Chapter 2

Materials and Methods

2.1. Reagents

2.1.1. *C. elegans*

2.1.1.1. *C. elegans* strains

See Table 2.1. for *C. elegans* strains that were used in this study. The *C. elegans* Genetics Center, USA (http:// www.cbs.umn.edu/CGC/) and the National Bioresources Project, Japan (http:// shigen.lab.nig.ac.jp/c.elegans/index.jsp) provided these strains.

2.1.1.2. Nematode Growth Medium (NGM)

NaCl	3 g
Peptone	2.5 g
Optional[a]: Agar	19 g
dd H_2O	to 1 L

The solution was autoclaved and cooled to 55°C before addition of:

Cholesterol solution (5 mg/ml in ethanol)	1 ml
1M $CaCl_2$	1 ml
1M $MgSO_4$	1 ml
1M KH_2PO_4, pH6.0	25 ml
Fungizone	800 µl

in the order as written, with mixing thoroughly after addition of each component. Solutions were sterile-filtrated through a membrane filter with a pore size of 0.2 µm.

[a] For preparation of agar plates, solution was poured into sterile Petri dishes.

2.1.1.3. M9 Buffer

1M KH_2PO_4	3 g
1M Na_2HPO_4	6 g
1M NaCl	5 g
ddH_2O	to 1 L

1 ml 1M $MgSO_4$ was added after solution had been autoclaved to sterilize.

Gene(allele)	Strain	L1 worms added	Screening temperature
Wild-type	N2	~10	20°C
rrf-3(pk1426)	NL2099	~15	20°C
arx-3	tm1681	~15	20°C
C26E6.3(ok1728)	RB1477	~15	20°C
emb-27(ax81)	DS88	~15	15°C
emb-27(g48)	GG48	~10	15°C
emb-27(ye143)	HY621	~15	15°C
emb-27(g48)	TJ1047	~10-15	15°C
emb-27(g48)	TJ1049	~15	15°C
emb-27(g48)	TJ1061	~15	15°C
mat-1(ax161)	DS77	~15	15°C
mat-1(ax144)	DS80	~15	15°C
mat-1(ye121)	HY604	~15	15°C
cdc-42(ok825)	RB942	~10	20°C
dhc-1(or195)	EU828	~15	15°C
dyn-1(ky51)	CX51	~15	20°C
sel-9(ar22)	GS107	~15	20°C
pes-1(leDf1)	UL768	~10-15	20°C
pfd-4(gk430)	VC1032	~15	20°C
fkh-10(ok733)	RB884	~10	20°C
pqn-19(ok406)	RB674	~15	20°C
C43E11.2	tm1937	~15	20°C
F57C7.2(ok661)	RB836	~15	20°C
pch-2(tm1458)	CA388	~15	20°C
R06F6.2(ok1664)	RB1457	~15	20°C
div-1(or148)	EU548	~10	15°C
div-1(or148)	EU550	~15	15°C
div-1(or345)	EU879	~15	15°C
div-1(or345)	EU880	~15	15°C
xpa-1(ok698)	RB864	~10	20°C
ubc-1(gk14)	VC18	~10	20°C
F58G6.1	tm1060	~10	20°C
him-6(ok412)	VC193	~15	20°C
R07E5.3(ok622)	RB810	~15	20°C
gta-1(ok517)	RB748	~10	20°C
K08E3.5(ok233)	MG278	~10	20°C
C17H12(ok548)	RB769	~10	20°C

Table 2.1. Conditions for high-throughput RNA interference by liquid feeding
For each *C. elegans* strain used in this study, genotype ('Gene(allele)'), the number of L1 worms that were added per well of a 96-well plate, and the incubation temperature for RNA interference screens ('Screening temperature') are shown.

2.1.1.4. Freezing buffer

1M KH_2PO_4	3 g
0.05 M K_2HPO_4	129 ml
0.05 M KH_2PO_4	871 ml
NaCl	5.85 g
Glycerin	30% (v/v)

2.1.1.5. Bleach solution

NaOH	250 µl
Sodium hypochlorite	100 µl
Autoclaved H_2O	to 1000 µl

2.1.2. Bacteria

2.1.2.1. Ahringer RNAi feeding library

Bacterial clones used for RNA interference (RNAi) experiments were selected from the Ahringer RNAi feeding library (Kamath *et al.*, 2003).

