# Report

# Fertility and Germline Stem Cell Maintenance under Different Diets Requires *nhr-114*/*HNF4* in *C. elegans*

Xicotencatl Gracida[1] and Christian R. Eckmann[1,*]
[1]Max Planck Institute of Molecular Cell Biology and Genetics, Pfotenhauer Str. 108, 01307 Dresden, Germany

## Summary

Animals can thrive on variable food resources as a result of autonomous processes and beneficial relationships with their gut microbes [1]. Food intake elicits major physiological changes, which are counteracted by transient systemic responses that maintain homeostasis in the organism. This integration of external information occurs through cellular sensory elements, such as nuclear receptors, which modulate gene expression in response to specific cues [2]. Given the importance of germline stem cells (GSCs) for the development of the germline and the continuity of species, it is reasonable to assume that GSCs might be shielded from the negative influence of environmental perturbations. To our knowledge, however, there are no mechanisms reported that protect GSCs from harmful dietary metabolites. Using *Caenorhabditis elegans* as a model, we report that the somatic activity of the conserved nuclear receptor *nhr-114*/*HNF4* protects GSC integrity from dietary metabolites. In the absence of *nhr-114* and on certain bacterial diets, otherwise somatically normal animals accumulate germ cell division defects during development and become sterile. We found that, in *nhr-114*(−) animals, the induction of germline defects and sterility depend on bacterial metabolic status, with respect to the essential amino acid tryptophan. This illustrates an animal-microbe interaction in which somatic nuclear receptor activity preserves the germline by buffering against dietary metabolites, most likely through a somatic detoxifying response. Overall, our findings uncover an unprecedented, and presumably evolutionarily conserved, soma-to-germline axis of communication that maintains reproductive robustness on variable food resources.

## Results and Discussion

### Loss of *nhr-114*/*HNF4* Induces Diet-Sensitive Sterility

Nuclear receptors (NRs) or nuclear hormone receptors (NHRs) are ligand-activated transcription factors and act as prevalent mediators of metazoan organ physiology [2]. For example, the broadly conserved hepatocyte nuclear factor HNF4 regulates metabolic homeostasis by coordinating glucose and lipid metabolism [3–5]. Interestingly, HNF4 orthologs are highly expanded in *C. elegans* [6]. In an RNA interference (RNAi) screen for genes necessary for germline development in *C. elegans*, we identified *nhr-114*, an ortholog of mammalian HNF4 (Figure 1A). Injection-mediated RNAi of *nhr-114*, but not its closest paralog *nhr-68*, causes high penetrance of sterility (no embryos are produced) (Figure 1B, and Figure S2 and Table S2 available online). Two *nhr-114* mutant alleles,

*Correspondence: eckmann@mpi-cbg.de

*gk849* and *ef24*, also cause sterility (Figures 1A and 1B and Table S1). Our transcript analysis predicts that *gk849* most likely produces no NHR-114 protein and that *ef24* might produce a protein with a truncated ligand-binding domain (Figure S1A). Importantly, *nhr-114* RNAi knockdown into *nhr-114(gk849)* or *nhr-114(ef24)* neither increased the penetrance of sterility nor changed the type of sterility (Supplemental Experimental Procedures), confirming that the sterility phenotype of *nhr-114*(−) animals arises from impaired *nhr-114* function. Altogether, this confirms a requirement of *nhr-114*/*HNF4* for fertility.

Unexpectedly, we found that *nhr-114* sterility is linked to bacterial diet. Although *nhr-114*(−) adult animals are sterile on *E. coli* OP50 food, the standard *C. elegans* laboratory diet [7], an *E. coli* HT115(DE3) diet abolished sterility and animals were fertile (Figure 1C). These bacterial strains derive from two major *E. coli* lineages: B (OP50) and K-12 (HT115) [8]. Although HT115(DE3) is commonly used in bacterial RNAi feeding experiments [9], these bacteria prevented *nhr-114*(−) sterility independently of double-stranded RNA (dsRNA) production (Figure S3B), suggesting that a number of similar phenotypes in large-scale RNAi screens may have been masked by this diet.

We tested additional bacteria commonly used for feeding *C. elegans* [10]. Although wild-type (WT) animals are fertile on any of these diets, *nhr-114*(−) animals were sterile when fed the *E. coli* B strain BL21(DE3) but fertile when fed *E. coli* K-12 strains or bacterial soil isolates (Figures 1C and S3A). Interestingly, we observed an intermediate penetrance of *nhr-114* sterility when feeding a 1:1 mixture of OP50 and HT115 bacteria (Figure 1C). This suggests that either HT115 provides metabolites that are lacking in OP50 or HT115 has a neutral role but OP50 produces harmful metabolites. Altogether, our results indicate that, in the absence of *nhr-114*, bacterial-strain-specific traits compromise the fertility of *C. elegans*, exposing a diet-sensitive sterility phenotype. In other words, *nhr-114*/*HNF4* function ensures fertility regardless of dietary variability.

