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RNAi-based biosynthetic pathway screens to identify *in vivo* functions of non-nucleic-acid-based metabolites such as lipids

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Abstract

The field of metabolomics continues to catalog new compounds, but their functional analysis remains technically challenging, and roles beyond metabolism are largely unknown. Unbiased genetic/RNAi screens are powerful tools to identify the *in vivo* functions of protein-encoding genes, but not of non-proteinaceous compounds such as lipids. They can, however, identify the biosynthetic enzymes – of these compounds- findings that are usually dismissed, as these typically synthesize multiple products. Here, we provide a method using follow-on biosynthetic-pathway screens to identify the endpoint biosynthetic enzyme and thus the compound through which they act. The approach is based on the principle that all subsequently identified downstream biosynthetic enzymes contribute to the synthesis of at least this one end product. We describe how to: systematically target lipid biosynthetic pathways; optimize targeting conditions; take advantage of pathway branchpoints; and validate results by genetic assays and biochemical analyses. This approach extends the power of unbiased genetic/RNAi screens to identify *in vivo* functions of non-nucleic-acid-based metabolites beyond their metabolic roles.

Introduction

The role of metabolites in biological functions other than metabolism is likely to be considerably underappreciated given the technical difficulties of functionally characterizing these compounds. For instance, lipids are major components of membranes, but their role in morphogenesis is largely unknown^{1, 2}. A key technical problem is the inaccessibility of nontemplate-derived compounds to specific *in vivo* targeting techniques such as gene knockouts or RNAi knockdowns that constitute the basis for the functional analysis of proteins. Although mass spectrometry (MS)- and NMR-based metabolomic approaches offer systems-level annotation and characterization of metabolites and their specific moieties, they cannot identify a causal relationship between a specific metabolite and its cellular function^{3–5}. Imaging techniques are not yet of high spatial resolution (e.g. the emerging MS-based techniques); they lack molecular specificity or interfere with function (e.g. of lipids on membranes)⁶; and the large panel of antibodies available for the detection of proteins, does

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not exist for lipids. Here we co-opt the successful strategy of nucleic acid targeting to investigate non-nucleic acid-derived metabolites such as lipids.

In a recent genome-wide RNAi tubulogenesis screen we identified a number of lipid-biosynthetic enzymes whose knockdowns generated the same specific polarity phenotype⁷. To find a putative common lipid compound through which these enzymes might act, we designed biosynthetic pathway screens and successfully identified ceramide glucosyltransferases (CGTs) as the endpoint biosynthetic enzymes, thereby revealing their single product glucosylceramide (GlcCer; the glycosphingolipid [GSL] backbone) as the common polarity-affecting lipid. Distinct biochemical functions of other identified enzymes, complemented by MS, characterized the compound being sought as a hydroxylated GlcCer (GlcCer-OH) with a C17-monomethylbranched-chain fatty acid (mmBCFA) and a saturated long-chain fatty acid (LCFA) of likely C22 length. No other existing technique could have identified this specific complex lipid's function by selectively removing it, nor would its biosynthetic enzymes have been identified in a forward mutagenesis/RNAi screen, as it required a double paralogue knockdown. This analysis demonstrates that this biosynthetic pathway screen approach can be used to query, in an unbiased manner, the *in vivo* cell biological functions of non-DNA-encoded metabolites beyond their roles as biosynthetic substrates or metabolic products.

The approach consists of a stepwise, systematic, although not comprehensive, targeting of all the molecules of the biosynthetic pathway of which the originally identified enzyme is a part. The same genetic/RNAi technique is used to search for a copy of the original phenotype with the goal of identifying the endpoint biosynthetic enzyme in the pathway, and thereby the underlying product species, and perhaps the exact chemical characteristics of the compound. The approach (1) eliminates potential bias by starting the inquiry with the analysis of the function (phenotype) rather than the molecule of interest (a 'forward' rather than a 'reverse' molecular analysis); (2) bypasses the problem of interconversion and compensation that creates a particular difficulty for the functional analysis of lipids^{8,9} and (3) overcomes the obstacle that one enzyme synthesizes many products and affects different functions. This method can be used not only to functionally characterize metabolic products of biosynthetic genes that have already been identified in time-consuming genome-wide RNAi screens (typically set aside as 'housekeeping' genes) but also to design 'lipid/metabolite' screens upfront by constructing RNAi libraries from their biosynthetic genes for the functional characterization of their products.

The strength of the approach lies in its potential to identify the mostly unknown non-metabolic (rather than the generally well-known metabolic) cellular roles of the multitude of specific metabolic compounds (rather than their enzymes). However, it also characterizes the biosynthetic functions of the enzymes *in vivo*, which, in turn, may not only identify novel biosynthetic functions but also expand or revise our knowledge about the biosynthetic pathways themselves, often only established *in vitro* or in a restricted number of species. Moreover, in combination with techniques such as liquid chromatography (LC)-MS and gas chromatography (GC)-MS, that determine compound structure, this approach will also identify species-specific different or altogether novel compounds, together with their functions. To detect such features and incorporate them into the analysis, biochemical

procedures are integrated into this overall genetic screen approach that otherwise relies on the particularly high conservation of metabolic enzymes between different species.

Applications of the protocol

Here, we provide a detailed technical protocol on how to design and carry out such a biosynthetic pathway screen, combined with genetic and biochemical analyses. We demonstrate the approach with lipids, specifically, membrane lipids, and illustrate it with our prior RNAi feeding screen on epithelial polarity in *C. elegans* (see refs.^{10–17} and <http://www.wormbase.org> for the expanding field of lipid biology and metabolomics in *C. elegans*). The concept of the approach should, however, be equally effective in other genetic model organisms (limited only by their accessibility to specific targeting procedures), and it should serve to identify the functions of other categories of lipids or other non-template-derived compounds, such as, for instance, sugars. With the advent of scalable knockout/knockdown techniques (RNAi and more recently clustered regularly interspaced short palindromic repeats (CRISPR) technology, forward screens, the method proposed here, can also be performed in mammalian cell lines. On the other hand, the use of live genetic screens in model organisms, not feasible in humans, is particularly well suited to the functional dissection of biosynthetic pathways and can serve as a direct means to investigate the non-metabolic functions of their well-conserved human orthologs. As such screens start from the phenotype, not the metabolite, they are not limited by species specificities of this metabolite, such as for instance fatty acid (FA) chain length; but, when combined with biochemical analyses, have the potential to identify the exact chemical composition of the causative metabolic end product.

Strengths and limitations of this method's ability to identify the causative end product

The biosynthetic-pathway screen approach relies on the copy of a signature phenotype that is generated by interference with one or several of multiple biosynthetic enzymes in a linear pathway (Figs. 1 and 2). The depletion of any intermediate (via its biosynthetic enzyme) in a specific pathway should (in principle) produce this phenotype, but inadvertently such screens also test conditions in which these same intermediates are increased, e.g., as accumulating substrates upstream of the catabolizing enzymes that are being tested via their depletion. Thus, seemingly paradoxically, the same phenotype may be present in animals that are depleted of a specific intermediate (by interference with its upstream biosynthetic enzyme) and in animals in which this same intermediate accumulates (secondary to interference with its downstream biosynthetic enzyme). However, in the latter scenario (accumulation of the intermediate), the phenotype is caused by the depletion of further downstream products, not by accumulation of this intermediate. This property of pathway screens, the simultaneous testing of intermediates as substrates and products, constitutes this approach's strength in uniquely addressing an inherent problem of metabolic analysis: the circularity of biosynthetic pathways and the possibility of compound interconnectivity (Figs. 1 and 2).¹⁸ It provides exceptionally strong evidence to demonstrate that it is the endpoint biosynthetic end product, not its upstream intermediates, that mediates the function under study. For instance, the same polarity defects were present in animals with loss and gain of ceramide (Cer) in our lipid biosynthetic pathway analysis (via depletion of Cer's upstream

and downstream enzymes, respectively), suggesting that the mediating lipid compound sought was a further downstream product, not Cer itself (in this case GlcCer)⁷.

Although the biosynthetic pathway screen approach addresses the problem of interconversion, it may not always solve it, nor can it overcome the problem of biosynthesis compartmentalization to different subcellular membrane systems. For instance, only the additional absence of the phenotype in a null mutant of its catabolizing enzymes would unequivocally exclude the possible contribution of the next step metabolite to the function that is being examined. This becomes an obvious challenge in cases in which a non-endpoint intermediate is suspected to execute the function of interest, which is indicated, for instance, by the absence of phenotypes in animals depleted of all its downstream metabolizing enzymes. Such a scenario may require the construction of null mutants of all immediate downstream pathway components, which may consist of several enzymes, including their paralogs. Moreover, in this particular case, strict validation of the intermediate by compound rescue of the phenotype would require the construction of a double (multiple) mutant of the immediate upstream (synthesizing) and downstream (catabolizing) enzymes. It is therefore particularly difficult to characterize functions of pathway hub compounds, such as Cer, which is a substrate of three and a product of four different metabolic reactions in *C. elegans*, most of which are catalyzed by several paralogous enzymes (Fig.2). Finally, the compartmentalization of lipid biosynthesis to different endomembrane and plasma membrane domains may cause restrictions to these biochemical rules, the functional consequences of which cannot currently be predicted. However, none of these problems can be addressed at present by any other available *in vivo* technique.

Experimental design

Procedures are provided for the scenario that one or several upstream FA biosynthetic enzyme genes have been identified in an unbiased screen as being required for a function of interest on the basis of their *C. elegans* RNAi phenotypes. As shown in Figure 3, a broad initial pathway screen (I; Steps 10–27) is designed to identify the underlying metabolite category/class and pathway branch; this is followed by an in-depth second screen (II; Steps 34 and 35) of this branch, aiming to identify the specific end product, followed (optionally) by a screen for derivatives of this end product (III; Steps 59–61), with the potential to identify its exact chemical characteristics. The direct identification of enzymes synthesizing complex lipids that are located further downstream in the pathway may make the broad screen (I) dispensable (see Supplementary Fig. 1 for complex lipid classes and their chemical compositions). The ancillary genetic and biochemical analyses serve to validate results, to connect results between different screen tiers and to exclude pathway branches, thereby reducing the number of enzymes requiring screening.

