

The Majority of Animal Genes Are Required for Wild-Type Fitness

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SUMMARY

Almost all eukaryotic genes are conserved, suggesting that they have essential functions. However, only a minority of genes have detectable loss-of-function phenotypes in experimental assays, and multiple theories have been proposed to explain this discrepancy. Here, we use RNA-mediated interference in *C. elegans* to examine how knockdown of any gene affects the overall fitness of worm populations. Whereas previous studies typically assess phenotypes that are detectable by eye after a single generation, we monitored growth quantitatively over several generations. In contrast to previous estimates, we find that, in these multigeneration population assays, the majority of genes affect fitness, and this suggests that genetic networks are not robust to mutation. Our results demonstrate that, in a single environmental condition, most animal genes play essential roles. This is a higher proportion than for yeast genes, and we suggest that the source of negative selection is different in animals and in unicellular eukaryotes.

INTRODUCTION

Almost all genes encoded in eukaryotic genomes are conserved over long evolutionary timescales. This high level of conservation suggests that almost all genes play important roles in the biology of the organism and further predicts that a loss-of-function mutation in any one of these highly conserved genes should have a deleterious effect. However, this is not what has been found in direct experimental studies. Large-scale studies to identify the loss-of-function phenotypes of each predicted gene have been done in vivo in several free-living organisms, including the prokaryote *E. coli* (Gerdes et al., 2003), the yeast *S. cerevisiae* (Giaever et al., 2002; Winzeler et al., 1999), and the animals *C. elegans* (Fraser et al., 2000; Kamath et al., 2003; Sönnichsen et al., 2005) and *D. melanogaster* (Dietzl et al., 2007). Although the technologies differed (in *E. coli* and *S. cerevisiae*, genes were disrupted by chromosome engineering, whereas in *C. elegans* and *D. melanogaster*, loss-of-

function phenotypes were generated by in vivo RNAi), the basic findings were very similar—in all of the genomes examined, only a minority of genes had a detectable loss-of-function phenotype. This finding is perplexing: the great majority of genes in *E. coli*, *S. cerevisiae*, *C. elegans*, or *D. melanogaster* have highly conserved orthologs in other species, and almost all of these genes show evidence of negative selection (Bierne and Eyre-Walker, 2004; Cutter et al., 2003; Koonin and Wolf, 2010; Pál et al., 2006; Papp et al., 2004; Stein et al., 2003).

Several models have been proposed to explain the difference between the high number of genes that appear to be functionally important based on conservation and the relatively low proportion of genes that have detectable loss-of-function phenotypes in direct experimental assays. These include models that suggest either that many genes do not have detectable loss-of-function phenotypes because genetic networks are robust to mutation (for example, due to redundancy or genetic buffering; Boone et al., 2007; Costanzo et al., 2010; Deutscher et al., 2006; Hartman et al., 2001; Kafri et al., 2005; Kitano, 2004, 2007; Krebs et al., 2011; Rutherford, 2000) or that many genes are required in other untested environmental conditions (Hillenmeyer et al., 2008). In *S. cerevisiae*, there is evidence to support several of these models: for example, many genes that do not have detectable phenotypes when yeast are grown in rich medium have strong fitness defects when assayed in either different environmental conditions (Hillenmeyer et al., 2008) or yeast having different genetic backgrounds (Costanzo et al., 2010; Dowell et al., 2010; Tong et al., 2004). Combined data from all such screens indicate that, in yeast, though the majority of genes have no detectable fitness defect under any given condition, almost every gene is individually required for normal growth in at least one environmental or genetic condition.

In *C. elegans*, more than 50 genome-scale RNAi screens have been carried out, and this is the largest data set available for any animal (all RNAi data from Wormbase [Harris et al., 2010]). The range of phenotypes examined over all 50 screens is very wide, ranging from strong phenotypes like lethality and sterility to more subtle developmental defects such as defects in vulval development and movement, and include screens carried out in sensitized backgrounds (e.g., in the RNAi hypersensitive strain *lin-35* [Lehner et al., 2006a; Wang et al., 2005]), mutant backgrounds (e.g., suppressor and enhancer screens [Byrne et al., 2007; Lehner et al., 2006b; Poulin et al., 2005]), different environmental conditions (e.g., DNA damage [Pothof et al., 2003]), and

using specific markers (e.g., for axonal guidance defects [Schmitz et al., 2007]). However, whereas in yeast, additional screens in different conditions or genetic backgrounds identified many new genes with detectable phenotypes, this has not been the case in the worm; almost all genes identified in any new screen had already been found to have a detectable phenotype in previous assays. It is possible that the 85% of *C. elegans* genes for which no phenotype has been identified despite numerous screens are conditional essentials, or redundant. However, there is a clear alternative: many genes may have loss-of-function phenotypes that are too subtle to be detected using standard genetic screening methodologies but that nonetheless have a major impact on selection over evolutionary timescales.

In worm and fly, the phenotypes examined for decades are almost always those that are detectable at the level of the individual organism and that are clearly visible within a single generation. In yeast, on the other hand, almost all systematic screens to identify loss-of-function phenotypes have measured fitness (Giaever et al., 2002; Hillenmeyer et al., 2008; Winzeler et al., 1999). Fitness is not measured at the level of individual yeast cells but is instead a population-level phenotype that is measured over multiple generations. Many genes that have barely detectable defects at the level of the individual cell, such as a very minor increase in cell-cycle time, have easily detectable fitness defects. We reasoned that many worm genes might likewise have subtle loss-of-function phenotypes such as very minor defects in brood size or in growth rate that would be essentially undetectable at the level of individual animals but would be detectable as population-level fitness defects.

Here, we describe two independent methods for directly measuring fitness in *C. elegans*. We use these methods to screen more than 550 randomly chosen genes and show that a majority of *C. elegans* genes affect fitness in laboratory conditions. This is far higher than the proportion of genes previously known to have loss-of-function phenotypes and suggests that animal genetic networks are, in general, not robust to mutation. This systematic examination of the connection between genotype and fitness in an animal reveals that, in a single environmental condition, a far higher proportion of genes affect fitness in animals than in unicellular eukaryotes.

