A p p e n d i x A

MITOCHONDRIAL β-OXIDATION OF THE ASCAROSIDE SIDE CHAIN

A.1 Introduction

Whereas the sequence and significance of peroxisomal β-oxidation is well characterized and understood, the bulk of ascaroside biosynthesis including precursor origins and participating enzymes is largely unknown.[1] Due to the fact that metabolites derived from tryptophan metabolism are incorporated into the biogenesis of indolated ascarosides, and other ascaroside structures suggest the possible incorporation of head groups derived from isoleucine and tyrosine degradation, we hypothesized that ascaroside biosynthesis may occur in the mitochondria, where amino acid catabolism occurs.[2-3]

Furthermore, although the digestion and truncation of long-chained lipids is a peroxisomal process, medium- and short-chained lipids are degraded in the mitochondria.[4] As it was previously observed by von Reuss *et al* that long-chained ascarosides undergo β-oxidation in the peroxisomes, we questioned whether ascarosides bearing shorter lipid side chains may be digested in the mitochondria in a similar fashion. In order to do so, we examined the ascaroside profiles produced by strains containing mutations in genes homologous to those involved in mammalian mitochondrial β-oxidation pathways. In this screen, we examined strains with loss-of-function mutations in *acdh-1* and *acdh-13*, two mitochondrial acyl-CoA dehydrogenases paralogous to the peroxisomal gene *acox-1*, and the enoyl-CoA-hydratase *ech-2*, a paralog of the peroxisomal gene *maoc-1*.

A.2 Results and Discussion

Compared to wild type N2, the ascaroside profile of the *ech-2* mutant was largely similar, demonstrating no major changes in ascaroside biosynthesis (Figure A.1). Levels of indolated ascarosides were affected to a larger degree in the *acdh* loss-of-function mutants. For example, the ascaroside profile of *acdh-13(ok3004)* demonstrated elevated levels of hbas and icas ascarosides (Figure A.2). Although HPLC-MS analysis of the mutant excretomes revealed the *acdh-1(ok1514)* mutant ascaroside profile to be largely wild type, a second mutant allele for the same gene *acdh-1(ok1489)* demonstrated greater penetrance and produced elevated levels of hbas and icas ascarosides, to an even larger degree than *acdh-13(ok3004)*. The *acdh-1(ok1514)* allele remains uncurated, but the lower penetrance observed may be the result of a deletion mutation that leaves the active site intact. Levels of hbas#3 produced by *acdh-1(ok1498)* were 12 times larger than wild type and each indolated ascaroside, including icas#3, icas#9, and icas#10, were increased by a factor of 3 to 5.

These results suggest that the *acdh-1* and *acdh-13* genes may be involved in the regulation of icas and hbas ascaroside production despite the precise mechanism remaining unknown. Previous research has shown that ACDH-1 expression depends upon nutritional state with expression upregulated in animals that are well-fed and downregulated in starved animals.[5-7] As such, ACDH-1 has been used as a *C. elegans* dietary sensor.[7] In summary, ACDH-1 may serve as a link between ascaroside production and the nutritional state of the worm. This also indicates that the mitochondria may be involved in regulating ascaroside production as the energetic needs of the worm changes due to food availability.