Criteria for starting a biosynthetic pathway screen (Pre-screen confirmation and analysis: Steps 1–9)

In principle, the identification of only one biosynthetic enzyme in a genetic/RNAi screen merits searching for the specific product that mediates its function. When the identified enzyme generates only one product, no further screening is necessary. More commonly, however, it generates multiple intermediates that require further analysis. The identification

of more than one biosynthetic enzyme whose losses produce the same phenotype is particularly reassuring; and the identities of these enzymes may already be informative for the screen design. Biosynthetic pathway screens are particularly useful for the analysis of ‘upstream’ (in relation to their endproduct) biosynthetic enzymes that contribute to the generation of multiple products and thus reveal little information with regard to the identity of the product mediating the investigated function. In our polarity screen, the losses of four lipid biosynthetic enzymes were found to generate the same specific phenotype, which strongly suggested they function through a shared lipid compound⁷. Three of them were common fatty-acid (FA)-biosynthetic enzymes, one of which was the first enzyme for all FA biosynthesis (*pod-2*/acetyl-CoA carboxylase(ACC)), itself giving no clue as to the nature of this compound (Fig. 1).

Several points should be checked before embarking on follow-on pathway screens (Fig. 3). First, the specificity and robustness of the phenotype is essential for the analysis that relies on its tracking. Lethality or slow growth, for instance, may be induced by interference with any of various lipids and are thus not useful for tracking a single pathway; nor is a phenotype with low penetrance or expressivity. Second, enzyme knockdowns should be validated in a corresponding germline mutant that is expected to display the same or a related phenotype, particularly if only one enzyme was identified. Third, it is useful to exclude upfront the possibility that the phenotype results not from the loss of a product downstream of the missing enzyme but rather from the accumulation of a substrate upstream of the missing enzyme, a possible alternate cause (various lipid storage diseases, for instance, are caused by toxic substrate accumulation)¹⁹. The identification of more than one biosynthetic enzyme, or of first-step biosynthetic enzymes, may already suggest that their (common) loss-of-function phenotype is not caused by an accumulating substrate. This can be ascertained by rescuing the phenotype with exogenously supplemented lipids, provided that the identified enzyme synthesizes a product that can be substituted (an expected scenario, as pathway screens are likely to be used to analyze early acting, upstream biosynthetic enzymes that generate simple FAs). Compound rescue simultaneously rules out the third, less likely, cause of a phenotype: a dual-specific function of the enzyme that is unrelated to its biosynthetic product.

Broad initial pathway screening with a focus on simple metabolites: identifying the metabolite class (Screen I: Steps 10–27)

Lipid biosynthetic pathways can be divided into three parts that build on each other (Fig.1): (1) short- and medium chain FA biosynthesis (upstream; top); (2) LCFA biosynthesis (middle); (3) complex lipid biosynthesis (downstream; bottom). Synthesis of both short- and medium-chain FAs and LCFAs involves four-step elongation cycles (condensation, reduction, dehydration and reduction, depicted for LCFA synthesis), with malonyl-CoA, which is synthesized by ACC, as chain extender. Medium-chain FAs and LCFAs can be further modified, for example, through desaturation by desaturases (right) and/or hydroxylation by hydroxylases (not shown). They then serve as precursors for complex lipids (bottom).

Not infrequently the loss of first-step, often rate-limiting, biosynthetic enzymes generate detectable phenotypes, which implicate the entire field of possible downstream products as candidate mediating compounds. The first-tier follow-on pathway RNAi screen searching for phenocopy should assemble an RNAi library that targets genes involved in a wide range of lipid-related processes, including synthesis, transport and regulation, with an emphasis on the (upstream) basic machinery of FA metabolism (the size of libraries typically ranges from ~100 to 300 molecules, scalable depending on ease of phenotypic evaluation⁷; Table 1 shows a collection of critical *C. elegans* FA biosynthetic enzymes). This analysis is targeted towards the identification of clues, and it should thus be broad rather than comprehensive.

Any positive hit will be both confirmatory with regard to the initially identified enzyme and newly informative with regard to the further screen design. For example, the identification of an elongase in a first-level follow-on pathway screen for an initial identification of ACC/*pod-2* (Fig. 1): (1) supports the functioning of ACC/*pod-2* via a downstream lipid compound; (2) identifies an LCFA component of this lipid compound and (3) may reveal – depending on what elongase was identified – whether this LCFA is branched or straight. Of nine *C. elegans* elongases, ELO-5 and ELO-6 have been characterized as producing branched-chain LCFAs (C15ISO and C17ISO)¹², and thus their identification would reveal the presence of a mmBCFA in the compound in question (Fig. 1)^{12, 20}.

Confirming the involvement of a specific pathway branch (Post screen I confirmation and analysis: Steps 28–30)

It is not necessary to probe every enzyme of the pathway, but predictions can be tested for further confirmation: for example, our first-level follow-on lipid biosynthetic pathway screens identified two polarity-affecting enzymes catalyzing successive steps of fatty acid elongation: the elongase ELO-3 and the 3-ketoacyl-CoA-reductase/KAR LET-767. Probing the enzyme catalyzing the reaction immediately downstream of KAR/LET-767 additionally identified the 3-hydroxyacyl-CoA dehydratase T15B7.2, thereby confirming pathway linearity (Fig. 1 and 3)⁷.

Identifying the phenotype of interest in animals depleted of a first step branch-specific enzyme will immediately direct follow-on screens to the corresponding branch. To increase confidence in the specific branch's involvement, findings can be validated by the analysis of the corresponding germline mutants and/or by rescuing the phenotype via a downstream lipid compound. As complex lipids - in contrast to FAs - may not be easily available for rescue, enzyme inhibitors can be used to evaluate whether they copy the phenotype of interest. Many small-molecule inhibitors have been developed to modify lipid biosynthetic enzyme activities^{21–25} (Tables 1, 2). For example, inhibitors of serine palmitoyltransferase include sphingofungins, lipoxamycin and myriocin. In *C. elegans*, they can generally be delivered by feeding. Inhibitors are often competitive substrates, and thus the same precautions apply for their supplementation as for lipid feeding (**steps 5 – 9**). Their efficiency at inhibition *in vivo* varies, thus, although phenocopy will be confirmatory, a negative result will not rule out the targeted enzyme.

Taking advantage of intermediate modifications and pathway branchpoints (Post-screen I confirmation and analysis: Steps 31 and 32)

Negative RNAi results, although informative, cannot by themselves exclude an enzyme as a pathway component. They could be caused by a number of reasons, including clone- or gene-specific RNAi resistance and redundancies, the latter being a typical scenario for biosynthetic enzyme genes, which often have paralogs (Tables 1 and 2). Although the screen design is guided by positive results, negative results can be used for branch exclusion (Fig. 3). In this case, further confirmation is necessary, with rigorous proof attainable only with germline null mutants; thus, it may require the construction of double, triple or even quadruple mutants. It is time-efficient to construct or obtain such mutants (many already available) if, with their help, large numbers of compounds can be excluded. This will be the case if the identified enzymes operate upstream in the pathway, thereby contributing to the synthesis of multiple compounds. For instance, the exclusion of desaturation has the potential to rule out a large proportion of simple, as well as complex, lipids, inclusive of all glycerolipids and phospholipids (Fig. 1). *C. elegans* contains three delta-9 desaturases, which are enzymes that carry out the first desaturation of 16:0 and 18:0 to 16:1n7 and 18:1n9 and thus generate mono unsaturated FAs with *fat-5* encoding a palmitoyl-CoA desaturase and *fat-6* and *fat-7* encoding stearoyl-CoA desaturases (Fig. 1). Therefore, if the *fat-6(tm331); fat-5(tm420) fat-7(wa36)* triple mutant, a severe loss-of-function mutant¹³, lacks the phenotype of interest, this will suggest that unsaturated FA-containing lipids do not mediate the function of the initially identified biosynthetic enzyme (although it will not, of course, exclude their function in other aspects of the same process). FAT-2, the only delta-12 desaturase in *C. elegans*, catalyzes the first reaction of polyunsaturated FA (PUFA) biosynthesis¹⁰. Lack of the phenotype of interest in the nonredundant strong loss-of-function allele *fat-2(wa17)*, which was demonstrated to be completely devoid of normal PUFAs¹³, would exclude PUFA-containing lipids, for instance eicosanoids. In our study on polarity, we could exclude phosphoinositides – which were prime candidates given their well-described roles in polarity – on the basis of absence of the polarity phenotype in *fat-2(wa17)*⁷. In principle, the same concept could be applied for any other type of compound modification (e.g., hydroxylation).

Pathway branchpoints can furthermore be exploited in this way to exclude entire branches. The enzymes that catalyze the first committed (and rate-limiting) steps of sphingolipid (SL) and glycerolipid/glycerophospholipid biosynthesis (Fig. 2a,b), respectively, are serine palmitoyltransferase and glycerol-3-phosphate acyltransferase or dihydroxyacetone phosphate acyltransferase. *C. elegans* contains three serine palmitoyltransferases (SPTL-1, 2 and 3) and three glycerol-3-phosphate acyltransferases (ACL-4, 5 and 6). The latter have been shown to acetylate both, dihydroxyacetone phosphate and glycerol-3-phosphate, which makes them sufficient targets for branch exclusion (Figure 2b, top)²⁶. The absence of the phenotype of interest in triple *sptl-1, 2, 3* or *acl-4, 5, 6* null mutants would indicate that the corresponding branches (SLs and glycerolipids/glycerophospholipids, respectively) are not involved in the investigated function. Before attempting to construct such triple-null mutants, it should be considered that they might be sterile or early lethal, limiting their usefulness for the tracking of phenotypes occurring at later stages. Moreover, lethal mutants will require balancers (re-introduction of the gene product by providing maternal lipids) and the concomitant depletion

of three or more paralogous enzymes is typically achieved by combining germline mutants with RNAi, thus depleting at least one paralog only incompletely. *cgt-1(qa1809)*; *cgt-2(tm1192)*; *cgt-3(RNAi)* animals, for instance, retain trace amounts of their product GlcCer²⁷. On the other hand, the depletion of several basic lipid biosynthetic enzymes and their products may generate only larval, rather than embryonic, lethality, and the *fat-2(wa17)* allele, although lacking all normal PUFAs, is viable¹⁰.