### Diet-Sensitive Sterility Is Due to Germ Cell Proliferation Defects

How are *nhr-114* loss and diet-sensitive sterility linked to postembryonic germline development? Because we observed no obvious somatic morphological defects in adult *nhr-114(gk849)* animals, we focused on germ cells, which are organized in a spatiotemporal manner in the hermaphrodite germline (Figure 2A). Most distally, a pool of mitotically dividing germ cells, which included germline stem cells (GSCs) [11], replenished the proximally differentiating gametes. Upon the reduction of *nhr-114* function (by RNAi or in mutants) and the exposure to an OP50 diet, prevalent cellular defects were observed: *nhr-114*(−) germlines were small and defective in their syncytial organization and lacked the typical row of differentiated oocytes although sperm was present (Figure 2B and Table S2). This suggests that a defect in oocyte production in hermaphrodites is a main cause for sterility. Consistent with a diet-sensitive sterile phenotype, *nhr-114* germlines looked normal when animals were fed
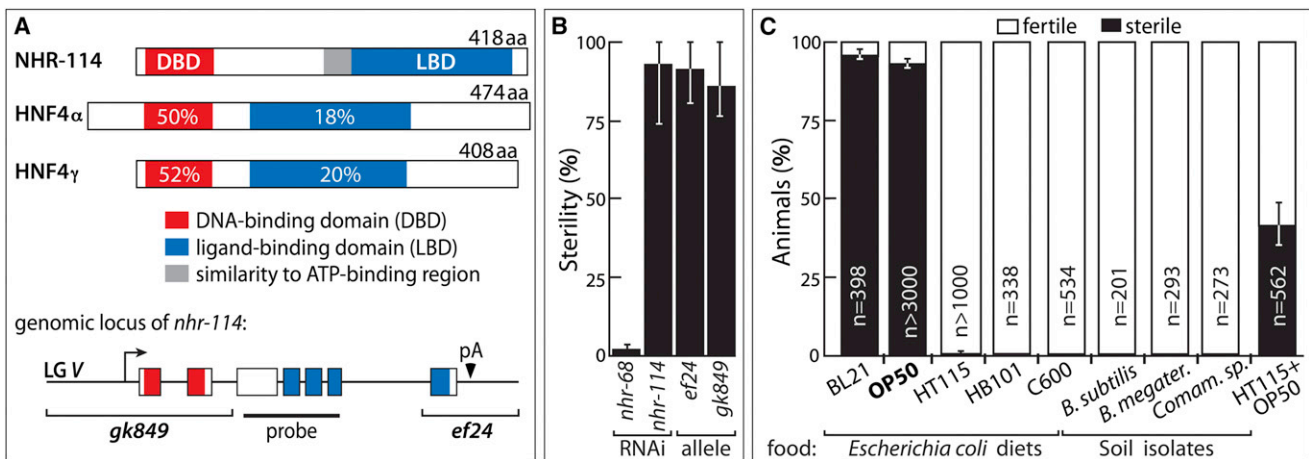
Figure 1. Fertility of *nhr-114*(−) Animals Is Sensitive to the Food Source

(A) The unique protein structure of NRs combines a DNA-binding domain with a ligand-binding domain. Nematode NHR-114 is most similar to HNF4 alpha (α) and HNF4 gamma (γ) in mammals, as determined by Protein BLAST. Amino acid identity (%) was determined by ClustalW2 multiple sequence alignments. *nhr-114* genomic locus maps to −6.22 on linkage group (LG) V. The arrow indicates the direction of transcription. Exons are shown as boxes. pA indicates the 3′end formation site. Deleted regions in *gk849* and *ef24* alleles and the hybridization probe for mRNA expression analyses are indicated.

(B) The penetrance of sterility on a standard OP50 diet is shown. *nhr-68(RNAi)* and *nhr-114(gk849)*, n > 1,300 animals each; *nhr-114(RNAi)* and *nhr-114(ef24)*, n > 3,000 animals. Error bars indicate range among experiments.