A.3 Figures

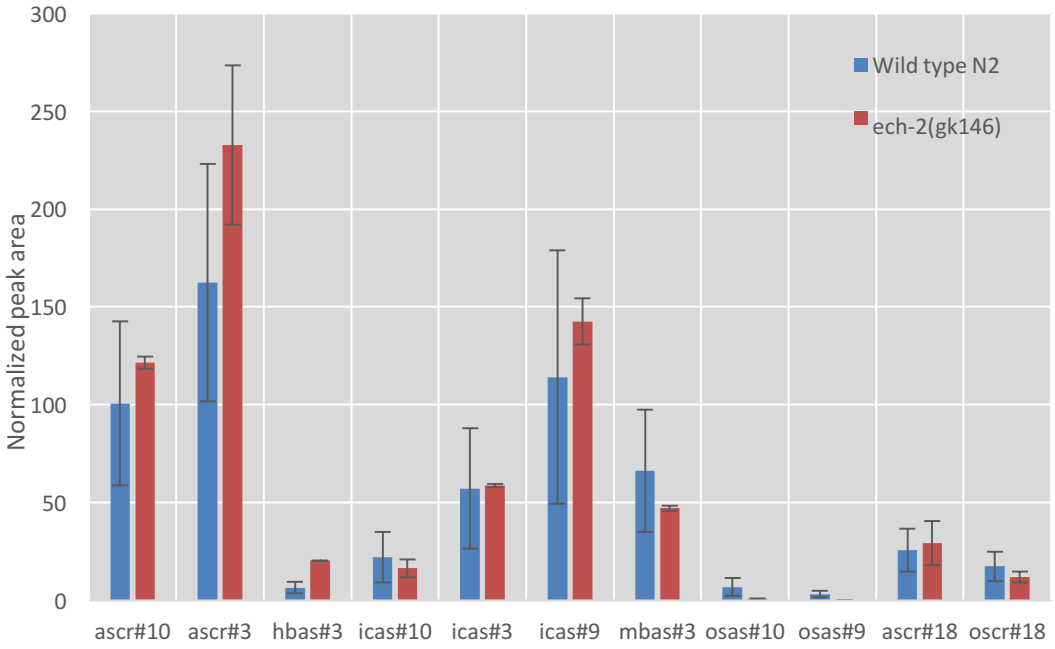

Figure A.1: Examining the Role of *ech-2*. The *ech-2* gene encodes a putative mitochondrial enoyl-CoA hydratase that is a paralog of *maoc-1*, the gene that encodes the protein responsible for hydrating the site of α,β-unsaturation during peroxisomal β-oxidation of the ascaroside lipid side chain. Loss of *ech-2* function produces a mostly wild type ascaroside profile. Excretomes were derived from mixed stage cultures. MS peaks are normalized to worm pellet mass. Averages are of two biological replicates.

