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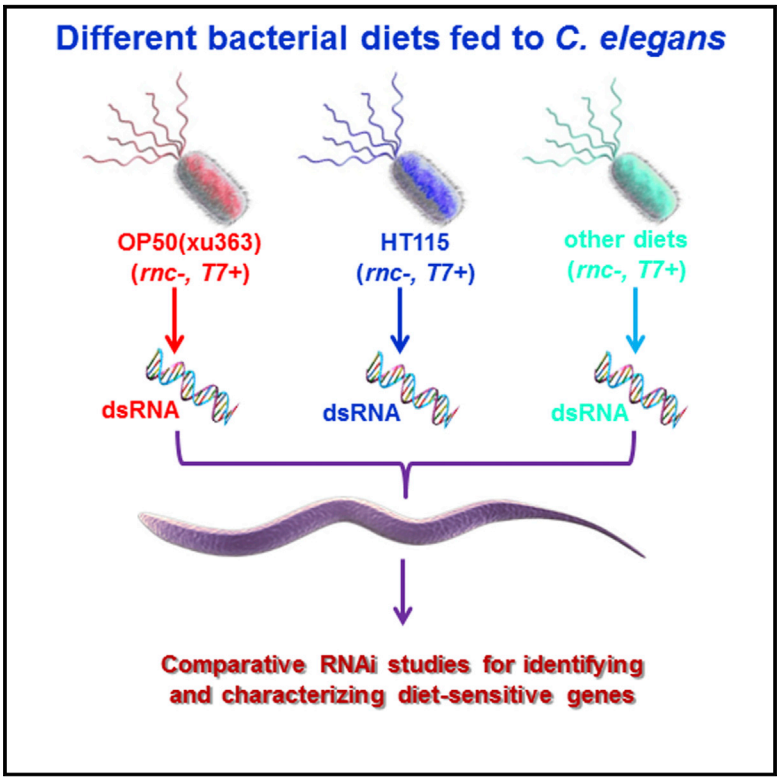
RNAi Interrogation of Dietary Modulation of Development, Metabolism, Behavior, and Aging in *C. elegans*

Graphical Abstract



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In Brief

Different diets differentially affect animal life. Here, Xiao et al. genetically engineer OP50 bacteria, the standard laboratory diet for *C. elegans*, to make it RNAi compatible. Using these RNAi-compatible OP50 bacteria as well as the standard RNAi strain, HT115, neuroendocrine and mTOR pathways are implicated in mediating differential dietary responses.

Highlights

- Different bacterial diets differentially affect *C. elegans* life
- We engineered the standard bacterial diet, OP50 strain, making it RNAi compatible
- This genetic tool enables RNAi interrogation of differential dietary signaling
- mTORC2 and neuroendocrine signaling are critical for differential dietary responses

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RNAi Interrogation of Dietary Modulation of Development, Metabolism, Behavior, and Aging in *C. elegans*

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SUMMARY

Diet affects nearly every aspect of animal life such as development, metabolism, behavior, and aging, both directly by supplying nutrients and indirectly through gut microbiota. *C. elegans* feeds on bacteria, and like other animals, different bacterial diets induce distinct dietary responses in the worm. However, the lack of certain critical tools hampers the use of worms as a model for dietary signaling. Here, we genetically engineered the bacterial strain OP50, the standard laboratory diet for *C. elegans*, making it compatible for dsRNA production and delivery. Using this RNAi-compatible OP50 strain and the other bacterial strain HT115, we feed worms different diets while delivering RNAi to interrogate the genetic basis underlying diet-dependent differential modulation of development, metabolism, behavior, and aging. We show by RNAi that neuroendocrine and mTOR pathways are involved in mediating differential dietary responses. This genetic tool greatly facilitates the use of *C. elegans* as a model for dietary signaling.

INTRODUCTION

Diet is one of the most critical environmental factors that affect animal life (Salonen and de Vos, 2014). All animals must receive nutrients from diet to support survival and reproduction. Diet also directly and indirectly affects development, metabolism, behavior, and aging (Nicholson et al., 2012; Pflughoeft and Versalovic, 2012; Yilmaz and Walhout, 2014). Different diets vary in the amount and composition of nutrients and thus elicit distinct dietary responses, depending on the genetic makeup and physiological state of the animal (Jones et al., 2012; Yilmaz and Walhout, 2014). Diet shift and aberrant dietary signaling have been linked to a growing list of human disorders such as obesity, diabetes, cancer, and cardiovascular diseases (Salonen and de Vos, 2014).

In addition to supplying nutrients, diet affects animal life indirectly through gut microbiota (Nicholson et al., 2012; Pflughoeft and Versalovic, 2012). For example, microbial organisms in the gut of the host animal digest fibers from the diet, which otherwise cannot be processed by the host, to produce short-chain fatty acids (Nicholson et al., 2012; Pflughoeft and Versalovic, 2012). These microbes also metabolize diet components to produce essential micronutrients such as vitamins (Nicholson et al., 2012; Pflughoeft and Versalovic, 2012). Such indirect dietary effects have also been associated with human diseases, ranging from diabetes and depression to autism (Nicholson et al., 2012; Pflughoeft and Versalovic, 2012). Despite the increasingly appreciated importance of diet and dietary signaling in health and disease, it has been challenging to characterize the underlying genetic basis due to the complicated mechanisms involved.

The nematode *C. elegans* is a popular model organism that has been widely utilized to investigate various biological processes. *C. elegans* feeds on bacteria that directly supply nutrients after being digested in the gut (Brenner, 1974). Utilizing chemicals from the surrounding environment, bacteria also produce essential micronutrients, such as vitamins, which cannot be synthesized by the worm (Yilmaz and Walhout, 2014). In this regard, bacteria fed to the worm serve as direct diet to provide macronutrients and also supply essential micronutrients, a role similar to that carried out by gut microbiota in mammals (Yilmaz and Walhout, 2014). Remarkably, worms and humans require a similar set of essential nutrients and also share similar basic metabolic pathways (Yilmaz and Walhout, 2014). These features together have led to the suggestion that *C. elegans* represents a great genetically tractable model system for the study of both direct and indirect effects of diet, including host-microbiota interactions (Yilmaz and Walhout, 2014).

The standard bacterial diet used to feed *C. elegans* in the laboratory is *E. coli* OP50, a B strain with which most experimental data have thus far been collected by the community, including

those related to gene expression, development, metabolism, behavior, and aging (Brenner, 1974). RNAi, a powerful genetic tool first developed in *C. elegans*, has been extensively applied to characterize nearly all biological processes in worms (Fire et al., 1998). These include many RNAi studies, particularly genome-wide RNAi screens. Yet, because RNAi cannot be performed with OP50, all RNAi data were acquired using the other *E. coli* diet HT115, a K-12 strain (Rual et al., 2004; Timmons et al., 2001). This was typically carried out by expressing dsRNA against a specific worm gene in HT115 and then feeding it to the worm (Timmons et al., 2001). OP50 and HT115 are two distinct types of bacterial diets that differ in both the amount and composition of nutrients and metabolites (Brooks et al., 2009; Reinke et al., 2010). Because of this, these two diets differentially affect gene expression in the worm, leading to differential modulation of nearly every aspect of worm life, including, but not limited to, development, metabolism, behavior, and aging (Coolon et al., 2009; Gracida and Eckmann, 2013; MacNeil et al., 2013; Maier et al., 2010; Pang and Curran, 2014; Soukas et al., 2009; You et al., 2008). As it has not been technically possible to perform RNAi on worms fed OP50, one cannot take advantage of the power of RNAi to systematically interrogate the genetic basis underlying diet-dependent differential modulation of worm biology. In addition, as OP50 and HT115 differentially affect worms, it is very difficult to compare the data sets collected from RNAi studies using HT115 with those accumulated in the literature using OP50. These difficulties pose great challenges not only for the studies aimed at characterizing differential dietary responses but also those intended to exclude differential diet effects.