(C) The fecundity of *nhr-114(RNAi)* adults on different bacterial diets is shown. In parallel, wild-type animals are 100% fertile on any of the given diets (n > 100 animals). Soil bacteria isolates include *Bacillus subtilis*, *B. megaterium*, and *Comamonas species*. Error bars indicate SEM.

See also Figures S1 and S2 and Tables S1 and S2.

diets that did not induce sterility (Figures 2B, S3A, and S3B). Similar germ cell division defects are also present in adult *nhr-114*(−) male germlines (Figure S4E).

Differential interference contrast (DIC) and immunostaining revealed that the distal region, which houses proliferative germ cells, is severely affected in *nhr-114*(−) animals on a diet of OP50 food (Figures 2B and S4B). In WT germlines, equally spaced nuclei are present with homogeneously distributed chromatin. By contrast, *nhr-114*(−) nuclei are of unequal size, cluster together, and are often fragmented (Figures 2C, 2D, and S4C), resembling mitosis- and cytokinesis-defective mutants [12, 13]. Moreover, cortical staining revealed cellular irregularities, including a high level of multinucleation (Figures 2C and S4C) and nuclear aberrations that also affect the most distal germline (Figures 2C and 3B), which overlaps with the presumed GSC pool [14, 15]. Altogether, these results indicate that *nhr-114* activity is required for proper germ cell divisions and for maintaining homeostasis for germ cells in varying diets.

To investigate at which stage the diet-sensitive sterile phenotype arises, we characterized larval germline development. At early larval stages, WT and *nhr-114(RNAi)* germlines are similar in both germ cell number and morphology (Figures 2D and S4D). However, at L3 onset, the proliferative germ cell pool expands rapidly in WT germlines; yet, it fails to expand and remains small in *nhr-114*(−) (Figure 2D). Concomitantly, 64% of *nhr-114*(−) gonads (n = 42) showed defective multinucleated germ cells appearing at the most distal end (Figure 2D), in close proximity to the stem cell niche [11]. Later in development, this distal multinucleation defect became more prevalent (81% at the L4 stage; n > 37). Thus, a simple explanation for the origin of the diet-sensitive sterile phenotype is that proliferative germ cells progressively accumulate cell-division defects, which reduce their competence for meiotic differentiation and gamete production. In the case of GSCs, these

defects compromise their ability to populate a robust proliferative pool as well.

An animal's nutritional status modulates GSC division dynamics [15–17]; well-fed or underfed conditions increase or decrease the GSC pool, respectively. Diet-sensitive *nhr-114*(−) sterility differs from this reversibility, and a strict developmental dependency is apparent when animals are transferred from OP50 to HT115 food. Although diet-sensitive sterility is prevented in early larval stages, L3 and older animals developed this type of sterility (Figure 2E), which correlated with the timely appearance of abnormal nuclei in the most distal germline (Figure 2D). Together, these observations show that the activity of *nhr-114/HNF4* is crucial for postembryonic germline development and that it preserves proper germ cell and GSC divisions.