RESULTS

The Majority of *C. elegans* Genes Are Required for Wild-Type Fitness in Laboratory Conditions

The great majority of *C. elegans* genes have no identified loss-of-function phenotype despite many systematic screens. However, these genes are, in general, highly conserved and under strong negative selection (Stein et al., 2003), suggesting that they do indeed play crucial roles in the development and function of the worm. How is it that these genes do not appear to have any loss-of-function phenotype? We hypothesized that many such genes have phenotypes that are too subtle to be detected in conventional screens but that might be detected in “fitness” assays analogous to those used in yeasts. We thus set out to investigate the connection between genotype and fitness directly in *C. elegans*, which has key advantages for this. First, worms can be maintained as selfing hermaphrodites, and thus

maintaining populations of isogenic animals over multiple generations is relatively trivial. Second, RNAi can be used to target any gene of interest in vivo (Fire et al., 1998; Kamath et al., 2001; Timmons and Fire, 1998). We thus set out to establish protocols to quantify the number of worms in a population over time so that we could examine the effect of targeting any gene by RNAi on the rate of population expansion and thus on fitness (Figure 1A). “Fitness” in this context is similar to that measured in yeast in either liquid growth or colony growth assays and is an aggregate property of the population; a defect in fitness could result from many different causes from changes in brood size, viability, or growth rate, or other more subtle defects in organism function.

We established two independent platforms for monitoring the rate of population expansion of *C. elegans* following RNAi (Figure 1B and Extended Experimental Procedures available online). The first is conceptually straightforward (Figure 1B, left). In outline, we count the number of animals in each RNAi experiment at various time points using a commercially available worm sorter (Union Biometrica, <http://www.unionbio.com>). To assess whether RNAi of any gene causes a fitness defect, we compare the worm number following RNAi of gene X to the worm number in a set of negative controls (bacteria expressing a number of nontargeting double-stranded RNAs; Extended Experimental Procedures). Any gene that is required for wild-type fitness will have a reduced population size compared to the nontargeting controls. Because this involves the direct monitoring of worm number, we call this the “direct” method. The second platform (Figure 1B, right) uses the same fundamental set-up but, rather than look at worm number directly, we monitor the rate at which bacterial food is exhausted by the expanding worm populations by examining optical density over time. The more worms that there are, the faster they will consume the bacteria; hence, the more bacteria remaining, the stronger the fitness defect. We convert measurements of change of bacterial density to fitness in a similar way to that used in the direct method: the fitness following RNAi against gene X is defined as the change in OD595 in cultures wherein gene X is targeted compared with the change in OD595 of nontargeting controls. This method gives a proxy of the actual worm number, and we call this the “indirect” method. We note that, during preparation of this manuscript, a similar method was recently described by the Poulin group (Elvin et al. 2010). In either method, we run these assays for 8 days, and this approximates to two to three generations of growth. The direct assay can accurately identify differences in population size of 20% after two to three generations, and this equates to a difference of as little as 5% per generation. Our assays can thus pick up very subtle perturbations of phenotype. The two key differences are in the method to measure worm number (direct versus indirect) and in the throughput. Though the direct method is conceptually far more obvious, the throughput is low; it is hard to assay fitness of more than ~100 genes per week by RNAi, as the worm sorter cannot deal with greater flow rates or worm densities. Because it is based uniquely on bacterial optical density measurement, the indirect method has a far higher throughput.

We first used both direct and indirect methods to measure the fitness defects of a set of 75 genes off chromosome III (Table S1).