To overcome these technical difficulties, here, we genetically engineered the OP50 strain to endow it with the capacity to produce and deliver dsRNA. Worms fed this strain are phenotypically indistinguishable from those fed OP50. Importantly, this strain produces and delivers dsRNA to worms as efficiently as HT115. Using this tool, we characterized diet-dependent differential modulation of development, metabolism, behavior, and aging in *C. elegans* and showed by RNAi that neuroendocrine and mTOR pathways are important for mediating differential dietary responses. The development of this genetic tool paves the way for conducting genome-wide RNAi screens to systemically dissect the genetic basis of dietary signaling using *C. elegans* as a model. As OP50 is the normal diet used by all *C. elegans* laboratories, this RNAi tool will also be of wide use in studies that aim to avoid differential diet effects.

RESULTS

Engineering an RNAi-Compatible OP50 Strain

As worms feed on bacteria, plasmids producing dsRNA targeting specific *C. elegans* genes can be readily transformed into bacteria, which can then be fed to worms (Timmons et al., 2001). This makes bacteria the most-convenient vehicle for dsRNA production and delivery. HT115 was selected as the host bacterial strain for two reasons (Timmons et al., 2001). First, it carries a Tn10 transposon insertion in the *rnc* gene that encodes an RNaseIII (Timmons et al., 2001). This results in the disruption of *rnc*. As RNaseIII cleaves dsRNA, its loss greatly enhances the yield of dsRNA (Timmons et al., 2001). Second, HT115 carries an IPTG-inducible T7 polymerase cassette derived from DE3 prophage, which was introduced through lysogenization (Timmons et al., 2001). The IPTG-inducible T7 polymerase cassette drives the production of dsRNA from plasmids in bacteria. OP50, however, lacks these two features. It is rnc^{+}, and introduction into this strain of the IPTG-inducible T7 polymerase cassette through DE3 lysogenization is also very difficult (see below). Thus, despite the fact that OP50 is the standard bacterial diet for worm culture and it is certainly desirable to conduct RNAi experiments on worms using OP50, it has not been possible to do so.

We took two steps to engineer OP50. First, we mutated its *rnc* gene (Figure 1A). In HT115, *rnc* was disrupted by a Tn10 transposon (Timmons et al., 2001). We thus took a similar strategy by introducing a Tn10 transposon into *rnc* through P1 phage-mediated transduction from an rnc^{-} host strain *E. coli* K8024 (Figure 1A). Similar to HT115, this strain (OP50(xu362)) is also tetracycline resistant, which is conferred by the Tn10 transposon.

We then attempted to introduce the IPTG-inducible T7 polymerase cassette into OP50(xu362) through DE3 lysogenization but without success, though positive controls worked efficiently (data not shown). We realized that OP50 is resistant to lambda phage infection, likely because it lacks functional phage receptors on its surface. However, transformation into OP50 of a plasmid carrying a K-12 strain-derived *malB* operon, which encodes lambda phage receptors, only conferred OP50 with limited phage receptibility that is insufficient to permit DE3 lysogenization (data not shown). To overcome this technical difficulty, we resorted to an alternative recombineering approach by introducing the IPTG-inducible T7 polymerase cassette into the *attB* site of OP50(xu362) through two steps of Red/ET-mediated recombination (Figure 1B). This cassette is also integrated into the *attB* site in HT115 (Timmons et al., 2001). In doing so, we hope to make the new strain OP50(xu363) comparable to HT115 in dsRNA production.

Indeed, OP50(xu363) produced dsRNA from plasmids at an efficiency similar to HT115 and did so in an IPTG-dependent manner (Figure 1C). As a control, the original OP50 strain cannot make dsRNA (Figure 1C). Like OP50, OP50(xu363) also formed a thin lawn of light yellow color on the surface of NGM plates (Figure 1D), though it grows at a slightly slower pace probably due to the disruption of *rnc* (Figure S1). Most importantly, worms fed OP50(xu363) were indistinguishable from those grown on OP50 in development, metabolism, behavior, and aging (see below), indicating that OP50(xu363) is well suited for culturing *C. elegans*.

We then examined whether dsRNA produced by OP50(xu363) can efficiently knock down gene expression in *C. elegans*. As a proof-of-principle test, we treated worms expressing a GFP transgene with OP50, OP50(xu363), and HT115, each of which carried a plasmid-encoding dsRNA against GFP (Figure 1E). GFP fluorescence in worms fed OP50(xu363) and HT115 was greatly diminished, whereas OP50 did not elicit any notable effect (Figure 1E). These results together indicate that OP50(xu363) is not only well suited as a diet for *C. elegans* but also an excellent vehicle for dsRNA production and delivery.

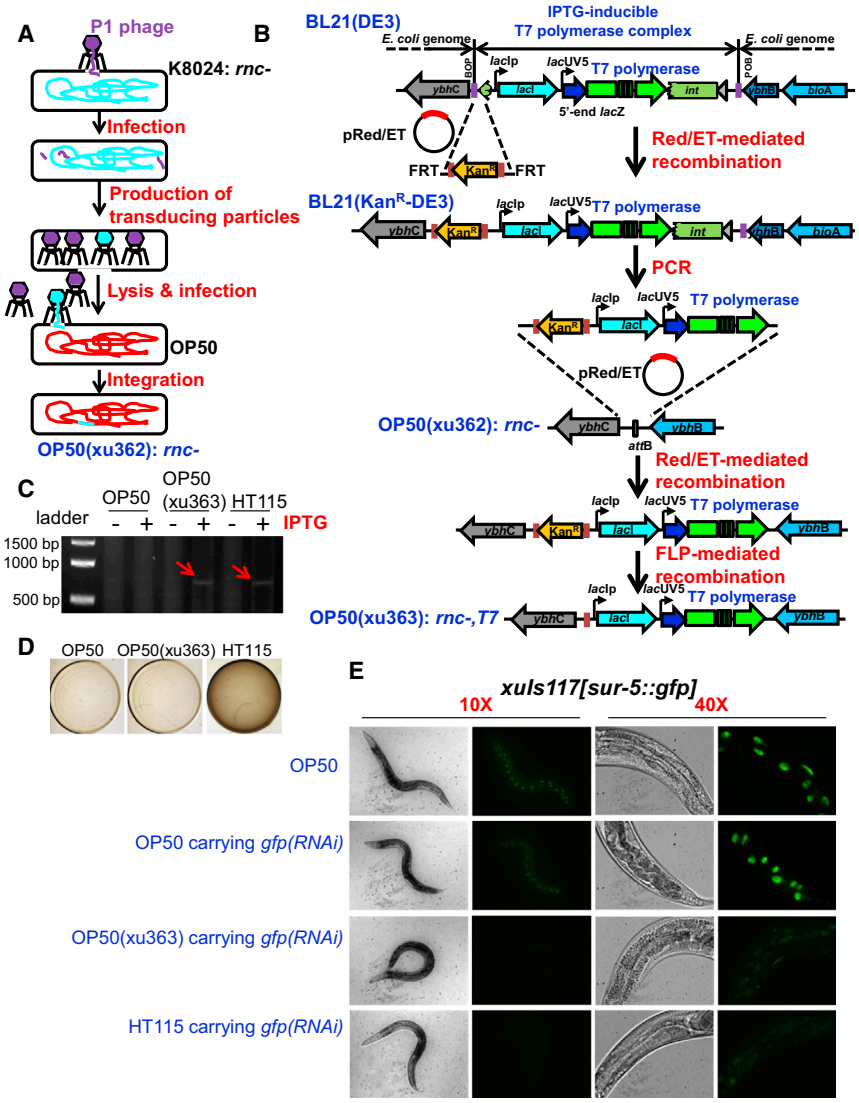

Figure 1. Engineering an RNAi-Compatible OP50 Strain

(A) The endogenous *rnc* function of wild-type OP50 bacteria was disrupted by insertion of a Tn10 transposon into *rnc* through P1 phage-mediated transduction, giving rise to OP50(xu362). Segments and particles in purple denote phage DNA. Segments in cyan and red denote genomic DNA from K8024 and OP50, respectively.