**Somatic NHR-114 Protects Proliferative Germ Cells against Dietary Metabolites**

To date, HNF4 orthologs have been implicated in the enteric tract as key metabolic regulators [18] but have not yet been implicated in germ cell development. Northern blot analysis revealed somatic and germ cell expression of *nhr-114* messenger RNA (mRNA) (Figures S1B–S1C). RNA in situ hybridizations showed that *nhr-114* mRNA is present in germ and intestinal cells (Figures S1D–S1E). To test whether *nhr-114* intestinal expression supports germline development and fertility, we generated animals expressing an epitope-tagged, RNAi-insensitive *nhr-114* transgene in the intestines (EV441). Similar to NRs that act as transcription factors, NHR-114 is enriched in intestinal nuclei (Figure S1F). Next, we reduced endogenous *nhr-114* by injection-mediated RNAi in EV441, leaving the RNAi-insensitive *nhr-114* transgene as the only source of *nhr-114* activity (Figure 3C). In this case, we quantified a reduction of DNA aberrations in the most distal germ cells and GSCs (Figure 3E). Consistent with this, we
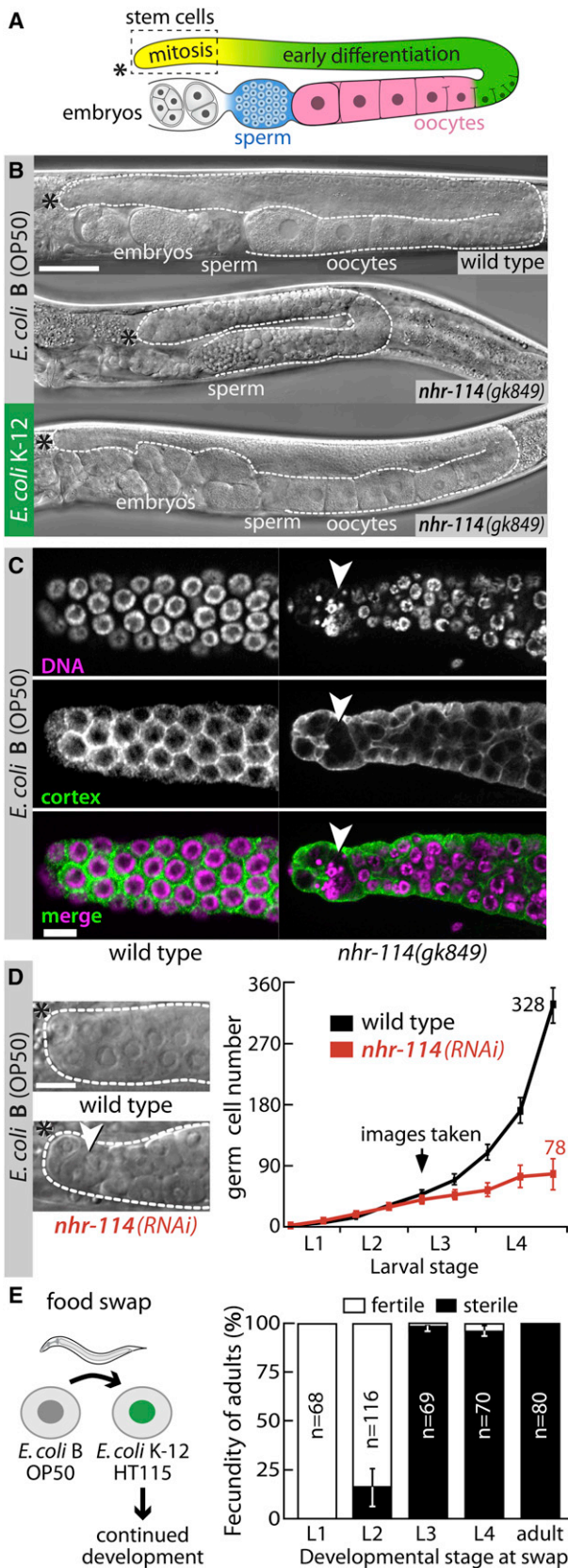
recovered fully fertile animals on an OP50 diet (84%, n = 511), suggesting that somatic NHR-114 activity in the intestine might be sufficient to prevent GSC division defects and sterility.

Additional evidence in support of this somatic role of *nhr-114* can be derived from RNAi experiments with the *rrf-1(pk1417)* mutant background, in which RNAi knockdown occurs mainly in the germline but occurs only weakly in somatic tissues [19]. A significant number of fertile *rrf-1(pk1417); nhr-114(RNAi)* animals were recovered (64%, n = 2150; Table S3), indicating that preservation of somatic *nhr-114* activity promotes fertility. Accordingly, defects in the most distal germ cells and GSCs were reduced (Figure 3E), which was also true for sterile animals, whose germlines had a larger, but not WT-size, mitotic region (Figure 3D). Similarly, *nhr-114* RNAi-treated EV441 germlines were also smaller than WT germlines, suggesting underproliferation (Figure 3C). Altogether, these observations suggest two distinct functions of *nhr-114*: a germ-cell-autonomous function for proliferation activity (as measured by the size of the mitotic region) and a germ-cell-nonautonomous function for proper germ cell divisions and germline integrity, which support fertility.

### Dietary Tryptophan Supplementation Suppresses Diet-Sensitive Sterility

What triggers *nhr-114/HNF4*-mediated somatic responses to prevent *nhr-114*(−) diet-sensitive sterility? We reasoned that different bacterial diets might provide varying levels of nutrients and metabolites, which would subsequently affect GSC proliferation, as reported for protein-rich diets in *Drosophila* and *C. elegans* [16, 20]. L-amino acid supplementation experiments on animals on a diet of OP50 food revealed that *nhr-114*(−) sterility is not sensitive to bulk amino acid levels but is sensitive primarily to tryptophan (Trp) (Figure 4A). A single supplementation of Trp to OP50 or BL21 is sufficient to suppress *nhr-114*(−) sterility and to recover fertility (Figures 4A–4B). Also, we observed a positive effect on fertility with tyrosine, but only when it was combined with other amino

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differentiated germ cells (green) remain connected through openings to a shared cytoplasmic region (syncytium), and growing oocytes start to cellularize.