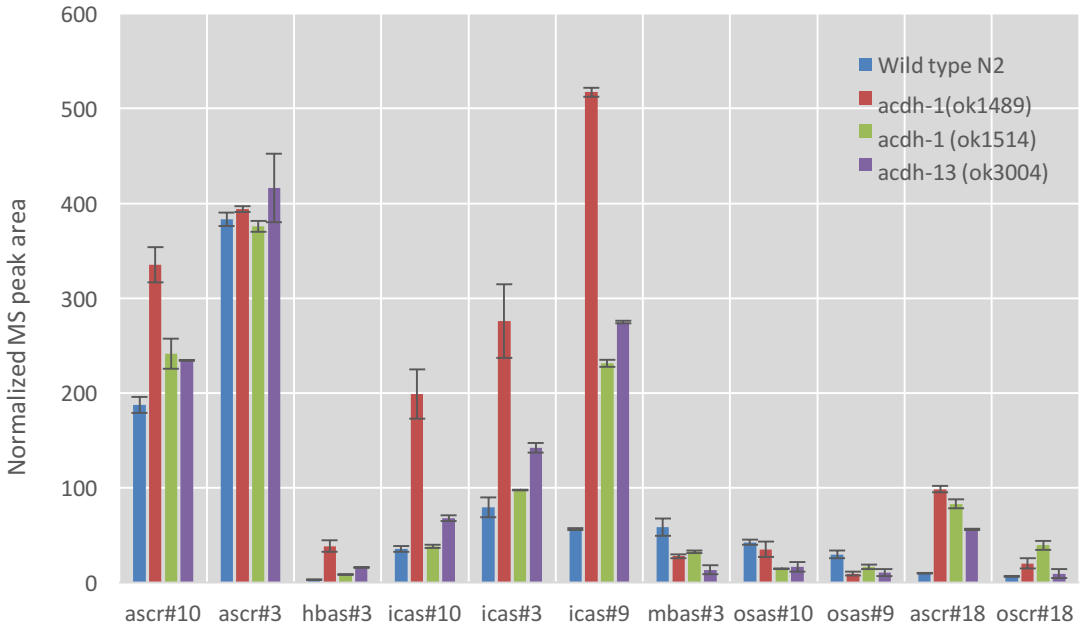

Figure A.2: Examining the Role of Acyl-CoA Dehydrogenases. The *acdh* genes are mitochondrial paralogs of *acox-1*, the gene that encodes the protein responsible for the first site of peroxisomal β-oxidation by introducing a site of α,β-unsaturation. Loss of function in *acdh-1* and *acdh-13* genes result in higher levels of hbas and icas ascarosides, with the *acdh-1(ok1489)* allele being more penetrant than the *acdh-1(ok1514)* allele. Excretomes were derived from mixed stage cultures. MS peaks are normalized to worm pellet mass. Averages are of two biological replicates.

A.4 References

1. von Reuss, S. H.; Bose, N.; Srinivasan, J.; Yim, J. J.; Judkins, J. C.; Sternberg, P. W.; Schroeder, F. C., Comparative metabolomics reveals biogenesis of ascarosides, a modular library of small-molecule signals in C. elegans. *J Am Chem Soc* **2012,** *134* (3), 1817-24.
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Appendix B

ADDITIONAL MUTANTS SCREENED FOR BIOSYNTHETIC DEFECTS

B.1 Introduction

Foundational ascaroside studies have demonstrated or indicated that ascarosides are constructed from metabolites from various primary metabolic pathways. For example, the ascarylose scaffold is a carbohydrate, the lipid side chains are modified by peroxisomal β-oxidation enzymes, and head groups attached to the 4'-position are derived from amino acid catabolism.[1-4] In our initial screens, we prioritized mutants involved in primary metabolic pathways and those homologous to genes involved in the biosynthesis of ascaroside components in other organisms.

B.2 Results and Discussion

The indole-3-carbonyl group attached to the 4'-position of ascarylose in the icas ascarosides is known to be derived from tryptophan based on feeding assays (Figure B.1).[5] Indole-3-carboxylic acid is also a well-known auxin, a plant hormone that directs cell growth. In *Arabidopsis thaliana*, it was found that tryptophan is converted first into indole-3-pyruvate by a tryptophan aminotransferase and subsequently, a YUCCA monooxengase converts the indole-3-pyruvate into indole-3-carboxylic acid (Figure B.2).[6-7] Although the indole-3-carboxylic acid auxin contains an extra carbon compared to the indole-3-carbonyl moiety on icas ascarosides, paralogous *C. elegans* genes may serve similar roles in the biosynthesis of

the icas head group. Cytochrome p450s are a family of enzymes that carry out monooxygenase functions, therefore we screened knockout mutants of five *cyp* genes: *cyp-13A7(gk31)*, *cyp-13B2(gk726)*, *cyp-14A2(gk289)*, *cyp-31A1(gk154)*, and *cyp-35A3(ok2709)*. HPLC-MS analysis of the mutant excretomes revealed no decrease in icas ascaroside biosynthesis (Figure B.3). In future, it might prove useful to examine the *fmo* gene family, which encodes flavin-containing monooxygenases with higher homology to the *A. thaliana* gene YUCCA1. Mutants are available from the CGC for *fmo-4(ok294)*, *fmo-3(ok354)*, and *fmo-2(ok2147)*.

The bacterium *Chromobacterium violaceum* and plants from the genus *Orobanche* are known to be able to synthesize indole-3-carboxylic acid, a process which involves the conversion of tryptophan into tryptamine, mediated by a decarboxylase enzyme.[8] We therefore screened three mutants that encode putative histidine decarboxylase enzymes based on homology: *hdl-1(ok956)*, *hdl-2(ok440)*, and *basl-1(ok703)*. HPLC-MS analysis of the liquid culture supernatant revealed that none of the three mutants screened resulted in a substantial reduction in icas production, suggesting that none of the enzymes tested are involved in the biosynthesis of the indole-3-carboxylic acid precursor (Figure B.4).