(B) An IPTG-inducible T7 polymerase cassette cloned from BL21(DE3) host strain was introduced into the *attB* site of OP50(xu362) through two rounds of Red/ET-mediated recombination, giving rise to the RNAi-compatible OP50(xu363) strain.

(C) OP50(xu363) and HT115 have a similar yield of dsRNA after IPTG induction. A *gfp(RNAi)* plasmid with the insert size ~700 bp was used in this study. Arrows point to the dsRNA band. dsRNA was prepared by treating total RNA prepared from bacteria with DNaseI to remove DNA followed by RNaseA to remove ssRNA. The average yield of dsRNA after IPTG induction is 945.7 ± 111.7 ng/ml bacterial culture for HT115 and 967.3 ± 171.6 ng/ml bacterial culture for OP50(xu363).

(D) OP50 and OP50(xu363) form a similar thin lawn of light yellow color whereas HT115 lawn is thicker in a darker color. The same amount of bacteria (OD_{600} = 2; 10 μl) was seeded on agar plates overnight before taking images.

(E) Effectiveness of GFP RNAi delivered by OP50(xu363). OP50(xu363) and HT115 expressing GFP dsRNA were fed to worms with an integrated transgene *xuIs117*, which carries a ubiquitously expressed GFP reporter *sur-5::gfp*. The original OP50 strain and one carrying the same GFP RNAi plasmid were used as negative controls (top two rows). Sample images were taken under a 10× (left) and a 40× (right) lens. Left: DIC. Right: SUR-5::GFP.

Also see Figure S1.

Diet-Dependent Differential Modulation of Development

We then interrogated diet-dependent differential modulation of worm biology by RNAi. We first looked at the developmental rate, which is differentially regulated by different diets (MacNeil et al., 2013; Maier et al., 2010; Soukas et al., 2009). RNAi is a commonly used approach for studying worm development. The inability to perform RNAi on OP50 poses an obstacle to investigating the genetic basis underlying diet-dependent differential modulation of development.

Worms developed at a faster pace from eggs to reproductive adults on HT115 than on OP50 and OP50(xu363) (egg to egg; Figure 2), an observation similar to that reported by others on HB101 versus OP50 (Avery and Shtonda, 2003; Soukas et al., 2009). A closer examination indicates that this diet-dependent effect was mainly observed at a late stage (L4 to egg), which includes multiple processes such as the development of L4 into adult and the development and function of the reproductive system (Figure S2). As we have not been able to distinguish them, it remains possible that the two diets may also affect some aspects of reproduction.

RICT-1, a specific, essential subunit of mTORC2 and its downstream effector SGK-1 are both required for diet-dependent differential modulation of developmental rate (Jones et al., 2009; Soukas et al., 2009). Mutations in *rict-1* and *sgk-1* slowed down worm development (L1–L4 and L4–egg stages) and, interestingly, eliminated the differential effects of HT115, OP50, and OP50(xu363) (Figures 2 and S2). RNAi of either gene using OP50(xu363) and HT115 was sufficient to induce a phenotype similar to mutants and also abolished the differential effects caused by the two bacterial diets (Figures 2 and S2). Although it remains unclear whether the two diets differentially affect development, these experiments demonstrate that RNAi using OP50(xu363) represents an efficient tool to knock down genes affecting worm development.

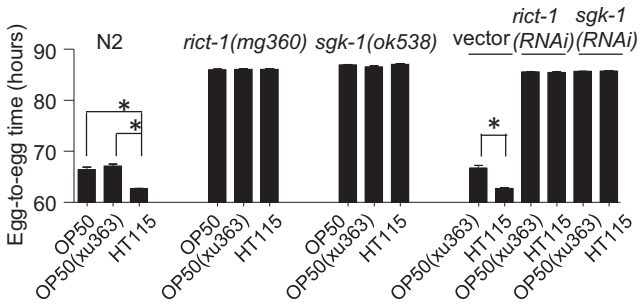

Figure 2. Diet-Dependent Differential Modulation of Development

Developmental rate experiments for wild-type N2 and mutants were performed at the same time. The egg-to-egg time was scored as previously described (Soukas et al., 2009), which spans from the time point at which the egg was laid by its parent to the appearance of the first egg laid by this animal. Assays were performed at 20°C. Error bars: SEM; n = 20; *p < 0.05; ANOVA test.

Also see Figure S2.

Diet-Dependent Differential Modulation of Metabolism

Metabolism is also regulated by diet in *C. elegans* (Watson and Walhout, 2014). Different diets induce distinct metabolic responses in *C. elegans* (Watson and Walhout, 2014). RNAi is commonly used in *C. elegans* metabolism studies; however, the inability to conduct RNAi on OP50, the standard worm diet, has limited our ability to characterize diet-dependent differential modulation of metabolism.

We examined the differential effects of diet on some common metabolic parameters such as respiration, ATP synthesis, and fat metabolism (Figure 3). mTORC2 is known to regulate these parameters (Jones et al., 2009; Soukas et al., 2009). Indeed, whereas oxygen consumption and ATP production were differentially modulated by OP50, OP50(xu363), and HT115 in wild-type worms, such differential diet effects were absent in *rict-1* and *sgk-1* mutant worms (Figures 3A and 3B). We then tested RNAi and found that RNAi of either gene mimicked the mutant phenotype and also abolished the differential diet effects (Figures 3A and 3B). In the case of fat metabolism, worms fed OP50 and OP50(xu363) stored a higher level of fat than those grown on HT115 (Brooks et al., 2009), displaying a differential diet effect (Figures 3C and 3F). Though loss of *rict-1* elevates fat storage, the differential diet effect persists in this mutant (Soukas et al., 2009), indicating that mTORC2 is not required for mediating such an effect (Soukas et al., 2009). We then tested *pept-1*, which encodes a peptide transporter and is known to regulate fat metabolism in worms (Brooks et al., 2009). Though *pept-1* mutant worms stored more fat, the differential diet effect was no longer observed in these mutant worms, supporting the notion that the differential effect of diet on fat storage requires *pept-1* (Figures 3D and 3F). This phenotype can be recapitulated by RNAi of *pept-1* using OP50(xu363) and HT115 (Figures 3E and 3F). Furthermore, neuroendocrine signaling has been suggested to play a role in *C. elegans* fat metabolism (Cunningham et al., 2014). *unc-31* encodes the worm ortholog of CAPS that functions in neurons and neuroendocrine cells and is required for DCV (dense core vesicle) exocytosis and hence neuroendocrine signaling (Livingstone, 1991; Speese et al., 2007). We thus checked *unc-31* mutants and found that, though mutant worms showed increased fat storage, the differential diet effect remained in the mutant (Figure S3). However, this does not necessarily indicate that neuroendocrine signaling has no contribution to the observed differential diet effect, as different classes of neuropeptides may have opposing effects. These experiments demonstrate RNAi as a powerful tool for characterizing the genetic basis underlying diet-dependent differential modulation of metabolism.