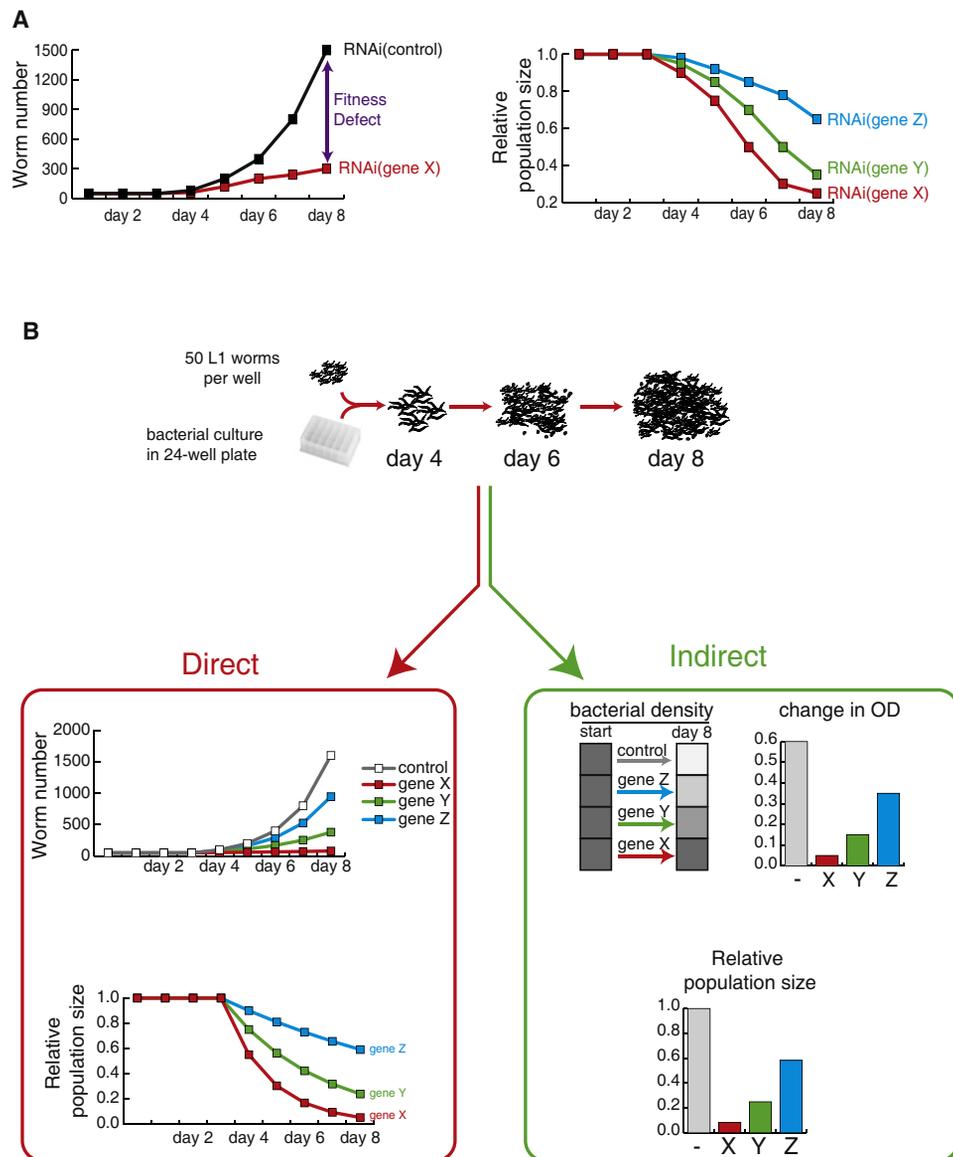


Figure 1. Schematic of Fitness Assays Using RNAi

(A) Outline of use of RNAi to examine impact of any gene on fitness. To examine the effect of targeting any individual gene by RNAi on fitness, we quantify the population size at different time points and compare this to the population size of parallel worm cultures grown in the presence of control nontargeting bacteria. The ratio of worm number between targeted populations and control populations is the relative fitness.

(B) Schematic of two independent methods to quantify fitness using RNAi. To assess population size and hence fitness, we use two independent methods. In the direct method (left), we inoculate 2 ml cultures of dsRNA-expressing bacteria with 50 L1 stage larvae and remove 50 μ l samples on days 5, 6, 7, and 8. The number of worms in each sample is counted using a commercially available worm sorter. In the indirect method (right), we set up identical cultures as the direct method but measure OD₅₉₅ at days 5 and 8. In this case, fitness is given by the relative change in OD₅₉₅ compared with the change in control cultures.

These included 20 genes that had previously known RNAi phenotypes (data from Wormbase [Harris et al., 2010]); the rest are all annotated as having wild-type RNAi phenotypes. We find that the fitness following RNAi measured by either direct or indirect measurements correlates tightly (Figures 2A and S1) and that the fitness defect of any gene is independent of the RNAi clone used for targeting (Figure 2B and Tables S2 and S3) and thus the fitness defect measured is a true property of the gene. We next examined the correlation between the fitness

defects that we monitor and data from previous RNAi screens. We find that 19 of 20 genes (95%) found to have an RNAi phenotype in any previous screen have a fitness defect in our assays (Table S1; $p < 0.05$), showing that our sensitivity is extremely high. Furthermore, the strength of phenotype detected in previous screens closely matches the strength of fitness defect that we measure (Figure 2C): genes that have lethal or sterile RNAi phenotypes have the strongest fitness defects, and those with more subtle phenotypes such as growth defects or

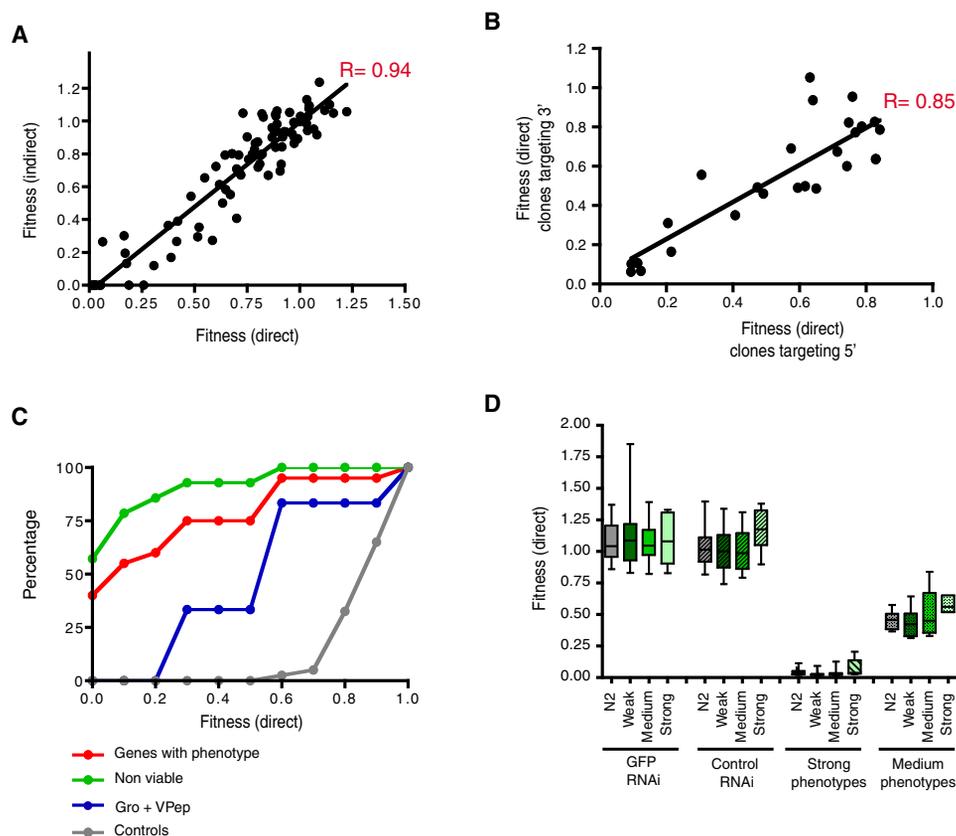


Figure 2. Comparison of Direct and Indirect Fitness Assays

(A) RNAi was carried out against each of 75 randomly chosen genes in three independent experiments, and the effect of targeting each gene was quantified either using the direct or indirect methods. The mean of the three experiments is shown, and the results of either method correlate highly ($r = 0.94$).

(B) RNAi-induced fitness defect is gene specific. Two independent nonoverlapping targeting clones were made for 23 genes in the test set. The direct method was used to assess the effect of either the 5' or the 3' targeting clone on fitness for each of the 23 genes. The fitness defect observed is very similar for both clones for almost all genes ($r = 0.85$), suggesting that the fitness defect observed is a property of the gene targeted and not the RNAi clone chosen.