Another subset of genes screened came from the *elo* gene family. The *elo* genes encode elongase proteins that catalyzes the lengthening of a fatty acid carbon chain. We screened three *elo* mutants: *elo-1(gk48)*, *elo-5(gk182)*, and *elo-6(gk233).* We found that *elo-1(gk48)* and *elo-5(gk183)* mutants produced higher amounts of the longer chain ascaroside ascr#18 and oscr#18, but did not significantly diminish the production of any particular ascaroside (Figure B.5).

B.3 Figures

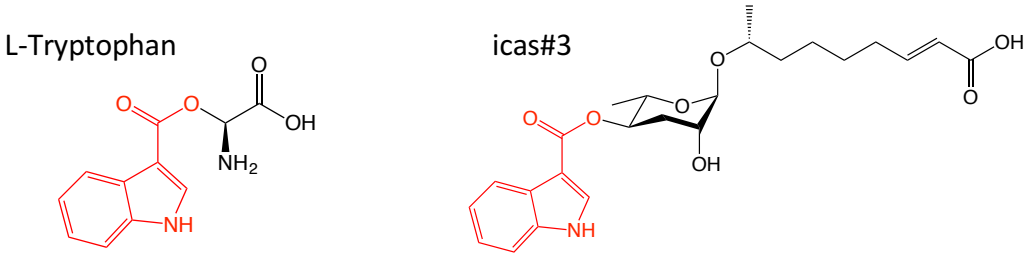

Figure B.1: The icas Ascarosides. The indole-3-carbonyl group attached to icas ascarosides is derived from tryptophan.

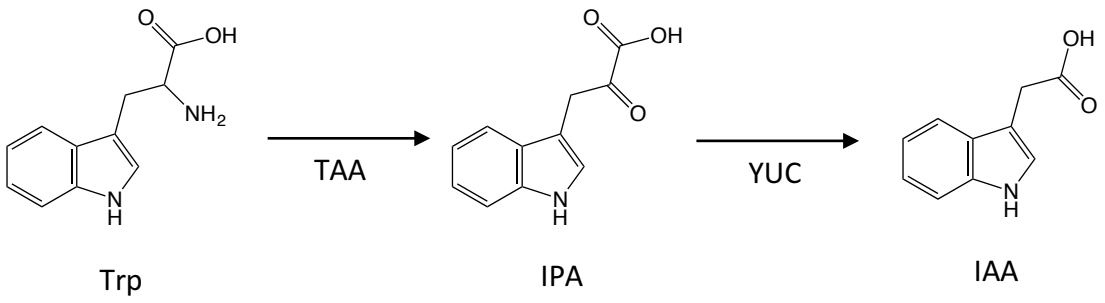

Figure B.2: Indole-3-carboxylic Acid Biosynthesis in *A. thaliana.* Tryptophan aminotransferase (TAA) converts tryptophan (Trp) into indole-3-pyruvate (IPA), which is then converted into indole-3-carboxylic (IAA) acid by YUCCA (YUC). (Modified figure from Won *et al.*[6])