Diet-Dependent Differential Modulation of Behavior

Food profoundly affects behavior, ranging from feeding, sensory, and motor behaviors to learning and memory (Avery and You, 2012; Hills et al., 2004; You et al., 2008; Zhang et al., 2005). Different diets induce distinct behavioral responses. Given the large number of behaviors affected by food, here, we decided to examine a few such examples. We first assessed the feeding behavior. Worms fed at a faster rate on OP50 and OP50(xu363) than on HT115 (Figure 4A), consistent with previous results (Maier et al., 2010). When feeding on HT115, worms periodically stopped feeding, a phenomenon called behavioral quiescence (Figure 4B) (You et al., 2008). By contrast, this phenomenon was hardly observed on OP50 or OP50(xu363) (Figure 4B). Such differential dietary responses were absent in *rict-1* and *sgk-1* mutant worms (Figures 4A and 4B), indicating that mTORC2 is required. In addition to mTORC2, feeding is regulated by neuroendocrine signaling (Avery et al., 1993; You et al., 2008). We therefore checked *unc-31* mutant worms. The differential effects of diet on pumping rate and feeding quiescence were no longer observed in *unc-31* mutant worms (Figures 4A and 4B). RNAi of *unc-31*, as well as *rict-1* and *sgk-1*, abrogated such differential diet effects (Figures 4A and 4B). These results are consistent with the view that mTOR and neuroendocrine-signaling pathways are required for diet-dependent differential modulation of feeding behavior.

Whereas worms spend most of their time on food, they occasionally leave the food patch to explore other areas (Bendesky et al., 2011; Milward et al., 2011; Shtonda and Avery, 2006). Different diets may differentially affect this food-leaving behavior (Shtonda and Avery, 2006). We therefore tested this possibility and found that worms fed HT115 left the food patch at a higher frequency than those fed OP50 and OP50(xu363), showing that diet differentially regulates food-leaving behavior (Figure 4C). TYRA-3, a G-protein-coupled catecholamine receptor, is known to regulate food-leaving behavior (Bendesky et al., 2011). Thus, we checked whether this receptor also mediates the differential diet effect. *tyra-3* mutant worms left HT115, OP50, and OP50(xu363) lawns at a similar frequency, indicating that the differential diet effect requires *tyra-3* (Figure 4C). By contrast, this differential effect does not require mTORC2, as it persisted in *rict-1* mutant worms (Figure S4). Because *tyra-3* is known to act in sensory neurons that are not very sensitive to RNAi (Bendesky et al., 2011), we performed RNAi on *Punc-119*:*sid-1*;*lin-15B* worms, in which RNAi efficiency in neurons is greatly improved (Calixto et al., 2010). RNAi of *tyra-3* mimicked the mutant phenotype and also abolished the differential diet effect (Figure 4C).

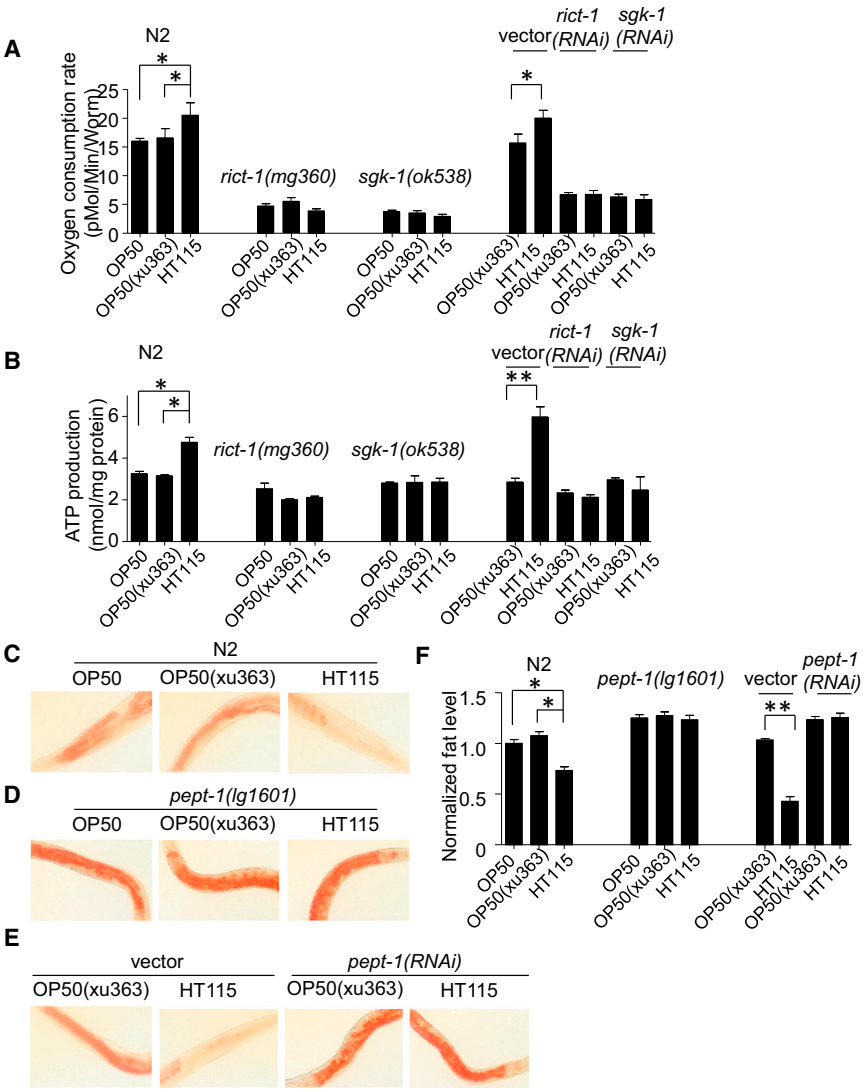

Figure 3. Diet-Dependent Differential Modulation of Metabolism

(A) N2 worms fed HT115 consume more oxygen than those fed OP50 and OP50(xu363). This diet-mediated differential effect was largely abolished in *rict-1*(*mg360*), *sgk-1*(*ok538*), and *rict-1* RNAi- or *sgk-1* RNAi-treated worms. Error bars: SEM; n = 5; *p < 0.05; ANOVA test.

(B) N2 worms fed HT115 produce more ATP than those fed OP50 and OP50(xu363). This diet-mediated differential effect was largely abolished in *rict-1*(*mg360*), *sgk-1*(*ok538*), and *rict-1* RNAi- or *sgk-1* RNAi-treated worms. Error bars: SEM; n = 5; *p < 0.05; ANOVA test.

(C–E) Representative Oil-Red-O (ORO) staining images of N2 (C), *pept-1*(*lg1601*) (D), and vector L4440 RNAi- and *pept-1* RNAi-treated worms (E) grown on OP50, OP50(xu363), or HT115.

(F) Bar graph summarizing ORO staining data. N2 worms stored less fat when grown on HT115. This diet-dependent difference was abolished in *pept-1*(*lg1601*) mutant and *pept-1* RNAi-treated worms. Error bars: SEM; n ≥ 30; *p < 0.05; **p < 0.01; ANOVA test.

Also see Figure S3.

Locomotion behavior is one of the most-prominent behaviors of *C. elegans*. Locomotion forms the foundation of nearly all worm behaviors (de Bono and Maricq, 2005). During locomotion, worms spend most of their time moving forward and occasionally change locomotion direction by switching from forward to backward movement (reversal) (Gray et al., 2005; Li et al., 2014; Piggott et al., 2011; Tsalik and Hobert, 2003; Zheng et al., 1999). Whereas it is well known that food has a profound effect on worm locomotion, it is unclear whether different diets differentially modulate this behavior. We quantified two simple locomotion parameters, locomotion speed and reversal frequency, and found that worms moved at a faster speed and exhibited a lower reversal frequency on OP50 and OP50(xu363) than on HT115 (Figures 5A and 5D). Apparently, different diets differentially modulate locomotion behavior. The differential effects of diet on locomotion speed rather than reversals persisted in *rict-1* mutant worms, indicating that mTORC2 is not absolutely required for mediating these diet effects (Figure S5). We also examined *unc-31* mutants because neuroendocrine signaling is well known to regulate locomotion and food affects neuroendocrine signaling (You et al., 2008). Though *unc-31* mutant worms are uncoordinated, they are not paralyzed at young ages (e.g., day 1), allowing us to record their locomotion behavior. Mutation in *unc-31* abolished the differential effects of diet on locomotion speed and reversals observed in wild-type worms (Figures 5B and 5E). Importantly, RNAi of *unc-31* in *Punc-119*:*sid-1*;*lin-15B* worms (Calixto et al., 2010) recapitulated the mutant phenotype and also eliminated the differential diet effects (Figures 5C and 5F). Thus, neuroendocrine signaling is required for mediating the differential effects of diet on locomotion behavior. These results together demonstrate RNAi as a powerful tool for characterizing the genetic basis underlying diet-dependent differential modulation of worm behavior.