(C) Fitness assays recover almost all genes previously known to have RNAi phenotypes. Twenty genes in the set of 75 random genes had previously been identified to have an RNAi phenotype using standard manual RNAi screening methods. Fourteen of these had nonviable (lethal or sterile) phenotypes, whereas six had either growth defects (Gro) or more subtle postembryonic defects (VPep). We show how our fitness assays recovered these known genes; data shown are mean data from three independent repeats of the day 8 time point using the direct population size assay. Each curve shows the percentage of previously known nonviable (green curve), Gro and VPep (blue curve), or all known genes (red curve) that are recovered at a given fitness defect cut-off; as a comparison, all 30 nontargeting controls are shown in gray. We find that, for example, more than 90% of nonviables, more than 30% of Gro and VPep, and 75% of all known genes have a measured fitness defect of 0.4 or stronger, for example.

(D) Engaging the RNAi machinery does not give a detectable fitness defect. RNAi was carried out in four different worm strains: wild-type (N2) or three transgenic lines expressing low, medium, or high levels of GFP in both intestine and muscle (PD4788 [weak], PD4753 [medium], and PD4790 [strong]; Figure S2). Each strain was exposed to bacteria expressing dsRNAs that target either GFP, a number of genes with strong fitness defects or medium strength fitness defects, and bacteria expressing a control nontargeting dsRNA. RNAi was carried out in independent triplicates and population growth monitored using a worm sorter. Data shown are population sizes on day 8 in comparison to the nontargeting controls; in each case, the box plot depicts the minimum value, lower quartile, median, upper quartile, and maximum value.

movement defects have weaker fitness defects. Finally, because the controls that we used throughout are bacteria-expressing nontargeting dsRNAs, which would not fully engage the RNAi machinery, we tested whether the fitness defects that we observe following the targeting of endogenous genes by RNAi might be an artifact due to an inherent fitness cost of fully engaging the RNAi machinery. We find that targeting GFP by RNAi has no effect on the growth of populations of worms whether or not the worms express GFP transgenically (i.e., whether or not the RNAi machinery is engaged; Figure 2D).

Furthermore, we can find pairs of endogenous genes that have highly similar levels of expression across development (Spencer et al., 2011) and for which RNAi knockdown is nearly identical but for which only one of the genes in the pair has a fitness defect (Figure S2 and Extended Experimental Procedures). This fitness defect cannot thus be explained by a difference in engagement of the RNAi machinery itself. We therefore find that engaging the RNAi machinery does not in itself result in a detectable fitness cost, and thus our results show that a fitness defect seen following targeting an endogenous gene by RNAi is gene

specific and is the direct consequence of knocking down the targeted gene.

Our assays detect fitness defects for 95% of genes with previously known RNAi phenotypes. In addition, we detect statistically significant fitness defects for an additional 20 genes that had never had any detectable RNAi phenotypes in any genome-scale screens (Table S1; $p < 0.05$). In total, we see that ~50% (39 of 75) of the genes tested have a statistically significant defect. To ensure that these were not due to any off-target effects, we made two independent nonoverlapping clones targeting 10 of these novel genes as well as 13 genes with previously known RNAi phenotypes. As shown in Figure 2B, we recapitulated the fitness defects for 17 of the 23 genes in both the independent clones tested. These data suggest that many worm genes that had previously been found to have no detectable loss-of-function phenotype have fitness defects following RNAi and thus that the true number of genes required for wild-type fitness is far higher than previously thought.

To explore this further, we used the higher throughput indirect method to screen ~550 genes for fitness defects (Table S4). These genes were chosen randomly off *C. elegans* chromosome III and span the entire length of the chromosome. Each RNAi knockdown was set up as biological duplicates, and we see an excellent correlation in fitness estimated in either replicate (Figure S3A). As in our initial test of 75 genes, we have excellent recovery of genes with previously known RNAi phenotypes (Figure 3A); of the 121 genes with previously known RNAi phenotypes, 96 (~80%) have a statistically significant fitness defect (Table S2; $p < 0.05$). In addition, as was the case with our initial smaller test set of genes, the strength of previously identified RNAi phenotype correlates well with our measured effect on fitness (Figure 3B).

Strikingly, as seen in our small test set of 75 genes, almost half (222 of 539; 42%) of all RNAi clones gave a statistically significant fitness defect (Figure 3A and Tables S4 and S5). If we restrict our analysis only to the set of genes that are present at single copy in the worm genome (i.e., are not members of gene families in which there could be substantial redundancy between paralogs [Tischler et al., 2006]), this number rises to more than ~60% (202 of 328) (Figure 3C). RNAi screens have a substantial false negative rate arising from combination of multiple sources, including errors in the RNAi library, difficulty in generating efficient knockdown for genes encoding proteins with long half-lives, and the fact that many genes require almost complete knockdown before any loss-of-function phenotype is visible. The most accurate measures of this false negative rate derive from genome-scale screens and suggest that the rate is ~40%, with a false positive rate of less than 1% (Kamath et al., 2003). We identify phenotypes for 42% of genes using our fitness assay, and thus we conclude that ~70% of all genes have a loss-of-function phenotype in laboratory conditions and, furthermore, that essentially all genes that are present in single copy in the worm genome have a phenotype.

Previous Genetic Screens Fail to Detect Most Loss-of-Function Phenotypes

In the worm to date, more than 50 genome-scale RNAi screens have been carried out, covering a wide range of phenotypes.

All previous RNAi-based analyses together detect phenotypes for ~15% of genes. We refer to these here as the “known” genes (Experimental Procedures). This number has barely changed, although increasing numbers of screens have been carried out (Figure S3B). We find that this set of known genes is highly skewed; they are highly enriched for having yeast orthologs and are also much higher expressed than random genes (Figure 3D; expression data from Ramani et al. [2009]). Most significantly, when we examine the fitness defects of these known genes, they are highly enriched for genes with very strong fitness defects; more than 75% of the previous known genes have an RNAi fitness defect of 0.4 or more. The genes that have detectable fitness defects in our assays that were previously not known to have detectable RNAi phenotypes (we refer to these as the “novel” genes) are different: most of these have weaker fitness defects than the known genes, their expression levels are not significantly higher than random, and they are not enriched for having yeast orthologs. Twice as many novel genes contain domains that are found in animal genomes as known genes (33 of 75 novel vs. 18 of 72 known; $p < 0.0001$), and 50% more have orthologs in other animal genomes, but not in yeasts (35 of 96 known vs. 64 of 119 novel; $p < 0.02$), suggesting that the novel genes are enriched for more recently evolved genes.