2.1.2.2. Luria-Bertani (LB) medium

Bacto-tryptone	10 g
Bacto-yeast extract	5 g
NaCl	10 g
Optional[a]: Bacto-Agar	15 g
ddH_2O	to 1 L

pH was adjusted to 7.2 and solution was autoclaved to sterilize.

[a] For preparation of agar plates, solution was poured into sterile Petri dishes.

2.1.2.3. 2 x Tryptone / yeast extract (TY)

Bacto-tryptone	16 g
Bacto-yeast extract	10 g
NaCl	5 g
dd H_2O	to 1 L

pH was adjusted to 7.2 and solution was autoclaved to sterilize.

2.2. Protocols

2.2.1. Maintenance of *C. elegans* stocks

C. elegans was maintained on NGM agar plates seeded with OP50 *E. coli* according to standard protocols (Brenner, 1974).

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2.2.2. Freezing and recovery of *C. elegans* stocks

For freezing, worms that were approaching starvation — L1 and L2 worms survive freezing best — were washed off plates in M9 buffer, pelleted by centrifugation at 1,000 rpm for 1 minute, and resuspended in an equal volume of M9 and freezing buffer. 1 ml of suspension was aliquoted per 1.8 ml cryovial. Cryovials were placed into freezing boxes filled with isopropanol to allow a gradual 1°C decrease in temperature per minute when placed at -80°C. Cryovials were stored at -80°C.

For thawing, cryovials were placed at room temperature and worms were spotted onto NGM plates seeded with OP50 *E. coli* as soon as all ice had turned to liquid.

2.2.3. High-throughput RNA interference liquid-feeding assay

All RNA interference (RNAi) experiments were performed by using bacterial feeding in liquid cultures in 96-well format.

Preparation of bacteria:

Bacterial glycerol stocks were replica plated onto LB plus 100 µg/ml ampicillin plates using a 96-pin replicating tool and grown overnight at 37°C. The day before starting the screen, bacteria were inoculated in 400 µl 2 x TY containing 100 µg/ml ampicillin in 2-ml 96-well plates and grown overnight to saturation (~15 hours) in a shaking incubator at 220 rpm at 37°C.

Induction of bacteria:

The following morning, expression of double-stranded RNA (dsRNA) was induced by addition of isopropyl-beta-D-thiogalactopyranoside (IPTG) to a final concentration of 4 mM to each well of bacterial cultures and incubated while shaking at 220 rpm for 1 hour at 37°C. Subsequently, bacterial culture medium was replaced by nematode growth medium (NGM). Therefore, bacterial cultures were pelleted by spinning at 3,500 rpm for 5 minutes, the supernatant discarded by quickly inverting the 2-ml 96-well plates, and pelleted bacterial clones were resuspended in 400 µl of NGM plus 100 µg/ml ampicillin and 4 mM IPTG.

Preparation of worms:

96-well RNAi liquid-feeding assays were started with L1 worms. Synchronized populations of L1 worms were obtained by filtration of mixed-stage populations through an 11 µm nylon mesh (MultiScreen^{TM} Nylon Mesh, Millipore) or by bleaching adult worms and allowing their eggs to hatch in M9 buffer.

Filtering: When synchronizing worms by filtration, it is recommended to use worm populations that are approaching starvation, to avoid dilution of dsRNA-expressing bacteria with OP50 *E. coli*. Meshes were sterilized before each filtration with 70% v/v ethanol. Worms were washed off plates in M9 buffer, and transferred into a mesh placed on top of a 2-ml 96-well plate. L1 worms were passed through the mesh by centrifugation at 1,000 rpm for 30 seconds. Synchronized L1s were collected and diluted to a concentration of approximately 10 L1s (for worm strains with a brood size similar to wild-type worms) or 15 L1s (for strains with reduced brood size as compared to wild-type) per 15 µl M9 buffer.