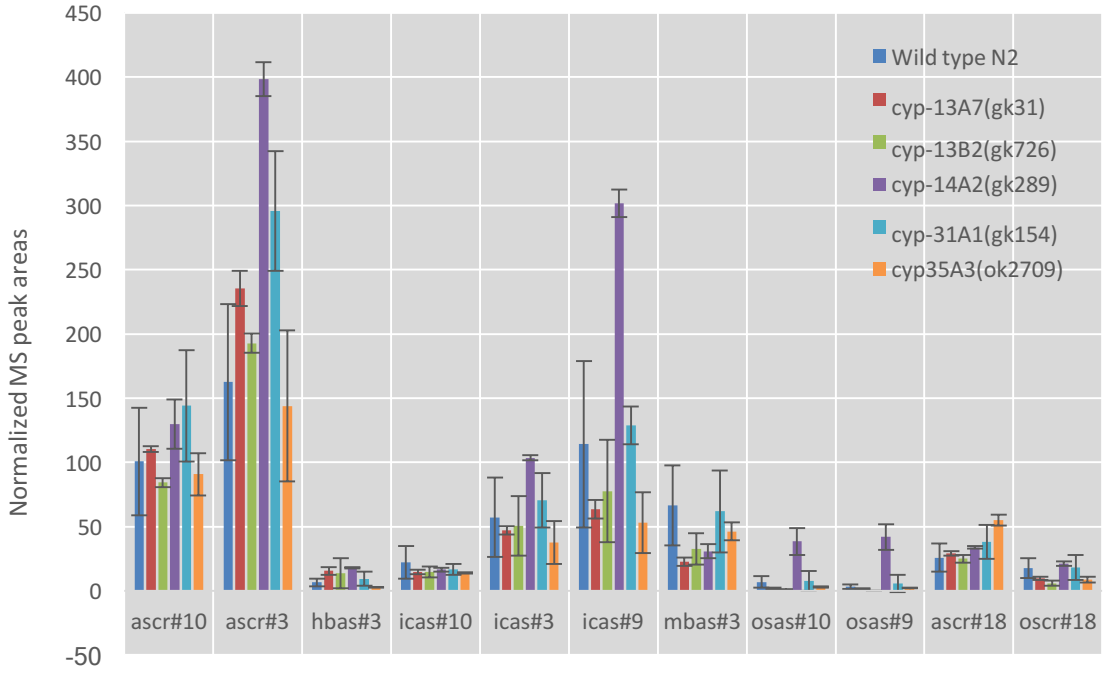

Figure B.3: The *cyp* Gene Family. The *cyp* family encodes for cytochrome P450 enzymes that can act as monooxygenases. Peaks are normalized to worm pellet mass. Averages and standard deviations are based on two biological replicates.

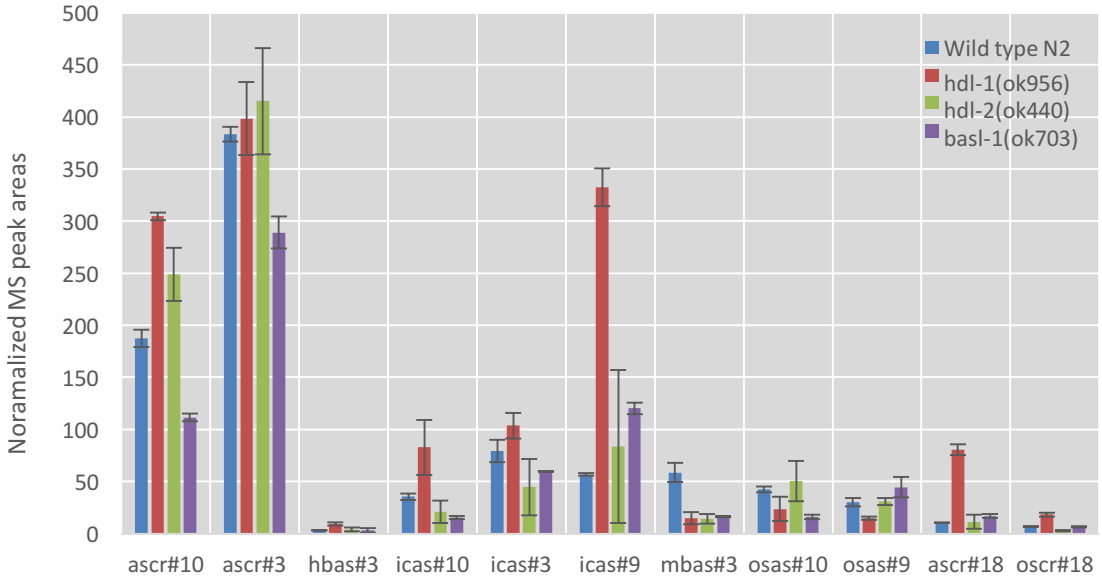

Figure B.4: Decarboxylases. In some bacteria and plants, decarboxylases have been shown to play a role in the biosynthesis of indole-3-carboxylic acid. Peaks are normalized to worm pellet mass. Averages and standard deviations are based on two biological replicates.