The finding that genes with very strong RNAi fitness defects are enriched for genes that are conserved between worm and yeast (Figure 3D) is consistent with a key prediction of the neutral theory of molecular evolution—that functionally more important genes should evolve more slowly than less important genes. It is also in keeping with previous studies in *C. elegans* that have shown that genes with RNAi phenotypes that are detectable in a single generation (often termed “essential” genes) tend to be more highly conserved than other genes (Cutter et al., 2003; Fraser et al., 2000; Kamath et al., 2003; Stein et al., 2003). However, because these previous screens failed to identify phenotypes for many of the genes that affect fitness in our assays, we thus examined whether we could detect any correlation between the level of functional importance of any gene (as we measure by its effect on fitness) and the rate at which it evolves, as measured by Ka/Ks (the adjusted ratio of nonsynonymous-to-synonymous changes in the coding sequence of each gene [Hurst, 2002; Yang and Bielawski, 2000]). Similar analyses have been carried in *S. cerevisiae*, wherein the effect of individual genetic perturbations on fitness has been systematically studied and quantified. Intriguingly, although there is a correlation in yeast between functional importance (as measured by effect of loss of function on fitness) and rate of evolution, it is extremely weak (Hirsh and Fraser, 2001; Wall et al., 2005; Wang and Zhang, 2009). We find that this is also true in worm; though one can see a trend in the data (as shown in Figure 4A), the correlation is weak (Figure S4), suggesting that, in animals (as in yeast), many other factors such as gene expression level (Hurst, 2002; Hurst et al., 2004; Wang and Zhang, 2009) make at least as significant contributions to the rate of gene evolution as the functional importance of the gene to the fitness of the organism. Despite the weakness of the correlation, we still find that Ka/Ks is a good predictor of the functional importance of any gene (Figure 4B): if we randomly pick pairs of genes, the gene with lower Ka/Ks has a stronger measured impact on fitness 55% of the time (random

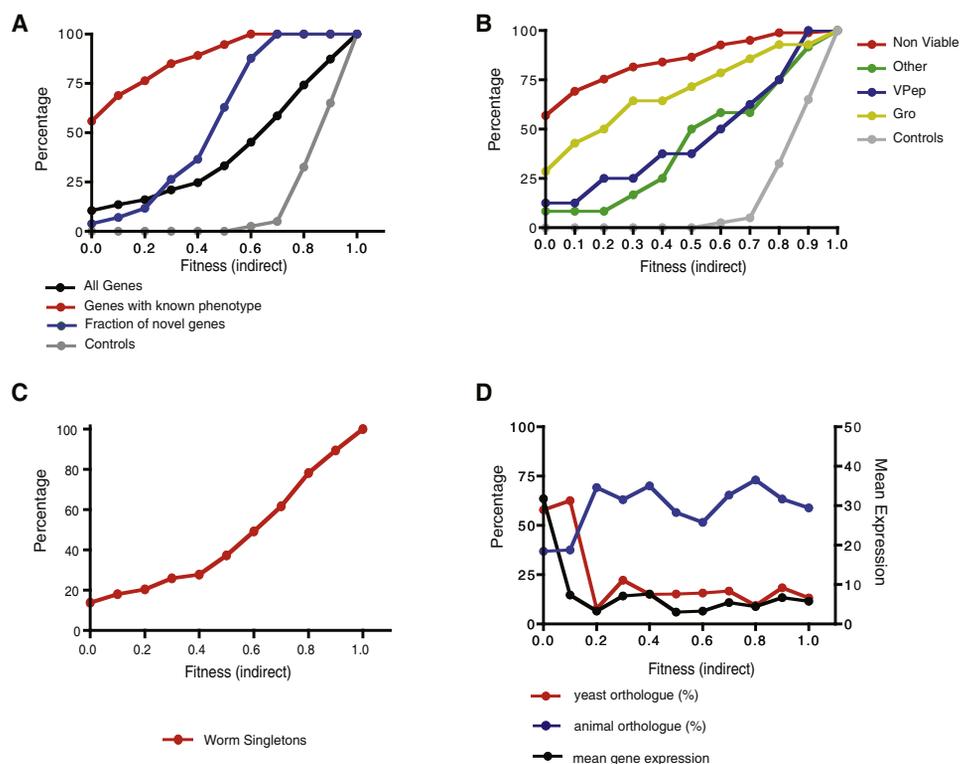


Figure 3. Many Genes with No Previously Detected RNAi Phenotype Affect Fitness

Approximately 550 genes were randomly chosen from *C. elegans* chromosome III to span the entire chromosome, and the indirect assay was used to quantify the size of fitness defect following targeting of each gene by RNAi.

(A) Distribution of fitness defects. Every gene has a fitness defect between 0 (complete fitness defect) and 1 (wild-type fitness). The curves show the recovery of different classes of gene: genes with previously known RNAi phenotypes are shown in red, all genes are screened in black, and control nontargeting assays are shown in gray. In each case, the curve shows the cumulative proportion of genes in each class that have a fitness defect of X or greater; for example, 100% of genes with known phenotypes and almost 50% of all genes screened have a fitness defect of 0.6 or greater. The proportion of genes in any bin that were previously not known to have a fitness defect is shown in blue. Most genes with strong phenotypes (e.g., in the range 0–0.1) were previously known, but most genes with weaker phenotypes (e.g., in the range 0.6–0.7) were not.

(B) Strength of RNAi fitness defect correlates with strength of previously known RNAi phenotype. Of the ~550 genes screened, 121 had previously known RNAi phenotypes that can be placed into four main classes based on phenotypic strength: nonviable (lethal or sterile) (red curve), Gro (Growth defects; yellow curve), VPep (subtle postembryonic defects; blue curve), and other (any other detected phenotypes). The graph shows the distribution of fitness defects for each class of gene. In each case, the curve shows the proportion of genes in each class that has a fitness defect of X or stronger.

(C) Fitness defects of single-copy genes. Of the 550 genes screened, 328 are single copy in the *C. elegans* genome (i.e., that do not exist in larger gene families); almost 70% of these have a significant fitness defect (0.7 or stronger) following RNAi.