Bleaching: An alternative approach for obtaining synchronized populations of L1 worms was the bleaching of gravid adults and allowing their eggs to hatch overnight in M9 buffer. Therefore, gravid adult worms were washed off plates in M9 buffer and collected by centrifugation at 1,000 rpm for 1 minute. Supernatant was aspirated off, and 1-2 ml bleach solution (depending on the size of the worm pellet) was added. Worms were incubated in bleach solution with occasional vortexing until dissolved and only embryos remained. To remove bleaching solution, eggs were resuspended in 10 ml M9 buffer and subsequently centrifuged at 1,000 rpm for 1 minute. This step was repeated three times. Worms were allowed to hatch overnight in M9 buffer with gentle rocking to allow aeration. The next morning, larvae were pelleted by centrifugation at 1,000 rpm for 1 minute and diluted to a final concentration of 10 to 15 L1s per 15 µl M9 buffer.

RNAi feeding experiments:

Worms in a final concentration of 10 to 15 L1s per 15 µl M9 buffer were pipetted into each well of a 96-well flat-bottom plate from a plastic tray by using a 12-well multi-channel pipette. To avoid settling of worms in the plastic tray, worms were pipetted up and down before aliquoting. Subsequently, 40 µl of resuspended bacterial culture were added to each well. For combinatorial RNAi feeding experiments, resuspended cultures of different bacterial strains were mixed to give a final volume of 40 µl.

Plates were incubated shaking at 150 rpm, 20°C, for 4 days (15°C for 6 days for temperature-sensitive genetic mutants; see Table 2.1. for screening conditions for all strains used in this study). To avoid evaporation of liquid from wells, 96-well plates were stacked in sealable plastic boxes and covered with a wet tissue.

Scoring of phenotypes:

After an appropriate incubation time (4 to 6 days), worms were screened under a dissecting microscope for sterility, embryonic lethality, growth, and developmental defects. Sterility and embryonic lethality were scored semi-quantitatively on a scale from 0 (wild-type) to 3 (100% sterile or embryonic lethal). In cases where sterility or embryonic lethality appeared enhanced after targeting both genes simultaneously by combinatorial RNAi or by RNAi in a genetic mutant as compared to phenotypes of each individual gene, phenotypes were verified by quantification (see below).

2.2.4. Testing post-embryonic additive RNAi phenotypes and known post-embryonic synthetic genetic interactions

To score post-embryonic phenotypes, L1 larvae were collected from the 96-well liquid-feeding assay 4 days after the screen was set up and allowed to develop further on 12-well NGM plates. Therefore, liquid-feeding cultures were filtered through an 11 µm nylon mesh (MultiScreen^{TM} Nylon Mesh, Millipore) by centrifugation and L1 larvae were spotted onto 12-well NGM plates containing 100 µg/ml ampicillin and 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG), seeded with bacteria expressing a non-targeting dsRNA (Ahringer library clone Y95B8A_84.g). Adult worms were scored after further incubation at 20°C for 72 hours. Since I was assessing second-generation (post-embryonic) phenotypes, I had to exclude genes that resulted in sterility, embryonic lethality, or larval growth arrest after RNAi. Only genes that were (according to the above criteria) amenable to analysis in both wild-type worms and the RNAi-hypersensitive *rrf-3* background could be included in this study.

2.2.5. Investigating dilution effects induced by combinatorial RNAi

To investigate the extent to which combining dsRNA-expressing bacteria leads to a reduced strength of RNAi phenotypes, I evaluated the average failure rate for the successful generation of a phenotypically detectable knockdown for single genes at a given dilution. I therefore added unrelated control dsRNA-expressing bacteria to bacteria expressing dsRNA against genes with previously known non-viable RNAi phenotypes at 2-, 3-, 4-, 5-, and 10-fold dilution. The Ahringer library clone Y95B8A_84.g, expressing dsRNA that does not target an expressed sequence in *C. elegans* and bacteria expressing dsRNA against *lin-31*, respectively, were used as control dsRNA-expressing bacteria.

2.2.6. Estimating the false-negative rate of combinatorial RNAi

Assuming each gene is an independent targeting event in combinatorial RNAi, I estimated the false-negative rate of combinatorial RNAi for identifying multigenic

interactions by calculating the detection rate of n-genic interactions to be x^n, where x is the detection rate of single-gene phenotypes at n-fold dilution.