Diet-Dependent Differential Modulation of Aging

Diet has a profound effect on aging (Kenyon, 2010). Dietary restriction (DR) delays the onset of aging and extends lifespan (Kenyon, 2010). In addition to the amount of diet, different kinds of diet differentially affect lifespan. In the case of *C. elegans*, it exhibits different lengths of lifespan on different diets (Yilmaz and Walhout, 2014). For example, worms can live a bit longer on HT115 than on OP50 (Maier et al., 2010), and genes differentially regulate the lifespan of worms fed on HT115 and OP50 (Maier et al., 2010; Pang and Curran, 2014; Soukas et al., 2009). Specifically, some genes shorten lifespan on OP50 but extend lifespan on HT115, or vice versa. Examples include *nmur-1*, *alh-6*, and genes in mTORC2 signaling (Maier et al., 2010; Mizunuma

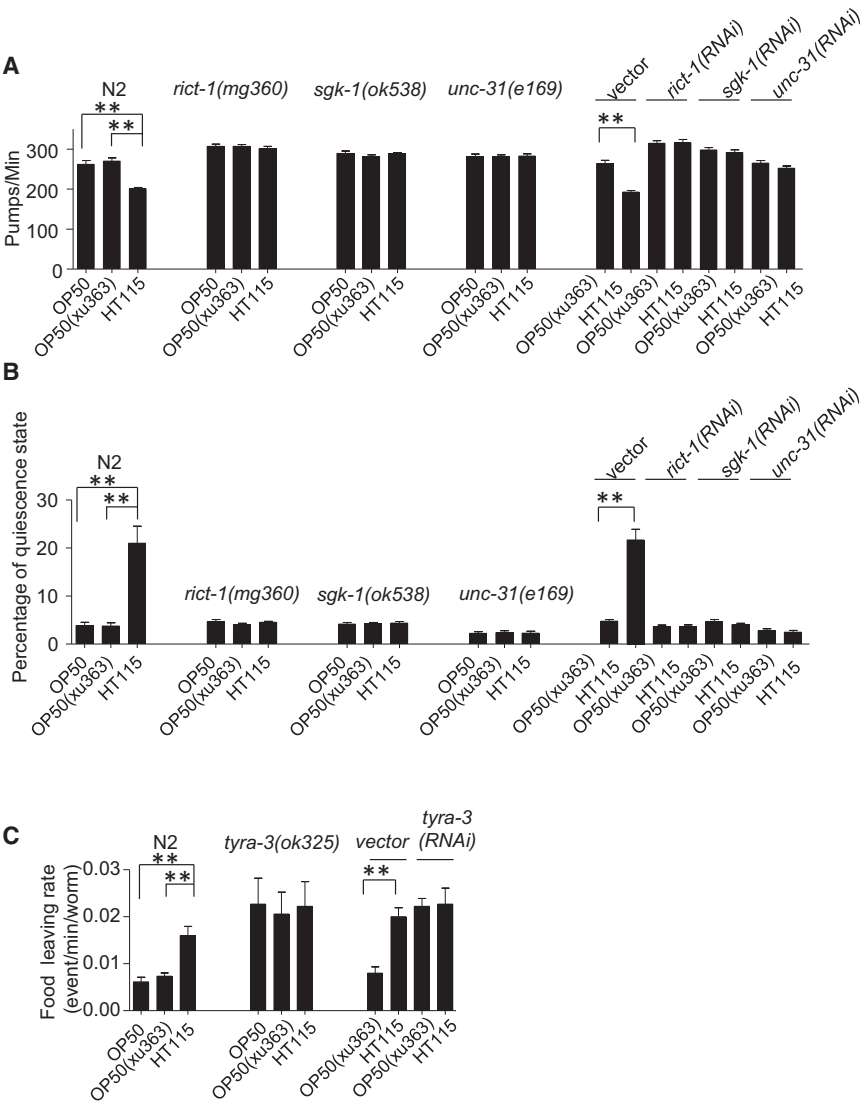

Figure 4. Diet-Dependent Differential Modulation of Feeding Behavior and Food-Leaving Behavior

(A) Worms feed at a faster rate on OP50 and OP50(xu363) than HT115. Pumping rate was quantified. The diet-mediated differential effect was abolished in *rict-1*(*mg360*), *sgk-1*(*ok538*), *unc-31*(*e169*), and *rict-1* RNAi-, *sgk-1* RNAi-, or *unc-31* RNAi-treated worms. Error bars: SEM; n = 20; **p < 0.01; ANOVA test.

(B) Worms show behavioral quiescence on HT115. For each animal, the percentage of time showing no pumping was quantified. The diet-mediated differential effect was largely abolished in *rict-1*(*mg360*), *sgk-1*(*ok538*), *unc-31*(*e169*), and *rict-1* RNAi-, *sgk-1* RNAi-, or *unc-31* RNAi-treated worms. Error bars: SEM; n = 20; **p < 0.01; ANOVA test.

(C) Worms fed HT115 leave food patch more frequently than those fed OP50 and OP50(xu363). This diet-mediated differential effect was largely abolished in *tyra-3*(*ok325*) and *tyra-3* RNAi-treated worms. RNAi was carried out on *Punc-119*:*sid-1*;*lin-15B* worms. Error bars: SEM; n ≥ 5; **p < 0.01; ANOVA test.

Also see Figure S4.

et al., 2014; Pang and Curran, 2014; Soukas et al., 2009). As RNAi is one of the most-commonly used approaches in aging studies, the lack of an ability to perform RNAi on OP50 poses a great difficulty in interrogating the genetic basis underlying diet-dependent differential regulation of longevity.

As a first step, we verified that worms fed OP50(xu363) lived a similar lifespan to those fed OP50 (Figure 6A). We then assessed whether RNAi using OP50(xu363) of genes in several common longevity pathways would give rise to expected lifespan results. These include DAF-2 (insulin/IGF-1 receptor), DAF-16 (FOXO transcription factor; downstream of DAF-2), HSF-1 (heat shock factor; stress and insulin/IGF-1 signaling), HSB-1(negative regulator of HSF-1), SKN-1 (Nrf transcription factor; stress and DR signaling), WDR-23 (negative regulator of SKN-1), PHA-4 (FOXA transcription factor; DR signaling), ATP-3 (ATP synthase subunit; mitochondrial signaling), SET-2 (H3K4 methyltransferase; epigenetic signaling), and RPS-15 (ribosomal subunit; protein translation) (Bishop and Guarente, 2007; Choe et al., 2009; Dillin et al., 2002; Hansen et al., 2007; Hsu et al., 2003; Kimura et al., 1997; Lin et al., 1997; Ogg et al., 1997; Panowski et al., 2007; Satyal et al., 1998; Tullet et al., 2008). In all cases, RNAi using OP50(xu363) yielded the expected lifespan phenotype as described in literature (Figures 6B–6J). Thus, OP50(xu363) appears to be a reliable vehicle for dsRNA production and delivery in lifespan analysis.