(D) Highly expressed core eukaryotic genes have stronger fitness defects. All 550 genes screened were placed into one of ten fitness bins based on their measured fitness defects (e.g., 0–0.1, 0.1–0.2, etc.). In each bin, the graph shows the percentage of genes with a homologue in *S. cerevisiae* (red curve) or in fly, mouse, human, or other nematodes (blue curve). In addition, the mean expression level of genes in each bin based on RNA-seq data is shown in black (Experimental Procedures).

expectation is 50%; as a comparison, this is 54% in *S. cerevisiae* [Wang and Zhang, 2009]. This rises as the difference in rank increases; for example, it gives a 60% prediction if the difference in rank is 30% or higher. Overall then, as in yeast, in an animal genome, the measured rate of gene evolution correlates only weakly with observed functional importance but can nonetheless be predictive.

In summary, we have undertaken a large-scale analysis of the connection between genotype and fitness in an animal. Previous experimental studies, either from classical mutagenesis or from RNAi-based analyses, suggested that only a small minority of worm genes affect the phenotype when they are mutated. In

contrast to these previous studies, we find that the majority of genes are required for wild-type fitness. Our data thus suggest that genetic networks are not robust to mutation, as has been previously proposed, but rather provide direct evidence that most genes in any animal genome play crucial roles in the normal functioning of the organism.

DISCUSSION

In this paper, we use RNAi to systematically assess the impact of loss-of-function perturbations on fitness in *C. elegans*. This is the first such study in any animal to our knowledge, and we show

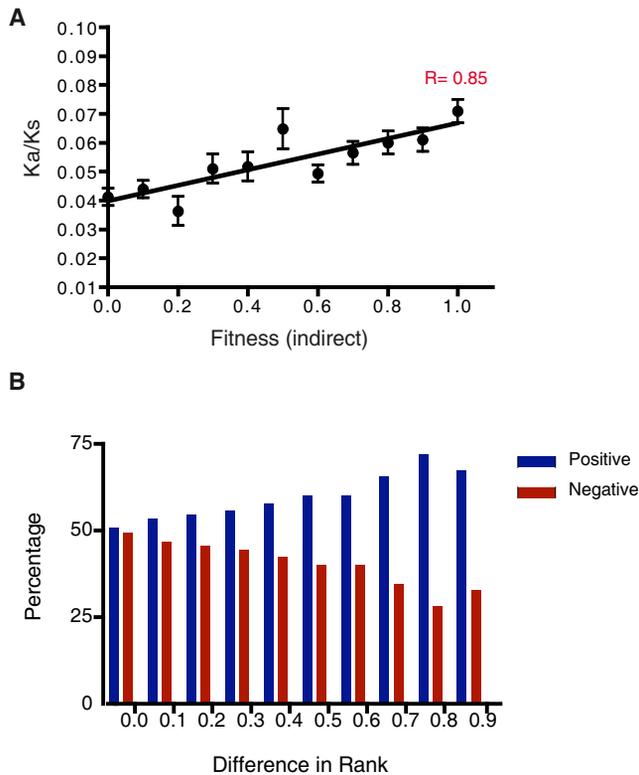


Figure 4. Level of Fitness Defect Measured in Laboratory Conditions Correlates Weakly with Level of Negative Selection

(A) All ~550 genes screened were placed into one of ten fitness bins based on their measured fitness defects (e.g., 0–0.1, 0.1–0.2, etc.). The graph shows the mean Ka/Ks for genes in each of the ten bins (Ka/Ks from a *C. elegans*–*C. remanei* comparison; see *Experimental Procedures* for details of calculation). Error bars are the standard error.

(B) Ka/Ks is predictive of relative fitness. Genes were ranked by Ka/Ks from lowest to highest, and random pairs of genes were compared. In each pair, we determined the size of the difference in rank (expressed on a scale from 0 to 1 in which 1 is the maximum possible rank difference) and whether the gene with lower Ka/Ks had a stronger fitness defect. If so, we classed this as a positive prediction. Data are shown for a million random pairs.

that the majority of genes affect fitness in laboratory conditions. This has two significant implications, which we discuss individually in the sections below.

Population-Level Phenotyping Reveals Functions for the Majority of Animal Genes

The proportion of genes that have a detectable loss-of-function phenotype is far higher than previously estimated. Previous studies either using large-scale RNAi screens (Kamath et al., 2003) or saturation mutagenesis analysis (Johnsen and Baillie, 1991; Kemphues, 2005) arrived at a similar estimate of the number of genes with detectable loss-of-function phenotypes at ~25%–30%. Thus, in *C. elegans*, as in *S. cerevisiae*, the majority of genes did not previously appear to have detectable loss-of-function phenotypes, and several models have been proposed to explain why so many highly conserved genes appear to be dispensable. These include the model that genetic

networks are inherently robust to mutation (Boone et al., 2007; Costanzo et al., 2010; Deutscher et al., 2006; Hartman et al., 2001; Kafri et al., 2005; Kitano, 2004, 2007; Krebs et al., 2011; Rutherford, 2000; Waddington, 1959) (and hence the absence of phenotypes for so many genes) or the view that many of the genes that have no phenotypes in laboratory conditions may have strong phenotypes in other environmental conditions (e.g., Hillenmeyer et al. [2008]). Our data show instead that, in the worm, the majority of genes are required for wild-type fitness in a single tested environment, and this result suggests an alternative explanation: that the absence of loss-of-function phenotypes for so many genes in previous studies in the worm is simply because the phenotypes for these genes are too subtle to be detected by the previous assays.

Many phenotypes that would be highly significant over evolutionary timescales would be undetectable in standard *C. elegans* genetic screens (whether using RNAi or mutagenesis), wherein the phenotypes examined are almost always those that are detectable at the level of the individual animal and that are clearly visible within a single generation. A mutation causing a mean brood size defect of 1%, for example, would have major consequences over the course of many generations. In standard RNAi screens, however, researchers manually assess phenotypes after just one generation and typically examine the progeny of only three individual animals (reviewed in Ahringer [2006]); no such screens could detect this subtle a difference, and indeed, animal-to-animal variation in brood size is ~10% even in wild-type worms (Hodgkin and Doniach, 1997). In the fitness assays described here, however, we look at two to three generations of growth and measure a mean phenotype of 50 initial animals; we also use quantitative phenotyping rather than subjective manual examination. We thus believe that we detect fitness defects for many genes whose loss-of-function phenotypes were too subtle to be detected in any previous screens, and we suggest that more sensitive population-level phenotyping will identify phenotypes for almost all genes in any animal genome. Finally, we note that the population-level phenotyping that we have carried out here has given a similar experimental result to that inferred from conservation: that the majority of genes are individually required for the development and function of the worm.

