehicle or 4.5 mM phenformin treatment, for wild type animals (N2), BX10 (*ads-1* mutant), BX259 (*acl-7* mutant) and BX275 (*fard-1* mutant). Mass spectrometer polarity, parent compound mass (m/z), retention time (RT) and molecular identity (Compound ID) for each detected lipid is shown, along with data for internal standards, metformin, and phenformin. Data are in non-normalized mass counts.

Acknowledgments- Experimental Design

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