In principle, any branchpoint further downstream in a biosynthetic pathway can be excluded in the same manner, although branchpoint exclusion may become difficult due to the ability of many complex lipids to interconvert. For instance, the possibility of mutual interconversion of phosphatidylethanolamine and phosphatidylserine precludes using the phosphatidic acid-processing phospholipid-biosynthetic enzymes phosphatidate phosphatase and cytidine diphosphate-diacylglycerol synthase for branch exclusion of specific phospholipids (Fig. 2). Note that most phospholipids could also be excluded by definitively demonstrating that interrupting the first steps of unsaturated LCFA biosynthesis does not generate the phenotype (see above).

Demonstrating pathway contiguity: connecting simple and complex metabolites (Post-screen I confirmation and analysis: Step 33)

Demonstrating contiguity between upstream and downstream (branch-specific) enzymes will confirm that the initially identified upstream enzymes exert their effects through this branch and are required for the synthesis of its products, while at the same time defining new functions for specific intermediates and delineating the biosynthetic pathway itself. For instance, our polarity screen revealed that mmBCFAs function in polarity, but it also demonstrated that they function as components of SLs⁷, which is a finding subsequently confirmed by Zhu et al.²⁸.

Evidence for pathway contiguity can be accrued genetically and biochemically. Genetic interactions between hypomorphs, for instance, are expected to show enhancement, and the existence of such interactions would demonstrate that the enzymes operate in the same function but could not prove that they interact in the same pathway. Nulls mutants, which are necessary to address this question rigorously, may not be available given the early essential requirement of many biosynthetic steps. If aspects of the investigated function can be temporally or spatially separated, this can be used to demonstrate functional dependency of upstream on downstream components. Our reversible polarity phenotype, for instance, allowed the temporal separation of its induction and reversion: pathway contiguity could be definitively demonstrated by showing that reversion of a phenotype (induced by depletion of an upstream, FA-biosynthetic enzyme) required the branch-specific downstream enzyme (here, an SL-biosynthetic enzyme)⁷. Rescue of the phenotype by downstream lipid compounds is another definitive demonstration of pathway contiguity. However, whereas the supplementation of simple FAs generally suffices to rescue upstream enzyme losses, exogenous complex lipids may not fully replace the biological functions of their endogenous counterparts (constraints for rescue include but are not limited to, uptake, species-specific compound composition and chirality, and spatial-subcellular-and temporal availability).

Finally, MS or other quantitative approaches can be used to demonstrate a concomitant relative decrease of the identified branch-specific enzyme's lipid products in animals depleted of their upstream FA biosynthetic enzymes. Given the possibility of interconnection and complementation between different lipid compounds, alterations of absolute levels of specific compounds must, however, be interpreted with caution.

In-depth screening of pathway branches with a focus on complex metabolites: identifying the metabolite species (Screen II and Post-screen II confirmation and analysis: Steps 34–58)

Once a specific branch for complex lipid biosynthesis has been identified, it is possible to assemble a more comprehensive RNAi library for this specific branch and add: (1) double and triple RNAi conditions to detect redundant enzymes and (2) RNAi sensitive conditions to detect mild effects (the size of libraries remains similar, although the number of targeted reactions is reduced; Table 2 shows a collection of *C. elegans* SL-biosynthetic enzymes with paralogs). Strong RNAi conditions can be achieved either by using RNAi sensitive strains, such as *eri-1(mg366)*, *rrf-3(pk1426)* or *eri-1(mg366)*; *lin-15B(n744)*, or by strengthening double-stranded RNA production via increasing the IPTG concentration in agar plates to induce the bacterial polymerase (see 'RNAi feeding plates' in Reagent Setup section). Given the abundance of paralogs (Tables 1, 2), the simultaneous perturbation of a redundant gene group using combinatorial RNAi is key. We identified the double knockdown of two CGTs as the endpoint biosynthetic enzymes in our study, and thereby GlcCer as the furthest downstream polarity-affecting lipid species⁷. A forward RNAi screen is unable to detect these genes, and a forward genetic screen is also unlikely to do so because of the high number of animals that would need to be screened to identify a double hit.

The endpoint biosynthetic enzyme is validated, and its product is shown to mediate the function of the other identified biosynthetic enzymes through a combination of germline mutant analysis, specific inhibitors, MS analysis, genetic interaction experiments and/or exogenous compound rescue, as described above (pathway contiguity, Fig. 3). Definitive proof is rescue of the phenotype generated by loss of upstream biosynthetic enzymes via the endpoint product. This may not always be possible owing to specific requirements for complex lipids for certain functions (see above, pathway contiguity). For instance, *C. elegans* uses a C17 branched-chained long-chain base on its SLs, which is not readily available for purchase. If the above suggested experiments leave doubt, such compounds must either be purified from *C. elegans* or be chemically synthesized. Occasionally, bacteria that produce species-compatible lipids have been used for rescue. For example, a C17iso-sphinganine has been isolated from *Spingobacterium spiritivorum* to rescue worms that are deficient in serine palmitoyltransferases²⁸, and mmBCFA-deficient worms can be maintained on *Stenotrophomonas maltophilia*¹² (these compounds are not present in OP50, the common *C. elegans* bacterial food source). Beyond demonstrating absence (reduction) of the presumed lipid end product, MS analysis of wild-type versus mutant animals depleted of the endpoint biosynthetic enzyme (and/or of other enzymes whose losses produce the phenotype) will detect species-specific features of pathway products, the potential appearance of novel compounds, and it will contribute to the chemical characterisation of the endproduct. Lipid extraction processes and MS instrument setup conditions differ for

different lipids- e.g., for SLs, neutral glycerol lipids and glycerolphospholipids^{29–34}. The procedures that are outlined here are targeted to the analysis of *C. elegans* SLs, and specifically to complex SL species, such as Cer, GlcCer and sphingomyelin.

Pathway screens for derivatives: identifying the specific compound (Screen III: Steps 59–61)

Complex lipids themselves may be precursors for functionally distinct derivatives. For example, GlcCer, the endproduct identified in our polarity screen, provides the backbone for the large family of GSLs. A lipid glycosylation screen performed in conjunction with a prior lipid biosynthetic pathway screen may thus characterize the specific sugar modification of the identified lipid, and thereby the exact chemical composition of the compound. We used this approach, together with MS analysis, to identify the specific polarity affecting GSL in our study⁷. In this particular case, three negative results permitted the exclusion of a requirement for further sugar modifications of the identified lipid endproduct, thereby defining it as GlcCer itself: (1) the failure to identify the phenotype in the subsequent glycosylation RNAi screen; (2) its absence in a presumed null mutant of beta4-mannosyltransferase, the first step sugar-biosynthetic enzyme for the arthro-series of GlcCer-derivatives; and (3) the absence of lactosylceramide and consequently the entire GSL lacto-series in *C. elegans* (demonstrated by MS analysis), restricting the *C. elegans* GSL repertoire to the arthro series. To further refine the structural characteristics of such an end product, additional chemical characteristics that may have emerged in the prior biosynthetic pathway screens via the requirement of modifying enzymes for the function under study, can be confirmed by MS analysis. For instance, the requirement of a FA hydroxylase and of specific LCFA biosynthetic enzymes for polarity allowed us to characterize the endpoint polarity-affecting lipid compound as a hydroxylated GlcCer with a C17 branched chain LCB and a LCFA with a length of likely C22⁷.

MATERIALS

REAGENTS

Lipid-related reagents—*Fatty acids* of different lengths, numbers and positions of double bonds, hydroxyl groups and branches (Sigma, Matreya, Larodan, Nu-Chek Prep Inc.)

Glycerolipids (mono-, di- and tri-glycerols; Sigma, Avanti Polar Lipids, Matreya, Larodan)

Phospholipids with different headgroups, such as phosphocholine, phosphoethanolamine, phosphoserine, phosphoinositol and phosphoglycerol (Avanti Polar Lipids, Matreya, Larodan)

Sphingolipids with different sphingoid bases and lipid moieties (Sigma, Biomol, Avanti Polar Lipids, Matreya, Larodan)

Lipid biosynthetic enzyme inhibitors: for examples of fatty acid-, glycerolipid- and phospholipid biosynthetic enzyme inhibitors and references, see Table 1, and for inhibitors of sphingolipid metabolic enzymes see Table 2 (Sigma, Biomol, Matreya, Toronto Research Chemicals)

Chloroform (Sigma, cat. C7559)

Ethanol (Sigma, cat. no. E7023)

DMSO (Sigma, cat. no. D5879)

C. elegans-specific resources and reagents—*C. elegans* mutant alleles are available through the *Caenorhabditis* Genetics Center (<http://www.cbs.umn.edu/CGC/>) and the National Bioresource Project (<http://www.shigen.lab.nig.ac.jp/c.elegans/index.jsp>)

RNAi sensitive strains: *rrf-3(pk1426)*, *eri-1(mg366)*, *eri-1(mg366)*; *lin-15B(n744)* and *eri-1(mg366)*; *lin-35(n745)*, available from the the *Caenorhabditis* Genetics Center (<http://www.cbs.umn.edu/CGC/>)

RNAi feeding libraries; Source Bioscience: Ahringer library (19,762 bacterial clones), <http://www.lifesciences.sourcebioscience.com/clone-products/non-mammalian/c-elegans/c-elegans-rnai-library/>), Vidal ORFeome library (11,804 bacterial clones, <http://www.lifesciences.sourcebioscience.com/clone-products/non-mammalian/c-elegans/c-elegans-orfeome-version-11/>)

OP50 strain of *Escherichia coli* (*Caenorhabditis* Genetics Center, <http://www.cbs.umn.edu/CGC/>)

Yeast extract (BD Biosciences, cat. no. 211931)

Peptone (BD Biosciences, cat. no. 211677)

Tryptone (Acros Organics, cat. no. 611845000)

Bacto Agar (BD Biosciences, cat. no. 214040)

NaCl (Sigma, cat. no. S7653)

MgSO₄ (Sigma, cat. no. M2773)

CaCl₂ (Sigma, cat. no. C3881)

KCl (Sigma, cat. no. P9333)

K₂HPO₄ (Sigma, cat. no. P3786)

KH₂PO₄ (Sigma, cat. no. P0662)

NaOH (Sigma, cat. no. S5881)

Na₂HPO₄ (Sigma, cat. no. S7907)

Ammonium acetate (NH₄OAC, Sigma, cat. no. 431311)

Acetonitrile (CAN, Sigma, cat. no. 34976)

Methanol (CH₃OH, Sigma, cat. no. 34860)

Acetic acid (CH₃COOH, Sigma, cat. no. A6283)

Cholesterol (Sigma, cat. no. C8667)

Ampicillin (Sigma, cat. no. A0116)

Cabencillin (Fisher Scientific, cat. no. BP2648)

Tetracyclin (Fisher Scientific, cat. no. BP912)

IPTG (US Biological, cat. no. I8500)

Sodium hypochlorite solution (4–6% wt/vol)

! Caution this reagent is hazardous and corrosive. Wear protective gloves and a lab coat.