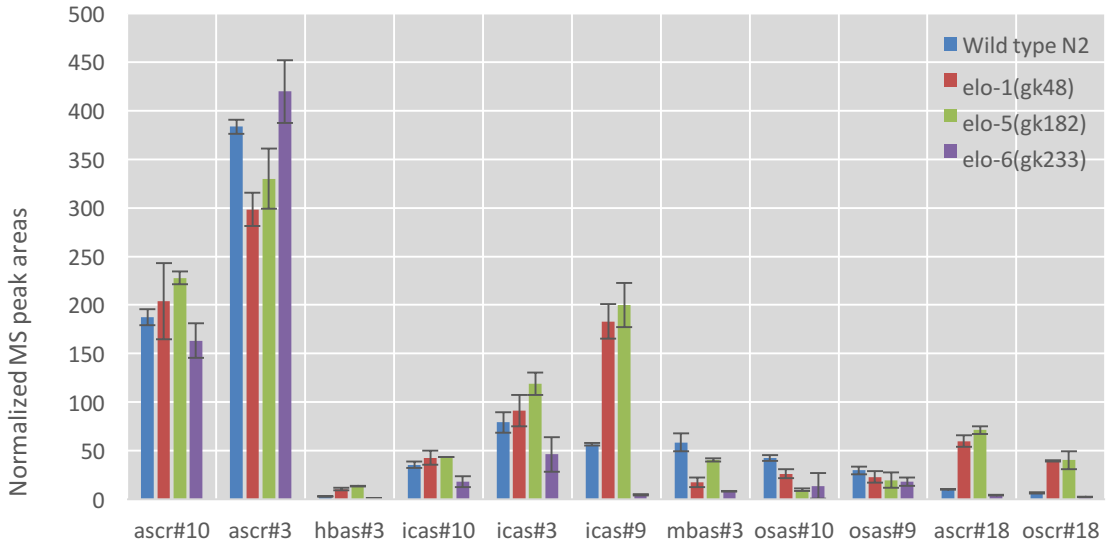

Figure B.5: The *elo* Gene Family. The *elo* genes encode fatty acid elongases. Peaks are normalized to worm pellet mass. Averages and standard deviations are based on two biological replicates.

B.4 References

1. von Reuss, S. H.; Bose, N.; Srinivasan, J.; Yim, J. J.; Judkins, J. C.; Sternberg, P. W.; Schroeder, F. C., Comparative metabolomics reveals biogenesis of ascarosides, a modular library of small-molecule signals in C. elegans. *J Am Chem Soc* **2012,** *134* (3), 1817-24.
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A p p e n d i x C

STRAINS, PLASMIDS, AND PRIMERS

Table C.1: Strains

Strain	Genotype	Source
N2	wild type, Bristol	CGC
RB859	*daf-22(ok693)*	CGC
PS7211	*daf-22(ok693); syIs456[Pvha-6::GFP::DAF-22]*	A. Akagi
PS7212	*daf-22(ok693); syIs457[Pmyo-3::GFP::DAF-22]*	A. Akagi
PS7213	*daf-22(ok693); syIs458[Pdpy-7::GFP::DAF-22]*	A. Akagi
FX06781	*acs-7(tm6781)*	O. Panda
PS7396	*acs-7(tm6781); syIs459[Pacs-7::ACS-7::GFP]*	A. Akagi
PS7447	*unc-119(ed3); syEx1592[Pacs-7::ACS-7::GFP; Pvha-6::mcherrySKL; unc-119(+)]*	A. Akagi
GH10	*glo-1(zu437)*	D. Gems
RB811	*glo-4(ok623)*	CGC
RB662	*apb-2(ok429)*	CGC
GH378	*pgp-2(kx48)*	CGC