Wild-Type Fitness Requires a Higher Proportion of Animal Genes Than Yeast Genes

Intriguingly, we find that the proportion of worm genes that are required for wild-type fitness appears to be substantially higher than the proportion of yeast genes required for normal growth. In yeast, simple colony growth assays find that ~30% of genes (~19% essential and ~12% slow growing) are required for wild-type fitness (Giaever et al., 2002; Winzeler et al., 1999); much more sensitive assays that are capable of detecting as little as a 1% difference in population size after 20 generations increase this number to almost 40% (Breslow et al., 2008). These assays in yeast are orders of magnitude more sensitive than the assays that we describe here for the worm and thus would be expected to identify phenotypes for a higher proportion of yeast genes if the fraction of genes affecting fitness was similar in both organisms. However, what we see is the reverse: in yeast, in

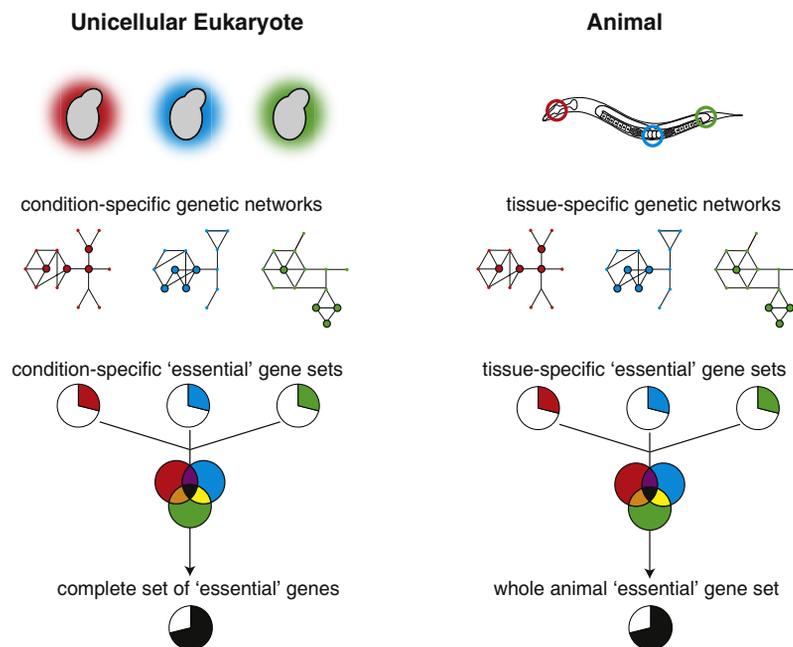


Figure 5. A Higher Proportion of Animal Genes Than Yeast Genes Are Required for Wild-Type Fitness in a Single Environmental Condition

In yeast, in any single environmental condition, only a minority of genes are “essential”—that is, required for wild-type fitness. However, in a different environment, a different set of genes is essential, and almost all genes are required for wild-type fitness in at least one environmental condition. An animal, however, has many different cell types, each of which has different sets of genes that are required for normal function. The differences in genetic networks between any two cell types are analogous to the differences in genetic networks in yeast cells growing in two different conditions. A defect in any cell type in an animal will lead to an overall defect in fitness in the whole organism. Thus, in an animal, a higher proportion of genes is required for fitness in any given environmental condition because the requirement for each gene is being simultaneously assayed in many different “cellular conditions.”

which the assay sensitivity is far higher, only ~40% of genes are required for wild-type growth, whereas in the worm, the number is greater than 70% (and rises to almost ~100% if we consider only genes for which there is no possibility of redundancy in function with other paralogs). This implies that there is a true qualitative difference in the connection between genotype and fitness in an animal and in a single-celled eukaryote—why is this? We believe that the answer can at least partially be found in data analyzing the fitness defects of yeast genes in multiple different environmental conditions.

The most sensitive fitness assays to date only identify fitness defects for ~40% of yeast genes even when yeast are grown in minimal media. However, many of the remaining 60% of genes, often termed “dispensable” or “nonessential” (Giaever et al., 2002; Winzeler et al., 1999), are absolutely required for yeast to survive in a different environment (Hillenmeyer et al., 2008) or in a different genetic background (Costanzo et al., 2010; Dowell et al., 2010; Tong et al., 2004). Thus, though most yeast genes do not have any detectable effect on fitness when assayed in one single condition, it appears that for each and every gene, a condition can be found in which it is individually required. In a complex organism with multiple different cell types, the situation is different. Most cells in any multicellular animal have different genetic networks and are effectively in as different conditions as two yeast cells exposed to different environmental conditions. Just as a yeast gene might have no detectable fitness requirement when yeast are grown in galactose but might have a strong fitness requirement when yeast are grown in glucose, a worm gene that might not be required for neuronal function might play a key role in the germline, for example. Crucially, defects in any one of these tissues affect fitness. In an animal, assessing whether a gene is required for fitness in a single environmental condition therefore inherently

tests whether that gene is required for wild-type function in any one of a large number of different coexisting “cellular conditions”; the equivalent in yeast would be assaying whether a gene is required for fitness not in one individual growth condition but in any one of a large number of different growth conditions (Figure 5). We believe that our finding that, in any single environmental condition, the proportion of genes that affect fitness is substantially higher in animals than in yeast has implications for the source of selective pressure that acts to maintain gene function.