Both mTORC1 and mTORC2 regulate lifespan. Whereas it is generally accepted that mTORC1 shortens lifespan, the role of mTORC2 in lifespan regulation is complex and somewhat controversial (Alam et al., 2010; Mizunuma et al., 2014; Soukas et al., 2009; Tullet et al., 2008; Xiao et al., 2013). Emerging evidence suggests that mTORC2 signaling regulates lifespan in a diet- and perhaps temperature-dependent manner (Mizunuma et al., 2014; Soukas et al., 2009; Xiao et al., 2013). We found that RNAi of the mTORC1-specific genes *daf-15* and *raga-1* both extended lifespan on either HT115 or OP50(xu363) (Figure 6K), consistent with a role of mTORC1 in shortening lifespan. By contrast, RNAi of the mTORC2-specific gene *rict-1* and its effector *sgk-1* shortened lifespan on OP50(xu363) but extended lifespan on HT115 (Figures 6L and 6M), supporting the view that mTORC2 signaling differentially regulates lifespan in a diet-dependent manner (Mizunuma et al., 2014; Soukas et al., 2009). To gather further evidence, we performed the converse experiments by overexpressing *rict-1* and *sgk-1* as a transgene in wild-type worms. Remarkably, whereas *rict-1* and *sgk-1* transgenes extended lifespan on OP50(xu363) (Figure 6N)

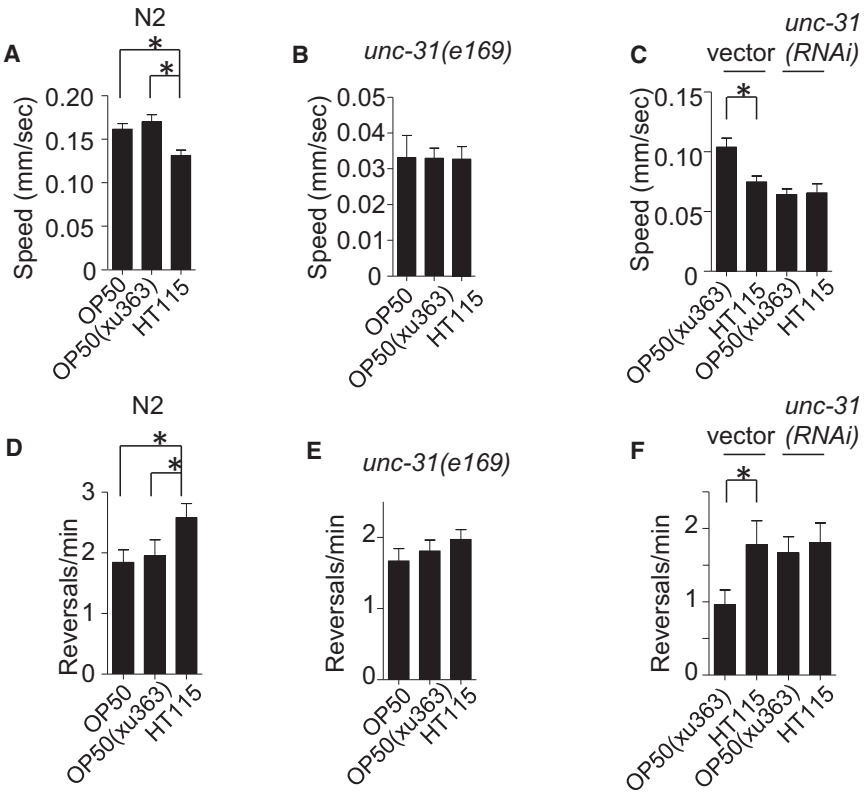

Figure 5. Diet-Dependent Differential Modulation of Locomotion Behavior

(A–C) N2 worms show a slower locomotion speed on HT115 than on OP50 and OP50(xu363). This diet-mediated differential effect is largely abolished in *unc-31*(*e169*) (B) and *unc-31* RNAi-treated worms (C). RNAi was carried out on *Punc-119*:*sid-1*;*lin-15B* worms. Error bars: SEM; n ≥ 10; *p < 0.05; ANOVA test.

(D–F) N2 worms show a higher reversal frequency on HT115 than on OP50 and OP50(xu363). This diet-mediated differential effect is largely abolished in *unc-31*(*e169*) (E) and *unc-31* RNAi-treated worms (F). RNAi was carried out on *Punc-119*:*sid-1*;*lin-15B* worms. Error bars: SEM; n ≥ 10; *p < 0.05; ANOVA test.

Also see Figure S5.

(Xiao et al., 2013), the same transgenes shortened lifespan on HT115 (Figure 6O), providing further evidence that mTORC2 signaling differentially regulates lifespan in a diet-dependent manner. These results highlight the value of the use of RNAi approach to characterize the genetic basis underlying diet-dependent differential modulation of longevity.

DISCUSSION

OP50 was selected by Sydney Brenner to culture *C. elegans* when he first introduced *C. elegans* as a genetic model organism (Brenner, 1974). Since then, OP50 has become the standard *C. elegans* diet in the laboratory. It is a uracil auxotroph strain that forms a thin lawn on the surface of culturing plates, making it convenient for visualization of worm morphology, development, and behavior under a microscope (Brenner, 1974). Over the past four decades, nearly all the data related to *C. elegans* have been collected using OP50. Since the 1990s, RNAi has become a powerful genetic tool that has revolutionized biomedical research (Fire et al., 1998). However, RNAi can only be performed on the other bacterial diet HT115, but not on the standard diet OP50. As OP50 and HT115 differentially modulate *C. elegans* biology, the inability to conduct RNAi experiments on OP50 not only limits the use of worms as a model for dietary signaling but also makes it very difficult to compare the data sets collected using genetic mutants on OP50 with those obtained by RNAi on HT115. The development of OP50(xu363) has now bridged this gap.

We showed that OP50 and HT115 differentially modulate many aspects of worm biology, including metabolism, behavior, aging, and perhaps development. The observed differential dietary effects are complex and unlikely all caused by metabolic alternations induced by the two diets (Heintz and Mair, 2014). Many other factors may contribute, including, but not limited to, sensory perception, behavioral regulation, and pathogenicity (Heintz and Mair, 2014). It should be noted that we only surveyed a few parameters to provide a proof of concept. Future efforts are needed to conduct a more-comprehensive analysis. Different bacterial diets also differentially regulate reproduction (Coolon et al., 2009; MacNeil et al., 2013). Worms fed OP50 and HT115, however, have a similar brood size (20°C; R.X., J.L., and X.Z.S.X., unpublished observation; MacNeil et al., 2013). This by no means indicates that these two diets do not affect reproduction under other conditions or other aspects of reproduction. For example, it has been reported that worms show a decrease in brood size on HT115 when assayed at a higher cultivation temperature (Maier et al., 2010). The observed diet-dependent differential effects on the development from L4 to reproductive adult might also have a contribution from reproduction. Mutations in the nuclear hormone receptor NHR-114 render worms sterile on OP50 but left them fertile on HT115 (Gracida and Eckmann, 2013), demonstrating that fertility is affected differentially by genes in a diet-dependent manner.

Genes, particularly those in nutritional and metabolic signaling pathways, are believed to mediate differential dietary responses (Watson et al., 2013; Watson and Walhout, 2014). Among them, mTORC2 signaling plays a key role. We also found this to be the case. Interestingly, we observed that mTORC2 is not required for mediating the differential effects of diet on fat storage or locomotion behavior, though its ablation does globally affect these two processes apparently in a diet-independent manner. Neuroendocrine signaling has been reported to regulate dietary responses. We showed that neuroendocrine signaling is also important for mediating some aspects of the differential