Sources:
Caenorhabditis Genetics Center (CGC, Minnesota)
Allison Akagi (A. Akagi, California Institute of Technology, California)
Oishika Panda (O. Panda, Cornell University, New York)

Table C.2: Plasmids

Name	Contents	Source
pAEA1	*Pvha-6::GFP::daf-22::unc-54 3'UTR*	A. Akagi
pAEA3	*Pdpy-7::GFP::daf-22::unc-54 3'UTR*	A. Akagi
pAEA4	*Pmyo-3::GFP::daf-22::unc-54 3'UTR*	A. Akagi
pAEA10	*Pacs-7::acs-7::GFP::unc-54 3'UTR*	A. Akagi
pAEA15	*Pvha-6::mCherrySKL::unc-54 3'UTR*	A. Akagi
HYM433	*Pvha-6::GFP::daf-22::unc-54 3'UTR*	H.Y. Mak
pU57	ACS-7 coding sequence with C-terminal His-tag	H. Le
pPH92	PSM GFP	Bargmann Lab
pPH93	PSM mCherry	Bargmann Lab
unnamed	*Pmyo-3::dsRed*	Sternberg Lab
pDP#MM051	*unc-119* rescue construct	D. Pilgrim
pRB1017	gRNA expression cassette	Fire Lab
pJA58	*dpy-10* gRNA plasmid	J. Arribere
#46168	*Peft-3::cas9-SV40_NLS::tbb-2 3'UTR*	Addgene

Sources:
Allison Akagi (A. Akagi, California Institute of Technology, California)
Ho Yi Mak (H.Y. Mak, Hong Kong University of Science and Technology, Hong Kong)
Henry Le (H. Le, Cornell University, New York)
Cornelia Bargmann Lab (Bargmann Lab, The Rockefeller University, New York)
Paul Sternberg Lab (Sternberg Lab, California Institute of Technology, California)
David Pilgrim (D. Pilgrim, University of Alberta, Edmonton, Canada)
Andy Fire Lab (Fire Lab, Stanford University, California)
Joshua Arribere (J. Arribere, Stanford University, California)
Addgene (Massachusetts)

Table C.3. Primers

Name	Purpose	Sequence
oAA001	Construction of pAEA5 (gRNA sequence)	TCTTGGACGCAACCGGTGCACGAG
oAA 002	Construction of pAEA5 (gRNA sequence)	AAACCTCGTGCACCGGTTGCGTCC
oAA 003	Construction of pAEA6 (gRNA sequence)	TCTTGGGAGGATCTACGGAGAACT
oAA 004	Construction of pAEA6 (gRNA sequence)	AAACAGTTCTCCGTAGATCCTCCC
oAA 005	Construction of pAEA7 (gRNA sequence)	TCTTGCGATTCCGAAGCTTCCGTC
oAA 006	Construction of pAEA7 (gRNA sequence)	AAACCGATTCCGAAGCTTCCGTCC
oAA 007	Construction of pAEA8 (gRNA sequence)	TCTTGCGATTCCGAAGCTTCCGTCGGGG
oAA 008	Construction of pAEA8 (gRNA sequence)	AAACCCCCGACGGAAGCTTCGGAATCGC
oAA 009	*acs-10* CRISPR knockout genotyping	GTTGCCCAGTTCTACTAGTCAACGAAATG
oAA 010	*acs-10* CRISPR knockout genotyping	GAATGACATTGGATCGAGAAAGGGTGTC