(B) Diet-sensitive effects on *nhr-114*(−) germline development. Differential interference contrast (DIC) images of adult hermaphrodites (n > 200 each) fed with indicated bacteria. The dotted line delineates the posterior germline. The asterisk marks the distal end. The scale bar represents 50 μm.

(C) Immunostaining of extruded gonads from OP50-fed adults (n > 50). In wild-type germlines, each germ cell contains a single nucleus (DAPI-stained) with a surrounding cytoplasm that is partially enveloped by a cell membrane and a cytoskeletal actin cortex with associated proteins such as anillin (anti-ANI-2 staining). The arrowhead marks an enlarged germ cell with multinucleation defects. The distal end is to the left. The scale bar represents 5 μm.

See also Figure S4A for the entire germline.

(D) Analysis of germline proliferation during development on a diet of OP50 food. On the left, DIC images of L3-stage germlines are shown. The asterisk indicates the distal end. The arrow indicates the most distal multinucleated cell. The scale bar represents 5 μm. On the right, germ cell numbers reflect gamete precursors per gonad arm of each stage.

(E) Food swap experimental design and fecundity analysis for the assessment of the reversibility of diet-sensitive *nhr-114*(−) sterility are shown. *nhr-114(RNAi)* animals were born on diets of OP50 food and subsequently transferred to HT115 food at the start of the indicated developmental stage and development proceeded until 2 days of adulthood. Error bars indicate SEM.
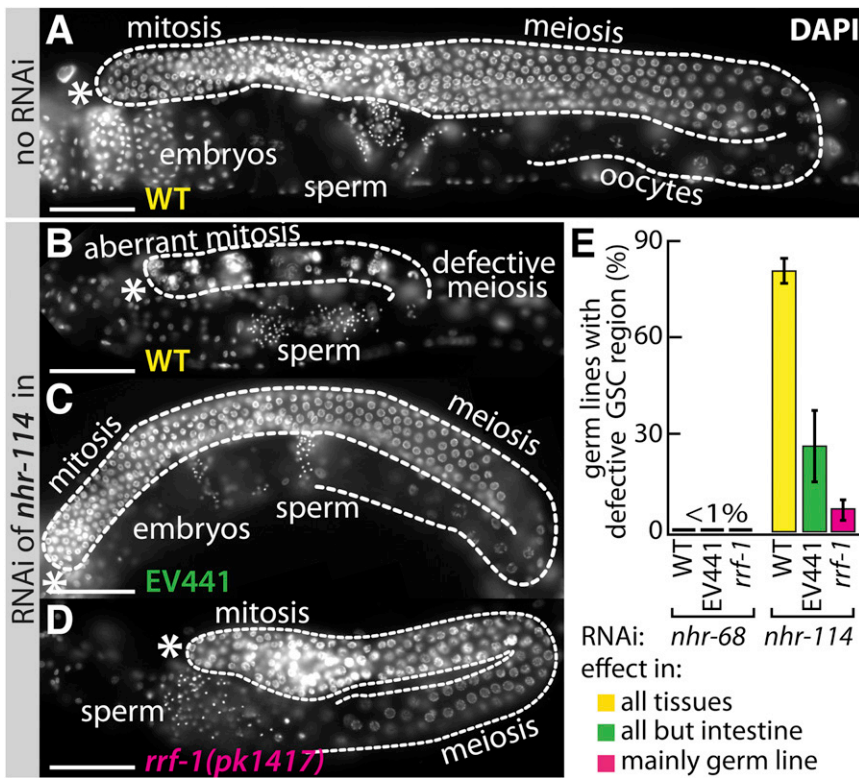
See also Figure S3.

**Figure 2. *nhr-114*(+) Activity Is Required for Germline Development**

(A) A scheme of an adult hermaphrodite gonad is shown in the top panel. The asterisk indicates the distal end. Mitotic (yellow) and incompletely

Figure 3. Somatic *nhr-114*(+) Expression Protects GSCs

(A–D) DAPI stainings of representative adult hermaphrodites (n > 150 each) fed with OP50 food and quantified in (E). The dotted line delineates the posterior germline. The asterisk indicates the distal end. The scale bar represents 50 μm. Morphological defects of germline nuclei upon tissue-restricted *nhr-114(RNAi)* knockdowns and germline size differences are apparent.