EQUIPMENT

Lipid-related equipment—LC-MS instrument, for instance an ABI 4000 Q trap mass spectrometer interfaced to a Dionex U3000 liquid chromatograph equipped with an Astec NH2 4.5 X 150 mm, 5µm column.

CentriVap benchtop vacuum concentrator (Savant)

Freeze-drier (Millrock Technology)

Analytical balance (Ohaus)

Probe sonicator (Fisher Scientific Model 505 Sonic Dismembrator)

Ultrasonic water bath (Fisher Scientific Branson B-5200R-1)

13 × 75 mm glass test tubes

***C. elegans*-and RNAi-screen-specific equipment**—Dissecting microscope (e.g. a Nikon SMZ-U) for phenotype analysis, with or without epifluorescence, depending on the features of the phenotype of interest (e.g. suitable for live analysis of fluorescently-labeled animals on their agar plates). Additional microscopes may be needed, such as one providing, for instance, differential interference contrast (Nomarski; for tracking cell lineage), or a confocal microscope (for analyzing subcellular morphology, e.g., a Leica TCS SL laser-scanning microscope). For detailed phenotypic analysis, high magnification, i.e., 630X or 1000X, is usually required.

Bench-top centrifuge for pelleting bacteria and worms (e.g., Eppendorf 5810R)

37°C shaking incubator to grow bacteria (New Brunswick)

Multichannel pipettes (20–200 µl) for dispensing media and bacterial suspensions

Thermowell sealing tape (aluminum; Corning Costar 6570)

Breatheasy sealing membrane (USA Scientific 9123–6100)

Petri Dishes 60 × 15 mm (Genesee, cat. no. 32–105)

Petri Dishes 100 × 15 mm (Genesee, cat. no. 32–107)

Petri Dishes 150 × 15 mm (Genesee, cat. no. 32–106)

6-well plates (35 × 18 mm, Thomas Scientific 6902A01)

12-well plates (22 × 18 mm, Thomas Scientific 6902A05)

24-well plates (15.5 × 18 mm, Thomas Scientific 6902A09)

48-well plates (9.8 × 18 mm, Thomas Scientific 6902A13)

96-well plates (for freezing RNAi clones, Fisher Scientific 08-772-2C)

96-deep-well plates (for liquid-culture of RNAi clones, Fisher Scientific 08-772-1B)

OmniTray plates (Nalge Nunc 242811)

96-pin replicator (Nalge Nunc 250520)

Conical tubes (50 ml; Fisher Scientific, cat. no. 14-432-22)

Collection tubes (15 ml; Fisher Scientific, cat. no. 05-527-90)

Reagent reservoirs (Corning Costar, 83–4870)

Roller

REAGENT SETUP

Lipid-related reagent setup

Lipid or inhibitor stock solution: Dissolve lipid or lipid mixture or enzyme inhibitors in an appropriate solvent. Solvents and concentration, as well as preparations vary with different compounds. Examples of stock solutions for some lipids and inhibitors are listed in Table 3.

HPLC mobile phases: Mobile Phase A = (97:2:1 CAN: CH₃OH: CH₃COOH) + 5mM NH₄OAC; mobile phase B = (99:1 CH₃OH: CH₃COOH) + 5 mM NH₄OAC. Mobile phases should be freshly prepared daily.

C. elegans-and *C. elegans* RNAi screen-specific reagent setup and solutions

LB liquid media: Add 5 g tryptone, 2.5 g yeast extract and 5 g NaCl per 1 liter of water, and autoclave the mixture. Store at 4 °C for 2–3 months. Use directly for growing OP50 bacteria, and add 0.6–1 ml of 100 mg/ml ampicillin for growing RNAi bacteria.

LB-antibiotics plates: Add 5 g tryptone, 2.5 g yeast extract, 5 g NaCl, 7.5 g agar per 1 liter of water and autoclave the mixture. Allow the medium to cool to 50–60 °C before adding 1 ml of 100 mg/ml ampicillin and 1 ml of 15 mg/ml tetracyclin stock solutions. Pour the medium into plates and leave them at room temperature until the agar sets. Store the plates at 4 °C for 2–3 months. Use for growing RNAi bacteria.

Nematode growth media (NGM) agar plates: For one liter, add 3 g NaCl, 2.5 g peptone, 17g agar and 975 ml H₂O, and autoclave the mixture. Allow the medium to cool to 50–60 °C, and then add 1 ml of cholesterol (5 mg/ml in ethanol), 0.5 ml of 1 M CaCl₂, 1 ml of 1 M MgSO₄, 25 ml of 1M Potassium phosphate buffer, pH 6. Pour into plates and leave them at room temperature until the agar sets. Store at 4 °C for up to 1 month. Use for growing worms.

RNAi feeding plates: For one liter, add 3 g NaCl, 2.5 g peptone, 17g agar and 975 ml H₂O and autoclave the mixture. Allow the medium to cool to 50–60 °C, and then add 1 ml cholesterol (5 mg ml⁻¹ in ethanol), 1 ml of 1 M CaCl₂, 1 ml of 1 M MgSO₄, 25 ml of 1M potassium phosphate buffer, pH 6, 2.4 ml of 200 mg/ml IPTG (final concentration: 2mM), and 0.5 ml of 50 mg/ml carbenicillin (final concentration: 25ug/ml). Pour into plates and leave them at room temperature until the agar sets. Make plates fresh 1–3 days before using for RNAi knockdown by feeding. Different IPTG concentration will produce milder or stronger RNAi effects and should be empirically determined for the phenotype under study.

M9 buffer: Dissolve 3 g KH₂PO₄, 6 g Na₂HPO₄, 5 g NaCl in 1 liter of water and autoclave the mixture. Add 1 ml of 1 M MgSO₄ after cooling to room temperature. Use the buffer for washing and aliquotting worms. This solution may be stored at room temperature for months.

Bleach solution: Add 25 ml of 10 N NaOH, 15 ml of household sodium hypochlorite solution and 60 ml water to make 100 ml. Bleach solutions lose activity over time. Freshly prepare the solution before using it for synchronizing worms.

CaCl₂, 1 M: Add 147.01 g of CaCl₂ to 500 ml of deionized water and stir until CaCl₂ is completely dissolved, and then bring the volume to 1 liter using deionized water, and autoclave the mixture. Allow the solution to cool to room temperature. This solution may be stored indefinitely at room temperature.

MgSO₄, 1 M: Add 246.48 g of MgSO₄ to 500 ml of deionized water, and stir it until MgSO₄ is completely dissolved, and then bring the volume to 1 liter using deionized water and autoclave the mixture. Allow the solution to cool to room temperature. This solution may be stored indefinitely at room temperature.

Potassium phosphate buffer, 1 M (pH 6.0): Add 30.1 g of K₂HPO₄ and 118.1 g of KH₂PO₄ to 500 ml of deionized water, and stir it until K₂HPO₄ and KH₂PO₄ are completely dissolved, and then bring the volume to 1 liter using deionized water and autoclave the mixture. Allow the solution to cool to room temperature. This solution may be stored indefinitely at room temperature.

NaOH, 10 N: Add 20 g of NaOH to 50 ml of deionized water, and stir it until NaOH is completely dissolved. This solution may be stored indefinitely at room temperature and in plastic containers.

Ampicillin stock solution (100 mg/ml): Add 1 g of ampicillin to 10 ml of deionized water to make stock solution. Sterilize the stock solution by filtering with a 0.22 µm filter and store 1-ml aliquots in 1.5-ml tubes at – 20 °C for 6–12 months.

Carbenicillin stock solution (50 mg/ml): Add 500 mg of carbenicillin to 10 ml of deionized water to make stock solution. Sterilize the stock solution by filtering with a 0.22-µm filter, and store 1-ml aliquots in 1.5-ml tubes at –20 °C for 6–12 months.

Tetracyclin stock solution (15 mg/ml): Add 150 mg of tetracyclin to 10 ml of ethanol to make stock solution. Sterilize the stock solution by filtering with a 0.22-µm filter, and store 1-ml aliquots in 1.5-ml tubes at – 20 °C for 6–12 months.

IPTG stock solution (200 mg/ml): Add 2 g of IPTG to 10 ml of deionized water to make stock solution. Sterilize the stock solution by filtering with a 0.22-µm filter and store 1-ml aliquots in 1.5-ml tubes at – 20 °C for 6–12 months.

Equipment setup

HPLC elution protocol: The protocol comprises the following steps: a 2-min column pre-equilibration in 9:1 A:B (vol/vol); sample injection; 2-min wash with 100% A; 12-min linear gradient to 100% B; and 1-min post-run column equilibration. The gradient profile is tabulated below, with a flow rate of 1 ml/min and an injection volume of 5 µl.

Time (min)	A%	B%
–2	90	10
0	100	0
2	100	0
14	0	100
15	0	100

PROCEDURE

PRE-SCREEN CONFIRMATION AND ANALYSIS: Validation of the phenotype in germline mutants

● TIMING 1d to 3 weeks

1. To cross the strain of the initial RNAi screen (a strain typically carrying a marker, e.g. a GFP label - used as example here) into the germline mutant strain under study, mate N2 male worms into the marker strain (could also generate a male stock of this strain).

2. Cross the resultant GFP⁺ male worms into hermaphrodites of the mutant (*a/a*) to generate *gfp*⁺; *a*⁺ (if *gfp* is integrated into the genome) or *a*⁺; *gfp* (if *gfp* is transmitted on an extrachromosomal array) F1 progeny and allow to self.
3. Clone the GFP⁺ F2 progeny to isolate homozygous GFP⁺ mutants (expected frequency: 1/12 if the GFP reporter is integrated and 1/4 if on an extrachromosomal array).
4. Evaluate whether the homozygous mutants display the same or similar phenotype as the corresponding RNAi animals (see <http://www.wormbook.org> for standard genetic procedures).