Name	Purpose	Sequence
oAA 011	Construction of pAEA4 (*myo-3* promoter)	CGACTCACTATAGGGCGAATTGGGTACCGG CTATAATAAGTTCTTGAATAAAATAATTTTC CC
oAA 012	Construction of pAEA4 (*myo-3* promoter)	GTTCTTCTCCTTTACTCATGGCGCGCCTCTA GATGGATCTAGTGGTCGTG
oAA 013	Construction of pAEA3 (*dpy-7* promoter)	CTCACTATAGGGCGAATTGGGTACCTCATT CCACGATTTCTCGCAACACATC
oAA 014	Construction of pAEA3 (*dpy-7* promoter)	CTCCTTTACTCATGGCGCGCCTTATCTGGA ACAAAATGTAAGAATATTCTTAAAAATTG
oAA 015	Construction of pAEA1 (*unc-54* 3'UTR)	CTGCACAGTCCAAGATTTGAAGGCCTGGCC GCTGTCATCAGAGTAAG
oAA 016	Construction of pAEA1 (*unc-54* 3'UTR)	CAGGAATTCGATATCAAGCTTATCGATGTA CGGCCGACTAGTAGGAAAC
oAA 017	Construction of pAEA1 (*GFP::daf-22*)	CTACCAAAACCCATAAAAAGGGCGCGCCA TGAGTAAAGGAG AAGAAC
oAA 018	Construction of pAEA1 (*GFP::daf-22*)	CTTACTCTGATGACAGCGGCCAGGCCTTCA AATCTTGGACTGTGC
oAA 019	Construction of pAEA1 (*vha-6* promoter)	GACTCACTATAGGGCGAATTGGGTACCCTC AACGTTGCCAGTGATGAATC
oAA 020	Construction of pAEA1 (*vha-6* promoter)	GTTCTTCTCCTTTACTCATGGCGCGCCCTTT TTATGGGTTTTGGTAGGTTTTAG

Name	Purpose	Sequence
oAA 065	Construction of pAEA10 (*acs-7* promoter)	GATTACGCCAAGCTTGCATGCGGCCGGCCT GCCACATCAAAAAGATTCGCTCAAC
oAA 066	Construction of pAEA10 (*acs-7* promoter)	CTTTGGCCAATCCCGGGGATCCGGCGCGCC TTCTGAAAATGTTGTGATCGAAAGG
oAA 067	Construction of pAEA10 (*acs-7* cDNA)	CATTTTCAGGAGGACCCTTGGATGATATTTC ACGGTGAACAACTTGAG
oAA 068	Construction of pAEA10 (*acs-7* cDNA)	CTCCTTTACTCATTTTTTCTACCGGCAATTTA GCCTTTTTTGCATCC
oAA 077	pAEA10 sequencing	CCAAGAGAAAGATCCTGATAATGTTG
oAA 078	pAEA10 sequencing	CGA TGA ATA GGG TAA GAA TAC AAT ATC
oAA 079	pAEA10 sequencing	GGAGAACTTTGGATTAAGGGTCCT
oAA 080	pAEA10 sequencing	GGT AAG TTT TCC GTA TGT TGC ATC
oAA 093	Construction of pAEA15 (mCherrySKL)	CAAAACCCATAAAAAGGATCCCCGGGATGG TGAGCAAGGGCGAGGAG
oAA 094	Construction of pAEA15 (mCherry SKL)	GCCGATGCGGCTAAGATCTGGTACCTTAGA GCTTAGACTTGTACAGCTCGTCCATGCCGC
oAA 095	pAEA15 sequencing	GTGTGGAATTGTGAGCGGATAAC
oAA 096	pAEA15 sequencing	CCTTGGTCACCTTCAGCTTGG
oAA 097	pAEA15 sequencing	GTCTTCGGTTTTCAGTCTTTAGTTC
oAA 098	pAEA15 sequencing	GAG AAA GAG CAT GTA GGG ATG TTG

Name	Purpose	Sequence
oHW36f	*acs-10* CRISPR deletion repair	CACTTGAACTTCAATACGGCAAGATGAGAA TGACTGGAAACCGTACCGCATGCGGTGCCT ATGGTAGCGGAGCTTCACATGGCTTCAGAC CAACAGCCTAT