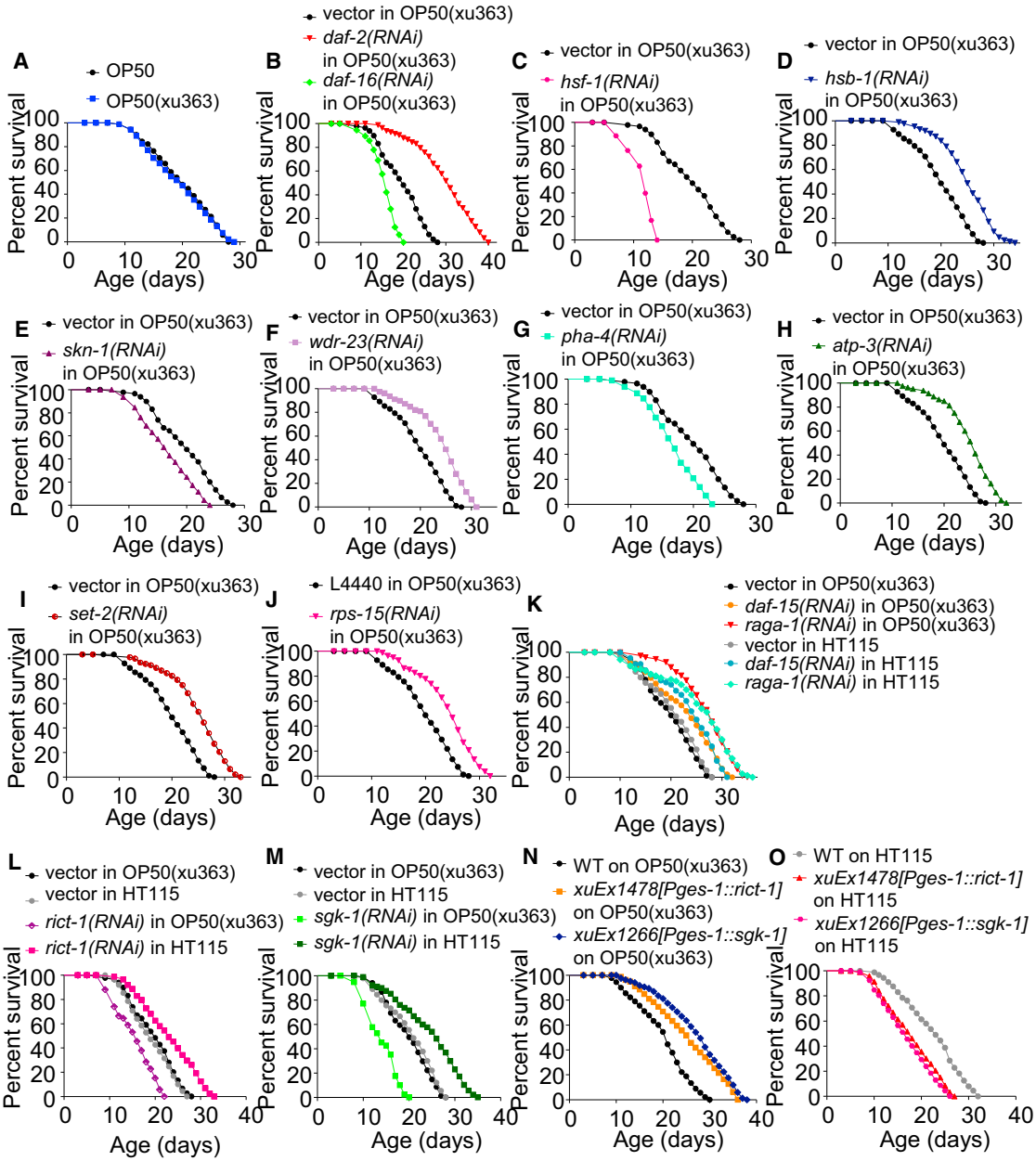

Figure 6. Diet-Dependent Differential Modulation of Longevity Revealed by RNAi Using OP50(xu363) and HT115

(A) N2 worms show similar lifespans on wild-type OP50 and OP50(xu363).

(B) *daf-2*(*RNAi*) extends the lifespan whereas *daf-16*(*RNAi*) shortens the lifespan of N2 worms grown on OP50(xu363).

(C and D) *hsf-1*(*RNAi*) shortens the lifespan whereas *hsb-1*(*RNAi*) (a *hsf-1*-negative regulator) extends the lifespan of N2 worms grown on OP50(xu363).

(E and F) *skn-1*(*RNAi*) shortens lifespan whereas *wdr-23*(*RNAi*) (a *skn-1*-negative regulator) extends lifespan of N2 worms grown on OP50(xu363).

(G) *pha-4*(*RNAi*) shortens the lifespan of N2 worms grown on OP50(xu363).

(H) *atp-3*(*RNAi*) extends the lifespan of N2 worms grown on OP50(xu363).

(I) *set-2*(*RNAi*) extends the lifespan of N2 worms grown on OP50(xu363).

(J) *rps-15*(*RNAi*) extends the lifespan of N2 worms grown on OP50(xu363).

(K) Knockdown of mTORC1 components *daf-15* and *raga-1* by RNAi in both HT115 and OP50(xu363) leads to lifespan extension.

(L and M) Knockdown of mTORC2 components *rict-1* (L) and its downstream effector *sgk-1* (M) by RNAi shortens lifespan when worms are grown on OP50(xu363) but extends lifespan when worms are grown on HT115.

(N and O) Transgenic expression of RICT-1 and SGK-1 extends the lifespan of N2 worms grown on OP50(xu363) (N) but shortens the lifespan of N2 worms grown on HT115 (O).

Also see Table S1.

effects of diet. Apparently, genetic regulation of differential dietary responses is highly complex, which likely involves many other genes and pathways. Nevertheless, it should be stressed that the current study only sampled a few regulatory genes as a proof-of-concept test. Future efforts are needed to conduct a more-comprehensive analysis. A few genome-wide RNAi libraries have already been generated using HT115 (Fraser et al., 2000; Kamath et al., 2003; Rual et al., 2004). Our work makes it possible to generate a genome-wide RNAi library using an OP50 strain, and sub-libraries (e.g., one covering all transcription factors) can also be readily made using our strain (R.X., J.L., and X.Z.S.X., unpublished observations). This would enable RNAi screens to systematically interrogate the genetic basis underlying diet-dependent differential modulation of worm biology.

Other *E. coli* strains (e.g., HB101) and bacteria (e.g., *Comamonas*) also differentially modulate *C. elegans* biology (Coolon et al., 2009; MacNeil et al., 2013; You et al., 2008). In addition, pathogenic bacteria such as some *Pseudomonas* and *Serratia* strains trigger innate immune responses in *C. elegans* (Aballay and Ausubel, 2002; Kim, 2013). Thus far, it has not been possible to conduct RNAi on worms fed these bacterial strains. The two-step strategy with which we developed to engineer OP50 should be applicable to other bacterial strains to render them compatible for dsRNA production and delivery. Genome-wide RNAi libraries may also be generated using these bacterial strains. This will greatly expand the use of *C. elegans* as a genetic model not only for dietary signaling and host-microbes interactions but also for innate immune responses.

EXPERIMENTAL PROCEDURES

Strains

Wild-type: N2. TQ2460: *xuIs117*[*sur-5*::*gfp*]. TQ3572: *xuEx1266*[*Pges-1*::*sgk-1*::*SL2*::*mCherry*]. TQ4776: *xuEx1478*[*Pges-1*::*rict-1*::*SL2*::*mCherry*]. The following mutant strains were obtained from the Caenorhabditis Genetics Center (CGC) and used in this study: *rict-1*(*mg360*), *sgk-1*(*ok538*), *pept-1*(*lg1601*), *unc-31*(*e169*), *tyra-3*(*ok325*), and *uIs57*[*unc-119p*::*YFP+unc-119p*::*sid-1+mec-6p*::*mec-6*];*lin-15B*(*n744*). Except *unc-31* RNAi clone that was designed individually (forward: 5′-CACAAGAATTCGATGGAGATGAGG-3′; reverse: 5′-GTGTTGTCACTGGAACTGTTTCC-3′), all other RNAi constructs were extracted from the Ahringer library and confirmed by sequencing before transformed into OP50(xu363).