CRITICAL STEP: Depending on the phenotype, high resolution microscopic analysis (e.g., differential interference contrast and/or confocal microscopic analysis) might be needed, in addition to screening using a dissecting microscope.

PRE-SCREEN CONFIRMATION AND ANALYSIS: exclusion of lipid substrate toxicity or lipid-unrelated mechanisms

● Timing 2–7d

5. Prepare lipid stock solution (see Reagent Setup).

?TROUBLESHOOTING

6. Mix lipid stock solution or solvent control with either OP50 or gene-specific HT115 RNAi bacteria (e.g., 5–40 µl + 120 µl, respectively, for each of a 6-well plate; see Step 24, RNAi screen setup). The concentration required for rescue needs to be titrated for each compound or compound mixture.

CRITICAL STEP: Some solvents interfere with RNAi and may therefore produce the false impression of phenotype rescue; it is therefore critical that these experiments be run with the appropriate solvent controls (and/or that results be confirmed in germline mutants).

?TROUBLESHOOTING

7. Seed the mixture of lipid/solvent and bacteria onto the RNAi or nematode growth medium (NGM) plates, and allow the bacteria to grow for 6–8 h.

CRITICAL STEP: Most lipids are not soluble in aqueous medium, thus they are not well distributed when they are directly applied to a bacterial lawn on NGM plates (some extent of precipitation of the lipid compounds might occur). The Watts lab has described an alternative method in which feeding plates containing lipids are made by mixing the lipids into the agar in the presence of tergitol, a nonionic surfactant³⁵.

?TROUBLESHOOTING

8. Seed or pick worms onto the lipid-supplemented NGM or RNAi plates.

?TROUBLESHOOTING

9. Score the phenotype.

? TROUBLESHOOTING

SCREEN I: BROAD BIOSYNTHETIC PATHWAY RNAi SCREEN: assembly of an RNAi feeding library

● **Timing 3d-1week (depending on familiarity with metabolic pathways)**

10. Expanding from the specific biosynthetic enzyme that was identified, assemble the initial library with the aim to target genes/transcripts involved in a wide range of lipid-related processes and with the emphasis on the basic machinery of FA metabolism. Taking advantage of the high structural conservation of biosynthetic enzymes throughout phylogeny, use several or all of the following approaches to assemble the library: Step 10A, assembly of a list of *C. elegans* lipid metabolism-related genes/proteins; Step 10B, assembly of a list of lipid metabolism-related genes/proteins of all species; Step 10C, assembly of a list of *C. elegans* orthologs of lipid metabolism-related genes/proteins of other species; and Step 10D, assembly of an expanded list of *C. elegans* orthologs of lipid metabolism-related proteins.

- (A) Assembly of a list of *C. elegans* lipid metabolism-related genes/proteins
 - i. Review experimental data on lipid metabolism in *C. elegans* navigating from wormbase (where parologs are immediately linked; <http://www.wormbase.org>) to generate a list of lipid-related genes in *C. elegans*.
- (B) Assembly of a list of lipid metabolism-related genes/proteins of all species
 - i. Analyze annotated pathway databases such as the LIPID MAPS (<http://www.lipidmaps.org/pathways/index.html>) and the KEGG pathways (<http://www.genome.jp/kegg/pathway.html>), which provide systematically aligned metabolic pathways, for a list of lipid-related molecules of all species (see en.wikipedia.org/wiki/List_of_biological_databases and <http://www.nature.com/scitable/content/genomics-databases-744357> for comprehensive lists of biological databases).
- (C) Assembly of a list of *C. elegans* orthologs of lipid metabolism-related proteins of other species.
 - i. Search databases such as AceView (<http://ncbi.nlm.gov/iebr/research/assembly/>) and iHOP (<http://www.ihop-net.org/UniPub/iHop/>) with links to multiple organisms, and species-specific databases, such as the *Saccharomyces* Genome Database (<http://www.yeastgenome.org>), Flybase (<http://www.flybase.org>), the Mouse Genome Database (<http://www.flybase.org>).

www.informatics.jax.org) and the Human Genome Database (<http://www.gdb.org>), to identify *C. elegans* orthologs of non-*C. elegans* proteins.

- (D) Assembly of an expanded list of *C. elegans* orthologs of lipid metabolism-related proteins.
 - i. Perform protein BLAST searches against the *C. elegans* databases to identify *C. elegans* orthologs of non-*C. elegans* proteins not traced with the above search engines, and perform searches inside the *C. elegans* database to identify more distantly related family members.
11. Pick RNAi clones of these candidate genes from the genome-wide RNAi feeding libraries onto LB plates containing the appropriate selective antibiotics, and grown overnight at 37°C.
12. Inoculate bacteria into 120 µl of LB media plus 100 mg/ml ampicillin in a 96-well plate, allow the bacteria to grow overnight in a shaking incubator at 220 r.p.m. at 37 °C, and then add 30 µl of 80% glycerol and keep it in a –80 °C freezer to generate a frozen stock of the library.

SCREEN I: BROAD BIOSYNTHETIC PATHWAY RNAi SCREEN: worm synchronization

● TIMING 3–6 d

13. Place 10–15 young adult hermaphrodites on each of ten 100-mm standard NGM plates seeded with OP50, and allow them to grow at 20 °C until the plate is full of gravid adults, but the worms are not starved (i.e., some of the OP50 lawn remains on the plate).
14. Wash the worms from the plates using M9 (1–3 ml per plate), and collect the M9 worm suspension into a 15-ml conical tube.
15. Centrifuge the collected worms at 1,500 r.p.m. for 5 min at room temperature and carefully decant the supernatant.
16. Add 3.5 ml of cold M9 and 1.5 ml of sodium hypochlorite solution to the worm pellet, mix by inversion and vortex the mixture.
17. Swirl the solution every minute or so until almost all adults have dissolved (it takes~ 6–8 min depending on the number of worms in each tube). Immediately fill the tube with M9 and centrifuge at 1,500 r.p.m. at room temperature for 2 min. Carefully decant the bleach solution without disturbing the worm embryo pellet.
18. Wash the embryo pellet using 5–10 ml of M9 buffer three times to remove bleach.
19. Resuspend the washed embryos in 5–10 ml of M9.
20. Allow the collected embryos to hatch in M9 by rotating them at 20°C for 12–20 h.

CRITICAL STEP Note that both the eggs from step 19 and L1 larvae from step 20 can be used directly for an L1-initiated RNAi feeding screen.

21. Plate the synchronized larvae in M9 onto NGM plates seeded with OP50 (depending on the amount of larvae, they can be plated onto several NGM plates to ensure a well-fed condition).
22. Incubate the plates at 20°C for 36–48 hours.
23. Wash the worms off the plates using M9 buffer three times before pipetting onto RNAi feeding plates for screening (for plate preparation, see step 26).

SCREEN I: BROAD BIOSYNTHETIC PATHWAY RNAi SCREEN: RNAi feeding and phenotype scoring

● TIMING 3–6 d

24. The day before setting up RNAi feeding experiments, inoculate bacteria from step 12 into 500–800 µl (50–200 µl final volume is required for each individual feeding experiment, depending on the type of plate used: 6-, 24- or 96 well) of LB media plus 100 mg/ml ampicillin in a 96-deep well plate, and allow them to grow overnight in a shaking incubator at 220 r.p.m. at 37 °C.

CRITICAL STEP: It is important to use freshly grown bacteria to achieve the full RNAi effect.

25. The following morning, induce transcription of double-stranded RNA by adding 50–200 µl of the bacterial cultures from Step 24 to each RNAi plate per well (containing IPTG, see ‘RNAi feeding plates’ in Reagent Setup section). For knock downs of multiple genes, add an equal volume of each bacterial feeding clone to the plates.

CRITICAL STEP: RNAi has an intrinsic variability, therefore, it is essential to set up replicates for every clone. In each experimental run, at least one positive and one negative control are included (Step 27).

26. After 10–12 h, for parentally induced RNAi (where the F1 generation is scored), pipette L3-L4 worms from Step 23 onto each RNAi well per plate (typically 5–10 worms in a volume of 10 µl of M9 buffer for six-well plates); for RNAi induced in the same generation, pipette eggs or L1s from Step 20 (typically 50–100 worms in a volume of 10–20 µl of M9 buffer for six-well plates).

CRITICAL STEP: Experiments may need to be repeated with larger number of worms if the brood size is low.

27. Scoring depends on the screen set-up and the nature of the phenotype of interest. To score morphological F1 phenotypes scored on a dissecting microscope, day 2 is optimal for scoring embryonic phenotypes, and day 3 is optimal for scoring larval phenotypes. If maternal effects are being scored, also inspect the F3 generation. Suitable bacterial control RNAi clones should be included for each set of RNAi experiments. The optimal positive control is a clone that copies the phenotype of interest, and the optimal negative control is a clone that produces

an unrelated phenotype or no detectable phenotype (e.g., targets a non *C. elegans* gene).

CRITICAL STEP: Steps 13–27 constitute one cycle of RNAi screening.

Depending on numbers and time required for scoring, several overlapping cycles of screening can be carried out per week.

? Troubleshooting

CONFIRMATION AND ANALYSIS OF RESULTS OF SCREEN I: demonstration of pathway linearity ● TIMING 3–7 d

28. Identify the enzyme catalyzing the reaction immediately upstream or downstream of the enzyme identified by RNAi phenocopy in the above screens (use resources indicated in Step 10).
29. Perform RNAi with this enzyme as described in Steps 13–27, to demonstrate copy of the phenotype.

?TROUBLESHOOTING

CONFIRMATION AND ANALYSIS OF RESULTS OF SCREEN I: Validation of branch selection ● TIMING 3 to 4weeks (depending on options chosen)

30. Probe the validity of a branch-specific positive result by either one or several of the following steps: Step 30A, confirmation of phenotype in germline mutants; Step 30B, demonstration of phenocopy by small molecule inhibitors; and Step 30C, rescue of phenotype via downstream lipid product(s).

(A) Confirmation of phenotype in germline mutants

- i. Use procedures described in Steps 1–4 to generate and analyze the branch-specific germline mutant.