(E) Somatic *nhr-114* is required for proper germline organization of animals grown on a diet of OP50 food. The average number of germlines with defective distal organization (abnormal nuclei within 20 germ cell rows) is plotted for the given genotypes. WT, wild-type; *rrf-1*, sterile *rrf-1(pk1417)* mutants; *nhr-114*, RNAi-insensitive EV441. *nhr-68* serves as a control for RNAi effects in these strains. Error bars indicate the range among independent experiments. n > 100 in each.

See also Figure S1 and Tables S2–S4.

acids (Figure 4A), which may be explained by microbial enzymes that act on aromatic amino acids [21]. To confirm the specificity of the L-isomer of Trp, we supplemented OP50 food with the D-Trp and we observed no significant reduction of sterility (Figure 4A), suggesting that L-stereospecific enzymes might metabolize Trp to other metabolites. These data show that Trp supplementation of less-optimal diets compensates for the loss of *nhr-114* function in an animal.

Trp is a precious nutrient; it is an essential amino acid and is the least abundant residue in proteins [22, 23]. Animal Trp derivatives (e.g., serotonin and melatonin) are key signaling peptides that play broad roles in animal physiology, feeding, and maintenance of gastrointestinal homeostasis [24, 25]. Furthermore, microbial Trp metabolites have been implicated in mammals as protecting against stress-induced lesions of the gastrointestinal tract [1, 21]. To investigate whether Trp metabolized by bacteria prevents diet-sensitive sterility, we fed *nhr-114*(−) animals dead OP50 bacteria supplemented with Trp or solvent. We recovered virtually no fertile animals in either case (Figure 4B), indicating that the "Trp effect" requires live bacteria. However, we recovered fertility in a time-dependent manner when Trp was supplemented prior to the killing of the bacteria (Figure 4B), supporting the notion that bacterial Trp metabolism, rather than animal uptake of Trp, suppresses *nhr-114*(−) sterility. These findings also raise the possibility that intestinal *nhr-114/HNF4* maintains GSC integrity and supports germline development by compensating for dietary variations in bacterial Trp metabolites among *E. coli* strains [22].

### Detoxification and Stress Responses in the Absence of *nhr-114/HNF4*

Because Trp supplementation can be substituted for *nhr-114* activity, we hypothesized that Trp-derived metabolites and

NHR-114 function converge on the expression of similar genes. Using microarray analysis, we identified Trp-induced gene expression changes by comparing mRNA profiles of WT animals fed OP50 diets supplemented with either Trp or solvent (WT+Trp versus WT) (Figure 4C). Using DAVID gene enrichment analysis [26, 27], we found that a Trp-supplemented diet induces the expression of genes with functional classes associated with detoxification and xenobiotic responses (see Table S5).

Next, we identified gene expression changes in the soma that are associated with a reduction of *nhr-114* activity. We identified 2,001 genes that are differentially expressed in sterile *nhr-114(RNAi)* animals in comparison to sterile *glp-1(q224ts)* animals that lack a germline (*nhr-114* versus *glp-1*) (Figure 4C). Moreover, 57 genes overlapped with the WT+Trp versus WT data set (Figure 4C). Of these, a major cluster of 33 genes displayed a reciprocal regulation pattern; e.g., they were downregulated in *nhr-114*(−) but upregulated in WT+Trp conditions (Figure 4C). Intriguingly, this cluster contains genes that are similar to mammalian HNF4α targets, such as cytochrome P450 oxidoreductases and UDP-glucuronosyltransferases [28, 29], plus additional detoxifying enzymes (C-type lectins or glutathione S-transferases) [30, 31]. Altogether, these correlative data suggest that a Trp-supplemented diet and *nhr-114* activity may mount similar stress responses that protect the developing germline (Figure 4D). Our results are consistent with a model in which intestinal NHR-114/HNF4 may act as a sensor of the bacterial metabolic status to mediate transcriptional detoxification responses that buffer against dietary variations in order to maintain GSC integrity and reproductive capacity.