Generation of RNAi-Compatible OP50(xu363)

Two genetic modifications were made in the OP50 genome. (1) The endogenous *rnc* gene of OP50 was disrupted by insertion of a tetracycline-resistant *miniTn10* transposon through P1 phage-mediated transduction from an *rnc*$^-$ host strain *E. coli* K8024 (a derivative of the K-12 strain K37 [derived from W3102; NIH strain collection]) with the *rnc14*::*miniTn10* allele (Takiff et al., 1989). The product from this step is termed OP50(xu362) with the genotype *ura*$^-$, *strR*, *rnc*$^-$, and the transposon insertion site has been verified by sequencing. (2) We introduced an IPTG-inducible T7 polymerase cassette into the *attB* site of *E. coli* bacteria OP50(xu362) through two steps of Red/ET-mediated recombination followed by a FLP-mediated recombination (Fu et al., 2010). Briefly, a kanamycin-resistant cassette flanked by two FRT sites was first introduced into the boundary region between *ybhC* and *lacI* genes of *E. coli* bacteria BL21(DE3) through Red/ET-mediated homologous recombination. Then, the genomic fragment carrying the whole T7 polymerase complex and the kanamycin-resistant cassette was amplified by PCR and inserted into the *attB* site of OP50(xu362) through a second step Red/ET-mediated homologous recombination. Lastly, the kanamycin-resistant cassette was removed through FLP-mediated recombination. The final product was verified by sequencing and termed OP50(xu363) with a genotype *ura*$^-$, *strR*, *rnc*$^-$, ΔattB::FRT-*lacI*-*lac*UV5p-T7.

RNAi Assay

Freshly streaked single colonies of HT115 and OP50(xu363) bacteria containing either empty vector L4440 or RNAi plasmid were grown up overnight (20–24 hr) at 37°C in LB supplemented with carbenicillin (100 μg/ml). As OP50(xu363) grows slightly slower than OP50 and HT115, it is recommended to pick colonies from freshly streaked agar plates. The next day, bacteria were diluted into fresh LB and cultured for a few hours, and subsequently, IPTG (200 μM final concentration) was added in liquid culture when bacterial density reaches ~OD_{600} = 0.6 to induce the production of dsRNA for ~4 hr at 37°C. Two days before experiments, freshly grown RNAi bacteria were seeded on RNAi nematode growth media (NGM) plates supplemented with carbenicillin (25 μg/ml) and IPTG (1 mM). All RNAi feeding assays were started from eggs throughout lifespan at 20°C. Note: RNAi conducted in adulthood alone may generate different results, and this may account for some of the discrepancies reported in the literature.

Developmental Rate Assay

All experiments were performed at 20°C using a protocol described previously (Soukas et al., 2009). Eggs were age synchronized by allowing 100 gravid adult worms to lay eggs for 30 min. At the L4 stage, 20 worms per strain per food source were transferred to individual NGM plates seeded with corresponding bacteria and continuously monitored every 30 min until the first egg was laid. Data were represented as the mean egg-to-egg time ± SEM.

Oil-Red-O Staining

All experiments were performed at 20°C. Oil-Red-O (ORO) staining was performed as described previously (O'Rourke et al., 2009; Soukas et al., 2009). Briefly, ~500 age-synchronized day 1 adult worms of each strain were washed off plates with 1× phosphate-buffered saline (PBS) buffer. Worms were washed twice and re-suspended in 375 μl PBS. Then, 500 μl freshly prepared 2× MRWB (160 mM KCl, 40 mM NaCl, 14 mM Na_2EGTA, 1 mM spermidine HCl, 0.4 mM spermine, 30 mM NaPIPES [pH 7.4], and 0.2% beta-ME) and 125 μl 16% paraformaldehyde were added to fix the worms for 30 min at room temperature (RT). After washing twice with Tris-Cl buffer (100 mM; pH 7.4), the worms were incubated in a reducing buffer (100 mM Tris-Cl [pH 7.4] and 10 mM DTT) for 30 min at RT and pelleted and washed twice with 1× PBS before re-suspended in 60% isopropanol for dehydration (15 min; RT). After all isopropanol was removed, the worms were incubated in 1 ml ORO staining solution (prepared as follows: 0.5 g ORO was dissolved in 100 ml anhydrous isopropanol and equilibrated for 2 days by stirring to make ORO stock solution. ORO staining solution was freshly prepared by mixing 60% ORO stocking solution with 40% distilled water and filtered through a 0.2-μm syringe filter) overnight at RT.

ORO-stained worms were directly mounted on glass slides with an agarose pad (2% agarose in 1× PBS), and images were acquired through an upright compound microscope (Olympus BX51) coupled with a digital color CCD camera. For lipid content quantification, color images were background subtracted and converted into gray scale images using ImageJ program. Signal intensities were then compared among worms grown under different conditions. At least 30 worms were assayed for each experiment.

Oxygen Consumption and ATP Production

All experiments were performed at 20°C. Oxygen consumption rate (OCR) was measured in real time using a Seahorse XF24 Analyzer (Sapir et al., 2014). Age-synchronized day 1 adult worms were rinsed off a 10-cm NGM plate and washed three times by gravity separation in M9 buffer (3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, and 1 ml 1 M $MgSO_4$ in 1 l distilled water). 50~300 worms were transferred into each well of a Seahorse assay plate, and OCR was measured according to the standard protocol recommended by the manufacturer. Final OCR was obtained by dividing the measured OCR by the number of assayed worms.

For ATP production measurement, ~1,000 age-synchronized day 1 adult worms were collected and washed three times with M9. After three freeze-thaw cycles, the worms were boiled in M9 for 15 min. After centrifugation, the

supernatant was assayed for ATP production (ATP Determination Kit; Life Technologies) and protein concentration (Bradford Protein Assay Kit; Thermo Fisher Scientific). Final ATP production was normalized to the amount of proteins.

Pharyngeal Pumping Rates and Quiescence State

All experiments were performed at 20°C. To assay pharyngeal pumping rate, we measured the duration required to complete 20 pumps. Twenty well-fed day 1 gravid adult worms were assayed on food for each experiment and approximately two or three measurements were recorded for each animal. Quiescence state was calculated by measuring the percentage of non-pumping duration within 1-min recording period. Twenty well-fed day 1 adult worms were assayed on food.

Food-Leaving Assay

All experiments were performed at 20°C. Food-leaving behavior was assayed as described previously (Bendesky et al., 2011). Briefly, 60-mm NGM plates were seeded with either 70 μl (conditioning plate) or 10 μl (assay plate) of freshly cultured bacteria. Ninety minutes after seeding the bacteria, ten day 1 adult worms were transferred into the conditioning plates for 30 min food conditioning. Then, the worms were transferred into the assay plates and incubated for 1 hr followed by 30 min videotaping of food-leaving events using a WormLab recording system (MBF Bioscience). The food-leaving rate was calculated as the number of leaving events per minute per worm. Each experiment was repeated at least five times.

Locomotion Analysis

All experiments were performed at 20°C. Locomotion behavior was assayed as previously described (Liu et al., 2013; Ward et al., 2009). Five minutes before tracking, 50 μl freshly grown bacteria was spread on an NGM plate to form a thin layer of bacterial lawn. A custom-developed single-worm tracking system was used to track and obtain speed data of worms. Each worm was recorded for 10 min, and the average speed was used for comparison. For reversal rate measurement, locomotion videos were manually analyzed for the number of reversal events within a 10-min recording period. At least ten worms were tracked for each experiment.

Lifespan Assay

All lifespan studies were conducted at 20°C as described previously (Hsu et al., 2009; Xiao et al., 2013). In all experiments, the first day of adulthood was scored as day 1. Worms were censored if they crawled off the plate, exploded, or bagged. All RNAi experiments for lifespan assays were started from eggs throughout lifespan. All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software) and IBM SPSS Statistics 19 (IBM). The p values were calculated using the log rank (Kaplan-Meier) method.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.04.024.

AUTHOR CONTRIBUTIONS

R.X., L.C., and E.A.R. performed the experiments and analyzed the data. D.I.F. generated reagents with R.X. R.X., J.L., and X.Z.S.X. wrote the paper.

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