(B) Demonstration of phenocopy by small-molecule inhibitors

- i. Dilute the inhibitor stock solution or solvent control with OP50 bacteria to the desired volume of, for instance, 150 μ l per well of a six-well plate (typically a 4 – 25 time dilution). The optimum concentration to produce the phenotype of interest needs to be titrated for each compound.
- ii. Seed the mixture of solution onto the NGM plates and let OP50 bacteria grow for 6–8 hours (see ‘NGM agar plates’ in Reagent Setup section)

CRITICAL STEP: Some precipitation may occur owing to water insolubility (see Steps 5–9, analysis of lipid compounds, for caveats with regards to lipophilic substances).

- iii. Seed eggs, larvae or adults of the strain used to carry out the initial screen onto the plates.
- iv. Score the phenotype.

? Troubleshooting

- (C) Rescue of phenotype via downstream lipid product(s).
- i. Use procedures described in Steps 5–9 to rescue the branch-specific enzyme defect with downstream lipids.
- CRITICAL STEP: This approach may not be an option, as only complex lipids (rather than FAs) will rescue branch-specific enzyme knockdowns-which may require species-specific compounds that are not readily identified or available (see Experimental design). Failure of rescue with complex lipids must thus be interpreted with caution.

CONFIRMATION AND ANALYSIS OF RESULTS OF SCREEN I: exclusion of lipid classes or branches ● TIMING 1–4weeks (depending on availability of mutants and their characterization); this is optional if a branch-specific enzyme was identified, but required in the absence of such a positive result)

31. Generate severe double or triple loss-of-function conditions for paralogous enzymes (ideally null mutants) to abolish enzymatic reactions for the purpose of pathway branch exclusions, using a combination of standard genetic procedures, RNAi and specific enzyme inhibitors (see Steps 1–4 for an example of a simple cross; Steps 13–27 for RNAi; Step 30B, for treatment with inhibitors; and <http://www.wormbook.org> for standard genetic procedures, including the introduction of genetic balancers into lethal mutants). Some double and triple mutants are already available at <http://www.cbs.umn.edu/CGC/> or they may have been generated for specific studies (e.g., *fat-6(tm331); fat-7(wa36)*, *fat-7(wa36) fat-5(tm420)*, *fat-6(tm331); fat-5(tm420)*, *acl-4(xh10) acl-5(xh9)*, *cgt-3(tm504); +/-DnT1*; *cgt-1(qa1809)/DnT1*; *cgt-2(tm1097)*, *cgt-3(tm504); +/-DnT1*; *cgt-1(qa1809)/DnT1*; *cgt-2(tm1192)* double and triple mutants^{13, 26, 27}; see Fig. 1).
 32. Examine the mutant for copies of the phenotype
- CRITICAL STEP: To definitively exclude a class or branch, MS analysis is additionally required to demonstrate absence of the product of the enzymatic reaction in question: see Steps 37–58 below for MS analysis (such data may, however, be already available for some of these mutants).

CONFIRMATION AND ANALYSIS OF RESULTS OF SCREEN I: Demonstration of pathway contiguity ● TIMING 2–4 weeks

33. Show that the function of upstream biosynthetic enzymes is mediated by downstream biosynthetic enzymes/compounds by one or several of the following approaches: Step 33A, genetic interactions; Step 33B, phenotype rescue via downstream branch product(s); Step 33C, biochemical analysis of lipids (MS).
- (A) Genetic interactions

- i. Create double loss-of-function mutants, mutant/RNAi-, double-RNAi- or mutant/inhibitor/RNAi animals whose alterations concomitantly deplete one FA and one complex lipid biosynthetic enzyme (see Steps 1–4 for an example of a simple cross; Steps 13–27 for RNAi; Step 30B, for treatment with inhibitors). To score for the expected enhancement, aim for mild loss-of-function conditions. Titrate mild RNAi conditions by reducing IPTG plate concentration and/or by diluting RNAi bacteria with parent bacteria until a penetrance of 0–50% is achieved.

- ii. Score the phenotype.

CRITICAL STEP: Consider on a case-by-case basis whether it is worth investing time into genetic interaction experiments, as haploinsufficiency, as well as dosage-dependent nonallelic noncomplementation, is thought to occur only rarely in *C. elegans*³⁶. We did, however, find them helpful when examining metabolic enzymes and dosage-dependent processes, and we have also used heterozygous germline mutants for this purpose⁷.

? Troubleshooting

(B) Phenotype rescue via downstream branch product(s)

- i. Refer to Step 30C (including caveats) and use procedures described in Steps 5–9 to rescue an upstream biosynthetic enzyme defect with a downstream branch-specific lipid.

(C) Biochemical analysis of lipids by MS

- i. Demonstrate similar lipid profiles with regard to the missing lipid compound in RNAi animals/mutants with defects in upstream versus downstream biosynthetic pathway enzymes (see Steps 37–58 below).

SCREEN II: COMPREHENSIVE PATHWAY BRANCH RNAi SCREEN: assembly of an RNAi feeding library ● Timing 3–7d

34. Construct this smaller library (compared to the broad library assembled for screen I) as comprehensively as possible and include the simultaneous depletion of paralogs to effectively interfere with at least all those enzymatic reactions that could generate an endproduct (rather than an intermediate) and thus the searched-for lipid compound (as described in Steps 10–12).

SCREEN II: COMPREHENSIVE PATHWAY BRANCH RNAi SCREEN: RNAi screen procedure ● Timing 6–12d

35. Synchronize worms, set up RNAi feeding experiments and score phenotypes, as described for screen I (Steps 13–27).

CRITICAL STEP: The addition of double and triple RNAi for the depletion of redundant enzymes and/or of strong RNAi conditions to optimize enzyme depletion is critical for this round of screening (see Experimental Design).

CONFIRMATION AND ANALYSIS OF RESULTS OF SCREEN II: Validation of the identified furthest downstream lipid biosynthetic enzyme and its product ● Timing 2–4 weeks

36. Validate the presumed endpoint lipid biosynthetic enzyme and its lipid compound and demonstrate that they constitute the endpoint biosynthetic enzyme/compound pair by several of the following procedures: Step 36A, scoring for phenocopy in the corresponding germline mutant; Step 36B, scoring for phenocopy by the specific inhibitor of the identified enzyme; Step 36C, screening for genetic interaction of the identified enzyme with any or all other enzymes of the pathway; Step 36D, rescue of the loss-of-function phenotype(s) of the endpoint biosynthetic enzyme and/or of any upstream enzymes by the presumed lipid end product; and Step 36E, MS analysis of the loss-of-function mutant/RNAi animal(s) that display the phenotype to demonstrate absence (reduction) of the presumed lipid end product.
 - (A) Scoring for phenocopy in the corresponding germline mutant
 - i. Use procedures described in Steps 1–4.
 - (B) Scoring for phenocopy by the specific inhibitor of the identified enzyme
 - i. Use procedures described in Step 30B.
 - (C) Screening for genetic interaction of the identified enzyme with any or all other enzymes of the pathway.
 - i. Use procedures described in Step 33A.
 - (D) Rescue of the loss-of-function phenotype(s) of the endpoint biosynthetic enzyme and/or of any upstream enzymes by the presumed lipid end product.
 - i. Refer to Step 30C and use procedures described in Steps 5–9.
 - (E) MS analysis of the loss-of-function mutant/RNAi animal(s) that display the phenotype to demonstrate the absence (reduction) of the presumed lipid end product.
 - i. See Steps 37–58.

CONFIRMATION AND ANALYSIS OF RESULTS OF SCREEN II: Lipid profile analysis ● Timing 1–3 weeks

37. *Preparation of wild-type and mutant/ RNAi worms depleted of the endpoint biosynthetic enzyme for lipid extraction.* Set up 150mm RNAi (for RNAi depletion) or NGM culture plates (for germline mutants) to grow a sufficiently large amount of worms for lipid extraction, aiming at a final worm pellet of at

least 200 μ l. If L1 larvae are to be analyzed (a likely scenario given the frequent L1 lethality of lipid biosynthetic enzyme mutants/RNAi animals), 600 – 800 such plates may be required. As controls, set up equal amounts of synchronized wild-type L1 larvae, which are grown on the same bacterial strain (*E. coli* HT115).

38. Collect worms by rinsing the RNAi plates with M9 buffer and letting animals sediment at 4°C.
39. Subject worm pellets to three cycles of freezing in liquid nitrogen for 30–60 minutes and thawing on ice for 30 min, followed by sonication (10 s each time, 3–5 times for each cycle).
40. Combine samples with the same genotype into one tube, lyophilize the worm suspension using a freeze-drier and measure dry weight using an analytical balance.
41. Add methanol, chloroform and water in a 2:1:1 ratio (e.g. 2/1/1ml) to each sample (~40–60 mg dried worm pellet).
42. Spike 100 μ l of internal standard mixture (e. g., a SL standard mixture).
43. Mix by vortexing for 5 minutes to achieve a single-phase cloudy solution.
44. Incubate the samples at 48°C overnight in a heating block.
45. Add 1.0 ml of 1M KOH into each sample after cooling to room temperature.
46. Sonicate the samples in a bath sonicator for 5 min.
47. Incubate at 37°C for 2 h.
48. Remove a 1-ml aliquot of the extract and dry it under vacuum for long-chain base analysis when necessary.
49. Add 600 μ l of glacial acetic acid to neutralize the remaining solution.
50. Add 4 ml of water and 2 ml of chloroform, and shake for 2 min.
51. Centrifuge at 1,000g for 10 min at 20°C and discard the upper layer.
52. Collect the lower layer for analysis, and reduce the volume to ~100 μ l using a CentriVap Benchtop Vacuum Concentrator.

PAUSE POINT The samples can be stored at –20°C for 2–3 months.

53. Reconstitute the samples to a final volume of 1.0 ml with a 9:1 mixture of HPLC mobile phase A and B (see Reagent Setup section).

CRITICAL STEP: Glassware rather than plastic should be used for all extraction procedure steps that use organic solvents, in order to avoid extracting molecules from the plastic that could interfere with the biochemical analysis of lipids.

54. *L C-MS.* Carry out HPLC using Dionex U3000 liquid chromatograph equipped with an Astec NH2 column (4.5 mm $\times$ 150 mm, 5- μ m particles); mobile phases

A and B are prepared as described in Reagent Setup section, and elution protocol is described in Equipment Setup section.