2.2.7. Identification of *C. elegans* gene duplicates

I used the INPARANOID algorithm (Remm *et al.*, 2001) (version 4.0) to identify genes that have been duplicated in the genome of *C. elegans* since divergence from *S. cerevisiae* and *D. melanogaster*, respectively. I therefore identified single orthologues in *S. cerevisiae* and *D. melanogaster* genomes that correlate to duplicate gene pairs in *C. elegans*.

2.2.8. Identification of *C. elegans* orthologues of *S. cerevisiae* gene pairs with synthetic lethal interactions

The INPARANOID algorithm (Remm *et al.*, 2001) (version 4.0) was used to identify *C. elegans* orthologues of all *S. cerevisiae* gene pairs that were reported to have synthetic lethal or sick (SL) interactions in at least one of three genome-scale screens (Davierwala *et al.*, 2005; Pan *et al.*, 2006; Tong *et al.*, 2004). I only tested for genetic interactions between gene pairs that both had a single orthologue in *C. elegans*.

2.2.9. Identification of synthetic genetic interactions using combinatorial RNAi

For the identification of synthetic genetic interactions between *C. elegans* gene duplicates, I excluded all genes that are targeted by bacterial clones from the *C. elegans* whole-genome RNAi library (Kamath and Ahringer, 2003) with inserts having more than 80% nucleotide identity over 200 bp with multiple predicted genes from the analysis. This is the threshold for cross-reaction used in Kamath *et al.* (2003). Furthermore, genes that resulted in first-generation larval growth arrest after RNAi were not included in any study for synthetic genetic interactions, since this strong phenotype cannot be enhanced any further.

When screening for genetic interactions using combinatorial RNAi, single-gene RNAi phenotypes (as references) were compared with combinatorial RNAi phenotypes side by side. To account for dilution effects arising from combining two dsRNA-expressing bacteria, equal amounts of non-targeting dsRNA-expressing bacteria (Ahringer library clone Y95B8A_84.g) were added to bacteria expressing dsRNA targeting the reference genes. This setup allowed combinatorial RNAi to be performed in triplicates within independent screens, and RNAi against each gene individually in duplicates within independent assays.

Screens for synthetic genetic interactions were performed at least twice independently in duplicate/triplicate within independent assays. Synthetic phenotypes needed to be unambiguous and reproducible in at least two independent RNAi experiments to be scored positive.

Qualitatively observed synthetic lethal phenotypes were further verified by quantification. Therefore, larvae, unhatched eggs, and adults from each RNAi experiment were manually counted. To facilitate counting of worms and dead embryos, respectively, NaN_3 was added to a final concentration of 25mM to each well before worms were spotted onto empty 12-well NGM plates. Quantitative phenotype data were subject to statistical analysis as described under ‘Statistical analysis of quantitative phenotype data under a multiplicative model’.

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2.2.10. Identification of synthetic lethal interactions using RNAi in genetic mutants

When testing for genetic interactions using RNAi to target a single gene in a *C. elegans* strain carrying a homozygous viable loss-of-function allele (see Table 2.1. and Appendix Table 5.2.), RNAi phenotypes seen in the genetic mutants were compared to the RNAi phenotypes of wild-type worms and to the phenotypes of the genetic mutants fed on bacteria expressing a dsRNA that does not target an expressed portion of the *C. elegans* genome (Ahringer library clone Y95B8A_84.g).

Screens for synthetic genetic interactions were performed at least twice independently in duplicate within independent assays. Synthetic phenotypes needed to be

unambiguous and reproducible in at least two independent RNAi experiments to be scored positive.

To exclude false synthetic phenotypic effects caused by non-specific additive effects of genetic mutants and RNAi phenotypes, respectively, when screening for synthetic phenotypes, mutant strains that showed enhanced phenotypes when targeting a second gene by RNAi as compared to the phenotypes observed in wild-type worms were also fed on additional RNAi clones that produce phenotypes similar to the putatively interacting gene in wild-type worms.