We propose that the *nhr-114* functions described here reflect the robustness of a developmental program against environmental variability. On the basis of the evolutionary and anatomically conserved expression of *nhr-114*, we propose that metazoan HNF4 orthologs may also protect germ cells from dietary insults. Although HNF4α roles for glucose metabolism and diabetes are well documented, roles of the highly related HNF4γ are poorly known [32]. Our study
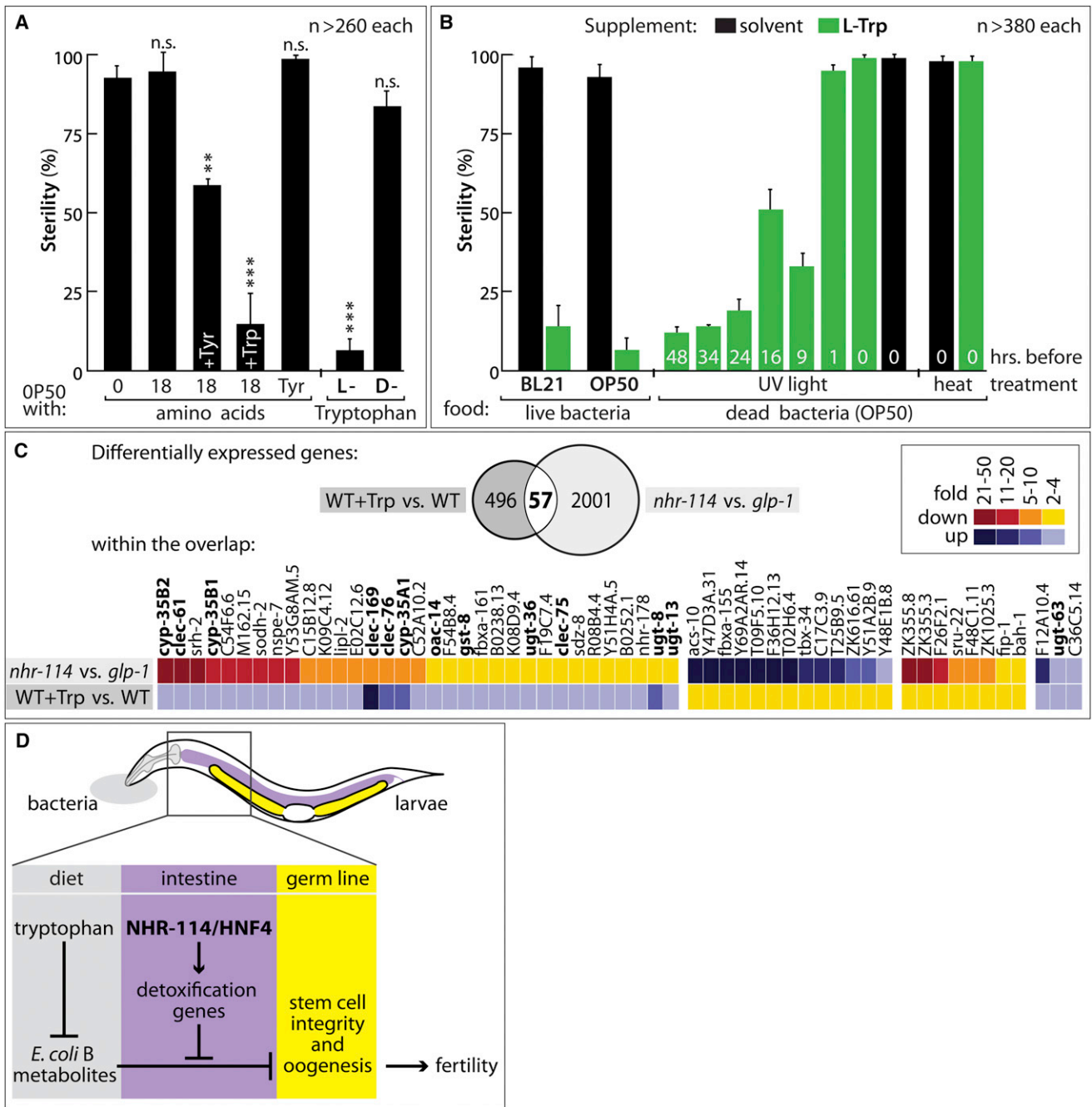
**Figure 4. Bacterial Tryptophan Metabolism Prevents *nhr-114*(−) Sterility**

(A) Summary of amino acid supplementation experiments on OP50-fed *nhr-114(RNAi)* animals. Mixtures of all L-amino acids minus tyrosine (Tyr) and tryptophan (Trp) are indicated as 18. Tryptophan stereoisomers are represented by L- and D-, respectively. **, p < 0.001; ***, p < 0.0001; n.s, not significant. A paired t test in respect to the solvent (water) was used.

See also Figure S3.

(B) The effect of bacterial metabolism on *nhr-114*(−) diet-sensitive sterility is shown. Tryptophan supplementation on two *E. coli* B strains (live bacteria) was fed to *nhr-114(RNAi)* animals. Standard OP50-seeded plates were supplemented with Trp and incubated for the indicated time before exposing the bacteria to UV light or boiling the bacteria. Control food plates are shown in black. Error bars indicate SEM.