55. Set up the following conditions for MS (ABI 4000 Qtrap): Ion Spray voltage: 4500V; ion spray temperature: 400°C; gas pressure: 5–20 p.s.i; program to monitor the $[M+H] \rightarrow 250.2$ u ion of the d17:1 *C. elegans* specific sphingoid base; ceramide/hydroxyceramide, hydroxyglucoceramide, and sphingomyelin/hydroxysphingomyelin compounds were monitored in three periods according to normal phase elution time of the internal standards.
56. Inject 5 µl of each 1 ml extract from Step 53.
57. Calculate concentrations for each compound from the response ratio of compound area/internal standard area of the corresponding period per dry weight of the sample.
58. Compare the lipid composition of mutant/RNAi animals with control animals.
CRITICAL STEP: It is not always useful to generate whole lipidomic profiles, as this may decrease the sensitivity for a lipid species of interest.

SCREEN III: METABOLIC PATHWAY RNAi SCREEN FOR DERIVATIVES (optional): Assembly of an RNAi feeding library ● Timing 3–7d

59. Construct an RNAi library from enzymes known or expected to biochemically modify the identified lipid end product—for example, enzymes functioning in lipid glycosylation (for library assembly see Steps 10–12). As described in Step 34 (and library size permitting) aim for concomitant depletion of paralogs and a broad range of depletion conditions.

SCREEN III: METABOLIC PATHWAY RNAi SCREEN FOR DERIVATIVES (optional): RNAi screen procedure ● Timing 6–12d

60. Synchronize worms, set up RNAi feeding experiments and score phenotypes, as described for screen I (Steps 13–27).

CONFIRMATION AND ANALYSIS OF RESULTS OF SCREEN III: validation of the endpoint lipid-modifying enzyme and chemical characterization of the endpoint lipid ● Timing 2–4 weeks

61. Proceed according to the corresponding Steps outlined for screen II (36–58).

?TROUBLESHOOTING

Trouble shooting advice can be found in Table 4

TIMING

PRE-SCREEN CONFIRMATION AND ANALYSIS:

Steps 1–4, phenotype validation in germline mutants: 1d to 3 weeks

Steps 5–9, exclusion of lipid substrate toxicity or lipid-unrelated mechanisms: 2–7d

SCREEN I: BROAD BIOSYNTHETIC PATHWAY RNAi SCREEN:

Steps 10–12, assembly of an RNAi feeding library: 3d to 1 week

Steps 13–23, worm synchronization: 3–6 d

Steps 24–27, RNAi feeding setup and scoring the phenotype: 3–6 d

CONFIRMATION AND ANALYSIS OF RESULTS OF SCREEN I (optional)

Steps 28 and 29, demonstration of pathway linearity: 3–7 d

Step 30, validation of branch selection: 3d to 4 weeks

Steps 31 and 32, exclusion of lipid classes or branches: 1–4 weeks

Step 33, demonstration of pathway contiguity: 2–4 weeks

SCREEN II: COMPREHENSIVE PATHWAY BRANCH RNAi SCREEN

Step 34, assembly of an RNAi feeding library: 3–7d

Step 35, RNAi screen procedure: 6–12d

CONFIRMATION AND ANALYSIS OF RESULTS OF SCREEN II

Step 36, validation of downstream lipid-biosynthetic enzyme and its product: 2–4 weeks

Steps 37–58, lipid profile analysis: 1–3 weeks

SCREEN III: METABOLIC PATHWAY RNAi SCREEN FOR DERIVATIVES (optional)

Step 59, assembly of an RNAi feeding library: 3–7d

Step 60, RNAi screen procedure: 6–12d

CONFIRMATION AND ANALYSIS OF RESULTS OF SCREEN III

Step 61, validation of the endpoint lipid-modifying enzyme and chemical characterization of the endpoint lipid: 2–4 weeks

ANTICIPATED RESULTS

In this era of fully sequenced genomes, biosynthetic enzyme genes are frequently identified in genome-wide screens (e.g., RNAi screens) that investigate specific cell biological functions. This information is often excluded from further analysis, because such ‘housekeeping genes’ typically contribute to the synthesis of a vast array of different compounds. Systematic follow-on pathway screens, as described here, are expected to identify the one specific metabolite that mediates their contribution to the investigated function, and to additionally map out the entire pathway leading to its synthesis. Such screens will therefore also identify multiple intermediate enzymes and their respective products as required for the function under study. With sufficient screen depth and the addition of screens for derivatives, biosynthetic pathway screens, together with MS-based biochemical analyses, are able to identify not only the compound species whose loss causes the functional defect, but also its exact chemical characteristics. Biosynthetic pathway screens can also be devised upfront as ‘metabolic RNAi screens’ to investigate the

contribution of nonproteinaceous compounds to a specific biological function. The prime purpose of these screens is the discovery of novel, non-metabolic functions of nontemplate-derived metabolites such as lipids (themselves no targets for knockouts or knockdowns), although they also extend the already well-established knowledge about their metabolic functions. It is hoped that such screens will harness the power of unbiased genetic/RNAi screens to open the field of metabolomics for the *in vivo* characterization of the largely unknown non-metabolic roles of biosynthetic intermediates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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uses specific enzymes for some reactions. In *C. elegans*, for instance, ELO-3 condenses straight chain- and FAs, and ELO-5 and ELO-6 condense branched-chain FAs, whereas LET-767 is used for the reduction of either. **Middle Right: Biosynthesis of unsaturated FAs.** Saturated FAs are desaturated to monounsaturated and polyunsaturated FAs (MUFAs and PUFAs) by a wide range of desaturase-(delta 5-, 6-, 9, 12 and omega-3) and PUFA elongase-(omega-3 and 6) activities to generate a variety of unsaturated FAs with different lengths and different numbers of double bonds. In contrast to higher plants and mammals, *C. elegans* possesses all of the enzymes required for the *de novo* biosynthesis of 20:4(*n*-6) and 20:5(*n*-3) FAs. Starting with the primer acetyl-CoA, the biosynthesis of the PUFA 20:5(*n*-3) by this route involves ~ 30 distinct enzymes and 70 reactions. **Bottom: Complex lipids.** Products serve as building blocks for complex lipids, with saturated FAs dominating sphingolipids (SLs) and desaturated FAs dominating glycerols and phospholipids. Corresponding metabolic enzyme genes with experimentally confirmed functions in *C. elegans* are indicated in red.

Figure 2. Conserved sphingolipid and phospholipid metabolic pathways. (a) Sphingolipid metabolic pathways

Left: *De novo* biosynthesis. The biosynthesis of sphingolipids (SLs) begins with the condensation of serine and palmitoyl-CoA. Three further synthetic steps are needed to produce ceramide (Cer) that links the sphingoid backbone (long chain base) with a FA chain and is the center of the SL metabolic pathway. **Right: Salvage pathways and complex SL biosynthesis.** The post-Cer metabolic steps are reversible. Ceramide serves both as substrate and as product for the biosynthesis and degradation, respectively, of complex SLs, such as sphingomyelin or glycosphingolipids. Deacylation of Cer yields sphingosine that can be

phosphorylated to sphingosine-1-phosphate. The irreversible degradation of the sphingoid backbone is catalyzed by a lyase that acts on sphingosine-1-phosphate. **(b) Major glycerophospholipid metabolic pathways. Top: Biosynthesis of phosphatidic acid**, which is an intermediate in the biosynthesis of phospholipids, by a series of reduction and acylation reactions. **Bottom Left: the Kennedy pathway**. Phosphatidic acid is dephosphorylated to yield diacylglycerol, which in turn is converted into phosphatidylcholine (PC) or phosphatidylethanolamine (PE) by the addition of a phosphocholine or a phosphoethanolamine head group, respectively. Phosphatidylserine (PS) can be formed by exchanging the head group from either PC or PE for serine. Mitochondrial PE is synthesized on location by decarboxylation of PS. **Bottom Right: Cytidine diphosphate diacylglycerol (CDP-DAG) pathway**. PS and phosphatidylinositol (PI) are synthesized from the precursor CDP-DAG by the enzymes CDP-DAG serine phosphatidyltransferase and CDP-DAG inositol phosphatidyltransferase (PI synthase), respectively. CDP-DAG is also used for the synthesis of phosphatidylglycerol and cardiolipin, which are phospholipids that are confined to mitochondrial membranes. Corresponding metabolic enzyme genes with experimentally confirmed functions in *C. elegans* are indicated in red.

Figure 3. Work flow chart for a tiered lipid biosynthetic pathway RNAi screen

Top to bottom: RNAi screens become successively constricted, zooming in onto the endpoint biosynthetic enzyme to identify the underlying common lipid compound. The broad initial pathway screen (I; Steps 10–27) aims to identify the underlying metabolite category/class and pathway branch and, typically, has a focus on simple metabolites. The second stage in-depth screen of an identified pathway branch (II; Steps 34 and 35) aims to identify the specific lipid end product and, depending on the identified enzymes, is focused on complex metabolites. An additional screen for derivatives of the identified end product (III; Steps 59–61) can be performed based on the same principles, and has the potential to

identify its exact chemical characteristics. Positive hits (phenocopy; left column) confirm initial findings, identify roles for intermediates, define components of the searched-for end product and guide the screen towards the downstream endpoint biosynthetic enzyme. Negative results (no phenocopy; right column), confirmed under severe loss-of-function conditions (e.g., nulls), exclude pathway arms and thus multiple compounds and narrow down follow-on screens, allowing in-depth screening that includes the simultaneous depletion of paralogs and a broader range of depletion conditions. If no downstream enzymes are identified, they redirect screens to repeat screening of a broader selection of molecules and/or screening with more severe depletion conditions, to the search for the causative compound among upstream components of the pathway.