Purifying selection can only act if mutations that reduce the function of that gene reduce the fitness of the organism. In yeast, in any single environmental condition, only a minority of genes appear to affect fitness when mutated, and thus in any one environment, most yeast genes are not under purifying selection. Direct evidence for this comes from experiments in which yeast are grown through many generations in highly nutrient controlled environments: genes that are essential for growth in one nutrient source are often lost through genomic deletion when yeast are maintained in a different nutrient source (Dunham et al., 2002; Gresham et al., 2008). Changes in the external environment or fluctuations in gene expression result in changes to the sets of genes that affect fitness, and thus, though purifying selection does not act on all yeast genes in any given environment, over long periods of time in varying environments, all yeast genes are under purifying selection. In an animal, on the other hand, as a result of there being multiple different cell-specific genetic networks in the same organism, the great majority of genes have loss-of-function phenotypes in a single environmental condition, and thus most animal genes are under continual purifying selection. We suggest that this implies that, whereas in yeasts, a significant source of selective pressure comes from a requirement to

cope with fluctuations in the external environment or in gene expression, in animals, selective pressure on gene function partly derives from the requirements for developing and maintaining the multiple different cell types that are required for the complex structure of an animal.

EXPERIMENTAL PROCEDURES

Strain Maintenance and Worm Handling

C. elegans strains were maintained on NGM agar plates seeded with OP50 *Escherichia coli* according to standard protocols (Stiernagle, 2006). All strains were supplied by the *Caenorhabditis* Genetics Centre (CGC), University of Minnesota, USA. L1 larval worms were purified from OP50 agar plates by washing them off using M9 and filtering them through an 11 μm filter. Filtered worms were then diluted in M9 media to reach a final concentration of one worm/ μl . See also Extended Experimental Procedures, Figure S5, and Table S6.

RNAi Methods and Calculation of Fitness

For RNAi fitness assays (both direct and indirect methods), dsRNA-expressing bacterial strains from the Ahringer library (Kamath et al., 2003) were inoculated for overnight growth in 2 ml of Luria-Bertani (LB) media with Carbinicillin (100 $\mu\text{g}/\text{ml}$ final concentration) in 24-well deep-well plates (Axygen); for each gene to be targeted, independent triplicate cultures were set up. After overnight growth, dsRNA expression was induced with IPTG (final concentration of 4 μM) for 1.5 hr before the cultures were spun down at 4,000 RPM for 5 min. Bacteria were resuspended in 2 ml Nematode growth medium supplemented with IPTG (4 μM final) and Carbinicillin (100 $\mu\text{g}/\text{ml}$ final). 100 μl of each fully resuspended culture was used to check the optical density of the starting culture for the indirect method. For both direct and indirect methods, ~ 50 L1 worms were added to each well and cultures were grown at 20°C for 8 days. For the direct method, the worm cultures were sampled every 24 hr from day 5 to day 8; 50 μl of a well-shaken sample was taken from each well, and worm number was counted using the Reflux module of the worm sorter. Raw data from the sorter were processed using Perl scripts and for each targeted gene, a fitness value was calculated as the ratio between the average worm number in the targeted cultures and the average worm number in cultures grown with nontargeting bacterial controls. For the indirect method, the OD595 of 100 μl of worm-free bacterial culture (obtained after allowing the worms to settle for 5 min) was measured using a spectrophotometer at 595nm; samples were taken on days 0, 5, and 8. The effect of targeting any gene on fitness was calculated as the ratio between the average change in OD595 from day 5 to day 8 in the targeted cultures and the average change in OD595 in cultures grown with nontargeting bacterial controls. For both direct and indirect methods, the controls were the same: they were bacterial strains expressing dsRNAs that did not target any endogenous *C. elegans* genes. We used bacterial strains expressing dsRNAs targeting either GFP or a *C. briggsae* gene with no ortholog in *C. elegans*. Four independent triplicates were set up for each of the negative controls and a mean value calculated. Fitness values for 30 independent repeats of these negative controls are distributed around a mean of 1.01 ± 0.11 (Figure S6), and thus fitness defects of > 20% are statistically significant ($p < 0.05$).

Construction of dsRNA-Expressing Bacterial Strains

We used essentially identical methods to those described in Fraser et al. (2000), Kamath et al. (2003), and Kamath et al. (2001). Two different primer pairs were designed for each gene using the software Primer3 (<http://primer3.sourceforge.net/>). One of the primer pairs was designed to amplify a region greater than 800 bp in the 5' end of the gene, and the other amplifies a region of similar length in the 3' end. We then checked with E-PCR (<http://www.wormbase.org/db/searches/epcr>) to ensure that they only amplify 1 product and these products do not overlap. Amplified products were further assessed using BLAST to make sure that there is no significant crossreactivity to other genes. Crossreactivity was defined as finding a region longer than 200 bp of the amplified product with an identity of 80% or higher to a secondary target. PCR was performed with 10 ng of *C. elegans*, genomic DNA PCR prod-

ucts were cloned into the EcoRV site of L4440, and the ligated products were transformed into HT115 bacteria.

Conservation Analysis

Orthologous gene pairs (*C. elegans*: *C. briggsae* and *C. elegans*: *C. remanei*) were obtained from InParanoid database, and their cDNA and protein sequences were retrieved from Wormbase. Ka/Ks ratios were calculated by using the codeml program in PAML package (version 4.3). Genes were first classified into bins based on fitness ratios (increments of 0.1), and the mean Ka/Ks ratio for genes in each bin was calculated.

Predictability of Relative Fitness from Ka/Ks

The procedure is similar to that outlined by Wang and Zhang (Wang and Zhang, 2009) and estimates how useful Ka/Ks is in predicting functional importance. We first ranked genes based on Ka/Ks and picked gene pairs at random, and for each pair, we asked whether the gene with the lower Ka/Ks also has a greater fitness defect, as measured by our indirect method. We repeated this process 1 million times and bin the difference in rank of the genes to fitness outcome (whether or not we correctly estimate the fitness defect to be greater for the lower ranked gene, as would be predicted).

Ortholog Distribution

Genes were classified into ten fitness bins (increments of 0.1), and in each bin, the genes were divided into two mutually exclusive groups: (1) those with a yeast ortholog (if they had a 1-1 yeast ortholog based on InParanoid [Berglund et al., 2008]) and (2) those with an animal ortholog (if there was no 1-1 yeast ortholog but there was a fly, mouse, or human or an ortholog in other worm species).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, six figures, and six tables and can be found with this article online at doi:10.1016/j.cell.2012.01.019.

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