(C) Number of differentially expressed genes from two different microarray experiments (gray underlined). The bottom panel shows a fold-change comparison of the 57 overlapping genes, which are distributed in four classes according to the direction of regulation. Contrarily to the 25% (14/57) random distribution assumed; 58% of these genes (33/57) form a cluster with functional annotations enriched in drug-metabolizing and detoxifying enzyme genes (shown in bold).

See Table S5 for analyses.

(D) A model of diet-sensitive *nhr-114*(−) sterility.

raises the possibility that gastrointestinal NRs, such as HNF4γ, may protect germ cell integrity from harmful effects of dietary expansion. Animal dietary expansion is proposed to evolve in coordination with the survival of the individual [33]. From our results, we propose that dietary expansion evolves also in coordination with survival of the GSCs and germ cell lineage. Moreover, this adaptation would counteract the ecological disadvantage of being restricted to specific diets and more confined environments.

## Experimental Procedures

### Strains and RNAi Knockdowns
Worm strains are derivatives of the WT Bristol strain N2 and were maintained at 20°C, as described previously [7]. Transgenic animals were maintained at 25°C. All adult animals were analyzed 24 hr after the mid-L4 larvae stage. See Supplemental Experimental Procedures for strain descriptions. For RNAi, 2 μg/μl dsRNA corresponding to mRNAs of *nhr-114* or *nhr-68* was injected into WT adult hermaphrodites.

### Bacterial Diets and Amino Acid Supplementation
Bacterial inocula were streaked on Luria Broth (LB), and HT115(DE3) bacteria were streaked on tetracycline-containing LB plates. A 20 ml LB overnight culture was harvested and resuspended in fresh medium to a final concentration of $OD_{600}$ = 25. Nematode growth media plates were prepared according to standard methods and spotted with 100 μl bacteria [7]. For supplementations, 12.5 mg/amino acid was dissolved in 1 ml of water and incubated for 30 min at 30°C in a thermoshaker at 1,000 rpm. For 6 cm plates, 100 μl of the solution was supplemented on top the OP50 lawn. L-amino acids are from Sigma-Aldrich (St. Louis, MO, USA) and Merck (Darmstadt, Germany), and D-Trp is from Carl Roth (Karlsruhe, Germany; # 7700.1).

### Transgenic *nhr-114* RNAi-Insensitive Animals
*nhr-114* ORF was codon-optimized with the *C. elegans* codon adaptor [34] to a 0.9 index, rendering the sequence insensitive to RNAi. A 5′-to-3′ construct was synthesized (GenScript, Piscataway, NJ, USA) (Acc65I::3×FLAGepitope::*nhr-114* ORF and a 3′ UTR::SL2 splicing acceptor sequence of *gls-1*::NLS::mCherry::BamHI) and cloned into pELT-2 downstream of the intestinal promoter *pept-1* (*opt-2*) [35]. Weak transcriptional activity of this promoter is reported in the hypodermis [36]. See also Supplemental Experimental Procedures.

### Immunocytochemistry and Whole-Mount Nuclei Staining
Extruded germlines were prepared as described previously [37]. Primary antibodies against ANI-2 (1:600) were a gift from Tony Hyman (Max Planck Institute of Molecular Cell Biology and Genetics [MPI-CBG], Dresden, Germany), nuclear pore complex (1:400, Mab414) was purchased from Covance (Munich, Germany), and the FLAG M2 epitope (1:300) was from Sigma-Aldrich. See Supplemental Experimental Procedures for nuclei stainings and detailed germ cell analysis.

### Microarray Analysis
Probes were custom designed for the *C. elegans* genome (WS228). Four different biological replicates were hybridized to single-color Agilent arrays. Unpaired t test analysis and Benjamini-Hochberg multiple testing corrections were performed. Differentially expressed genes had a fold change cutoff of 2.0 and an unpaired t test p value cutoff of 0.05 for WT+Trp versus WT and 0.01 for *nhr-114* versus *glp-1* (WT+TRP versus WT; 314 upregulated, 182 downregulated; *nhr-114 versus glp-1*: 1057 upregulated, 944 downregulated). Data analysis is displayed in Table S5.

### Statistics
Values represent the mean. Error bars represent SEM; a range is given in Figures 1B and 3E. Statistical significance was calculated with an unpaired two-tailed Student's t test.

### Accession Numbers

Microarray data have been deposited in the NCBI Gene Expression Omnibus under accession number GSE43864.

## Supplemental Information

Supplemental Information contains Supplemental Experimental Procedures, four figures, and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.02.034.

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