Table 1Critical *C. elegans* fatty acid biosynthetic enzymes

Gene Name	Sequence name	Activity *	Inhibitors
pod-2	W09B6.1	Acetyl-CoA carboxylase	Quizalofop ²¹ , CP-61043 ²¹ , CP-640186 ²²
	T28F3.5	Acetyl-CoA carboxylase	
fasn-1	F32H2.5	Fatty acid synthase	Cerulenin ²¹ , Thiolactomycin ²¹ , C75 ²¹
phi-46	F32H2.6	Fatty acid synthase	
elo-1	F56H11.4	Long chain fatty acid elongase	
elo-2	F11E6.5	Long chain fatty acid elongase	
elo-3	D2024.3	Long chain fatty acid elongase	
elo-4	C40H1.4	Long chain fatty acid elongase	
elo-5	F41H10.7	Long chain fatty acid elongase	
elo-6	F41H10.8	Long chain fatty acid elongase	
elo-7	F56H11.3	Long chain fatty acid elongase	
elo-8	Y47D3A.30	Long chain fatty acid elongase	
elo-9	Y53F4B.2	Long chain fatty acid elongase	
hpo-8	T15B7.2	3-hydroxyacyl-CoA dehydratase	
let-767	C56G2.6	3-keto acyl CoA reductase	
art-1	C15F1.6	Trans-2,3-enoyl-CoA reductase	
acs-1	F46E10.1	Long chain fatty acid CoA ligase	
acs-2	F28F8.2	Long chain fatty acid acyl-CoA ligase	
fat-1	Y67H2A.8	Omega-3-desaturase	
fat-2	W02A2.1	Delta-12-desaturase	
fat-3	W08D2.4	Delta-6 fatty acid desaturase	
fat-4	T13F2.1	Delta-5 fatty acid desaturase	
fat-5	W06D12.3	Palmitoyl-CoA (delta-9-) desaturase	
fat-6	VZK822L.1	Stearoyl-CoA (delta-9-) desaturase	CAY10566 ²³ , CVT-11127 ²²
fat-7	F10D2.9	Stearoyl-CoA (delta-9-) desaturase	

List is not comprehensive but includes critical biosynthetic reactions and closely related family members/paralogs (grouped by color) of biosynthetic enzymes. Genes with unknown names are left blank

* <http://www.wormbase.org>

Table 2Critical *C. elegans* sphingolipid metabolic enzymes and inhibitors

Gene name	Sequence name	Activity *	Inhibitors ^{24, 25}
sptl-1	C23H3.4	Serine palmitoyltransferase	Myriocin and its derivatives, sphingofungin B & C, cycloserine, beta-choroalanine
sptl-2	F43H9.2	Serine palmitoyltransferase	
sptl-3	T22G5.5	Serine palmitoyltransferase	
	Y37E11AM.3	3-Ketosphinganine reductase	
	Y54E5A.1	Dihydroceramide desaturase	GT11 and analogues, Dihydroceramide analogues
	F33D4.4	Dihydroceramide desaturase	
	C25A1.5	Sphingolipid fatty acid hydroxylase	
hyl-1	C09G4.1	Ceramide synthase	Fumonisin and related compounds
hyl-2	K02G10.6	Ceramide synthase	
lagr-1	Y6B3B.10	Ceramide synthase	
asah-1	K11D2.2	Acid ceramidase	Alpha-ketoamides, Ceramide analogues, Desipramine
	F27E5.1	Acid ceramidase	
	Y55D5A.3	Acid ceramidase	
	W02F12.2	Alkaline ceramidase	
cgt-1	T06C12.10	Ceramide glucosyltransferase	d,l-PDMP, D-DPMA, D-threo-PPPP, N-butyldeoxynojirimycin, OGT2378
cgt-2	F20B4.6	Ceramide glucosyltransferase	
cgt-3	F59G1.1	Ceramide glucosyltransferase	
gba-1	C33C12.3	Beta-glucocerebrosidase	D-gluconolactone, conduritol B epoxide, cyclophellitol, conduritol B aziridine
gba-2	C33C12.8	Beta-glucocerebrosidase	
gba-3	F11E6.1	Beta-glucocerebrosidase	
gba-4	Y4C6B.6	Beta-glucocerebrosidase	
	C29E4.10	Orthologous to human galactocerebrosidase	
sms-1	H21P03.3	Sphingomyelin synthase	MS-209, D609
sms-2	F53H8.4	Sphingomyelin synthase	
sms-3	Y22D7AL.8	Sphingomyelin synthase	
	F53B1.2	Sphingomyelin synthase-related protein	
	W07E6.3	Sphingomyelin synthase-like domain-containing	
asm-1	B0252.2	Acid sphingomyelinase	SR33557, Desipramine, GW4869, Scyphostatin, Manumycin A, SM analogues
asm-2	ZK455.4	Acid sphingomyelinase	
asm-3	W03G1.7	Acid sphingomyelinase	
	ZK856.5	asm-like phosphodiesterase	
	T27F6.6	Sphingomyelinase family protein	
sphk-1	C34C6.5	Sphingosine kinase	N,N-dimethylsphingosine, LCL204, L-threo-sphinganine, SG12, SG14, SKi, phenoxodiol
	T10B11.2	Ceramide kinase	F-12509A
spl-1	Y66H1B.4	Sphingosine phosphate lyase	FTY720, 1-desoxysphinganine-1-phosphonate, 2-vinylsphinganine-1-phosphate
tag-38	B0222.4	Sphingosine phosphate lyase	

Gene name	Sequence name	Activity*	Inhibitors ^{24, 25}
	Y104H12D.3	Sphingosine phosphate lyase	

List is not comprehensive but includes critical biosynthetic reactions and closely related family members/paralogs (grouped by color) of metabolic enzymes. Genes with unknown names are left blank.

* <http://www.wormbase.org>

Table 3

Examples of lipid and inhibitor stock solutions

Compound name	Lipid numbers/Short name	Company	Catalog number	Solvent	Concentration of stock solution
Lipids					
Palmitic acid	C16:0	Sigma	P5585	Ethanol	20 mg/ml
Stearic acid	C18:0	Sigma	S4751	Chloroform	100 mg/ml
Oleic acid	C18:1(n-9)	Sigma	O1383	Chloroform	50 mg/ml
Linoleic acid	C18:2(n-6)	Sigma	L1012	Ethanol	100 mg/ml
Arachidonic acid	C20:4(n-6)	Sigma	A3555	Chloroform	50 mg/ml
Docosahexaenoic acid	C22:6(n-3)	Sigma	D2534	Chloroform	50 mg/ml
Lignoceric acid	C24:0	Sigma	L6641	Chloroform	50 mg/ml
13-methylmyristic acid	iso-C15	Sigma	M7031	DMSO	400mM
15-methylpalmitic acid	iso-C17	Sigma	M6531	DMSO	400mM
Sphingomyeline from Bovine Brain		Sigma	S7004	Chloroform:Methanol (1:1)	50 mg/ml
C18 Sphinganine	d18:0	Biomol	SL-125	Ethanol	10 mg/ml
C16 Ceramide	d18:1/16:0	Biomol	SL-115	Ethanol	2 mg/ml
C6 Ceramide	d18:1/6:0	Avanti	860506P	Ethanol	10 mM
C12-glucosylceramide	d18:1/12:0	Avanti	860543P	Ethanol	10 mM
Ceramide/Sphingoid internal Standard mixture I *		Avanti	LM-6002	Ethanol	25uM each of 10 compounds
Inhibitors					
Myriocin		Biomol	SL-226	Methanol	2 mg/ml
Fumonisin B1		Biomol	SL-220	Water	25 mg/ml
Fumonisin B2		Biomol	SL-219	Water	25 mg/ml
N-(n-Butyl) deoxygalactonojirimycin	C ₄ DGJ	Toronto Research Chemicals	B690500	Water	80 mg/ml
N-(n-Nonyl) deoxygalactonojirimycin	C ₉ DGJ	Toronto Research Chemicals	N649900	Methanol	10 mg/ml
N-Butyldeoxynojirimycin hydrochloride	C ₄ DNJ	Toronto Research Chemicals	B691000	Water	100 mg/ml
D,L-threo-1-Phenyl-2-decanoylamino-3-morpholino-1-propanol•HCl	PDMP	Maureya	1719	Ethanol	100 mg/ml

* Available directly as solution mixture from Avanti

Table 4

Troubleshooting table

Steps	Problem	Possible reason	Solution
5–9	Exogenous lipid supplementation does not rescue the phenotype	The phenotype is too severe	Use mild RNAi conditions or a mild reduction-of-function mutant
		The lipid species used for rescue resides upstream of the identified enzyme	Choose a lipid species downstream of the identified enzyme, e.g. its direct product
		Amount too low	Increase the final lipid concentration on the plate
		Lipid uptake is poor (can test by using labeled lipids)	Can try microinjection or mix lipids into the agar in the presence of tergitol ³²
		Supplied lipids do not have organism-specific structure	May require lipid extraction from the animal and feed back
		Lipid biosynthetic process itself is linked to function	Overexpress the corresponding lipid biosynthetic enzyme; an alternative approach to prove pathway contiguity and/or the link between lipid and cellular process may be required
27	Phenotype not consistently present in animals depleted of enzymes in a linear stretch of the pathway (or absence of phenotype in any other unexpected scenario)	The specific transcripts are RNAi resistant	Examine the corresponding germline mutant
		RNAi too mild	Use RNAi sensitive strains
		Existence of maternal effect	Score phenotype of the third generation
		The gene functions redundantly with other genes	Knock down paralogs/redundant genes by double/triple RNAi/mutant analysis
		Phenotype is masked by sterility or early embryonic lethality	Mild, post-embryonic or tissue-specific RNAi can be tried. Mild RNAi conditions can be empirically determined for each clone by titrating IPTG concentrations over a wide range of concentrations and by diluting RNAi bacteria with control RNAi bacteria. RNAi can be initiated at any stage after completion of embryogenesis by placing eggs or larvae or adults on RNAi plates for evaluating the same generation. RNAi can be performed in a tissue-specific RNAi strain.
29	Depletion of any additional downstream lipid biosynthetic enzyme by any method available produces no phenotype	The lipid product of the furthest downstream identified lipid biosynthetic enzyme is the searched-for compound	This can be demonstrated by rescue of the phenotype in a double mutant/RNAi animal of the corresponding upstream and downstream biosynthetic enzymes with this lipid compound
30(B)(iv)	The small-molecule inhibitor fails to copy the phenotype resulting from the loss of the corresponding biosynthetic enzyme	Inhibitor concentration or uptake too low	Increase concentration; try microinjection
	The range of effective dose of inhibitor is narrow (no effect with lower, and early lethality with higher, dose)	The inhibitor has weak potency and lacks specificity	Can attempt to carefully titrate inhibitor concentration; check ability of the inhibitor to enhance the (mild) loss of the corresponding biosynthetic enzyme
33A(ii)	The expected enhancement between incomplete loss-of-function conditions of two enzymes is not detected	Phenotype(s) is too strong	Try different combinations of mutant alleles and RNAi conditions