2.2.11. Statistical analysis of quantitative phenotype data under a multiplicative model

To be able to unambiguously identify SL interactions, quantitative phenotype data were subject to statistical analysis. Therefore, measurements of brood size and embryonic viability following RNAi were normalized to measurements obtained when worms were fed on bacteria expressing dsRNA against control genes that give no detectable phenotypes (‘wild-type brood size’ and ‘wild-type embryonic survival’). In cases where measurements for brood size and embryonic viability exceeded 100% of wild-type brood and viability, values were set to 100% of wild-type values.

I analysed quantitative phenotype data under a multiplicative model. In this model, the null hypothesis for the contribution of independent genetic loci to a phenotype is that the double mutant RNAi phenotype equals the product of mutant phenotypes associated with each individual gene. Thus, to examine whether the combinatorial RNAi phenotypes were truly synthetic or merely additive, I compared the quantitative phenotypes following combinatorial RNAi with the calculated products of measurements for both individual genes of a pair. I therefore multiplied duplicate brood size and embryonic survival measurements obtained after RNAi against each gene individually (n=2) in two independent experimental setups (n=2) in all possible pairwise combinations. That way I obtained sixteen values (n=4x4) that represented the expected brood size and embryonic survival measurements if genes do not interact. This array of

calculated products was compared to six measurements (n=3x2) obtained after combinatorial RNAi in two independent experiments.

I used a Student's t-Test (two-tailed distribution, two-sample equal variance) to determine whether the observed values differed significantly from the expected values. I considered genes to be synthetic lethal if p-values were below 5.0×10^{-2}.

2.2.12. Evolutionary analysis

The INPARANOID algorithm (version 4.0) was used to identify *C. elegans* orthologues of *C. briggsae* genes (Remm *et al.*, 2001). If both *C. elegans* duplicates had a single identifiable orthologue in *C. briggsae*, this implies that the duplication predates the divergence of *C. elegans* from *C. briggsae*. Protein sequences were aligned using the CLUSTAL W program to determine the percentage of identity between gene duplicates (Thompson *et al.*, 1994). Numbers of synonymous nucleotide substitutions per synonymous site (Ks) and non-synonymous nucleotide substitutions per non-synonymous site (Ka) were estimated using the Maximum Likelihood Method (Goldman and Yang, 1994; Muse and Gaut, 1994).

2.2.13. Comparative analysis of synthetic genetic interactions compiled from literature

Previously known genetic interactions were extracted from BIOGRID (for *S. cerevisiae*; Stark *et al.*, 2006), WormBase (for *C. elegans*; www.wormbase.org), and FlyBase (for *D. melanogaster*; Crosby *et al.*, 2007), respectively, and the INPARANOID algorithm (version 5.1) was used to identify single orthologues between these species.

2.2.14. Imaging

For imaging, worms were pipetted from the 96-well RNAi liquid-feeding assay onto microscope slides. One drop of 25mM NaN_3 was added to worms in liquid before adding a coverslip. Worms with adult lethal phenotypes were very fragile and thus could

not be covered with a coverslip for imaging. Zeiss Stemi SV11 microscope plus Axiovision software version 7.0 were used to capture images.

2.2.15. Amplification of DNA fragments by polymerase chain reaction (PCR)

To confirm the identity of RNAi clones, gene-specific DNA fragments were amplified by PCR and verified by sequencing. For each clone, a separate PCR reaction was performed. Therefore, a small amount of bacterial clones was added to ~ 5 µmol of each primer, 1x Bioline NH_4 reaction buffer, ~37.5 mM $MgCl_2$, 20 mM dNTPs (Boehringer) and 0.2 µl *Taq* DNA polymerase (Bioline) in a 25µl reaction.
PCR Machines were preheated to 94°C for five minutes, followed by 34 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 2 minutes. PCR reactions were stored at -20°C until use. Sequencing of PCR products was performed using standard protocols at The Wellcome Trust Sanger Institute using 5 µmol of sequencing primers for each reaction.

Primers:
L4440 forward primer: 5'—AGCGAGTCAGTGAGCGAGGAAGC—3'
L4440 reverse primer: 5'—GGTTTTCCCAGTCACGACGTTG—3'
Sequencing primer: 5'—TCGAGGTCGACGGTATCG—3'