A Mechanistic Basis for the Coordinated Regulation of Pharyngeal Morphogenesis in Caenorhabditis elegans by LIN-35/Rb and UBC-18–ARI-1

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Abstract

Genetic redundancy, whereby two genes carry out seemingly overlapping functions, may in large part be attributable to the intricacy and robustness of genetic networks that control many developmental processes. We have previously described a complex set of genetic interactions underlying foregut development in the nematode Caenorhabditis elegans. Specifically, LIN-35/Rb, a tumor suppressor ortholog, in conjunction with UBC-18–ARI-1, a conserved EZ/E3 complex, and PHA-1, a novel protein, coordinately regulates an early step of pharyngeal morphogenesis involving cellular re-orientation. Functional redundancy is indicated by the observation that lin-35; ubc-18 double mutants, as well as certain allelic combinations of pha-1 with either lin-35 or ubc-18, display defects in pharyngeal development, whereas single mutants do not. Using a combination of genetic and molecular analyses, we show that sup-35, a strong recessive suppressor of pha-1–associated lethality, also reverts the synthetic lethality of lin-35; ubc-18, lin-35; pha-1, and ubc-18 pha-1 double mutants. SUP-35, which contains C2H2-type Zn-finger domains as well as a conserved RMD-like motif, showed a dynamic pattern of subcellular localization during embryogenesis. We find that mutations in sup-35 specifically suppress hypomorphic alleles of pha-1 and that SUP-35, acting genetically upstream of SUP-36 and SUP-37, negatively regulates pha-1 transcription. We further demonstrate that LIN-35, a transcriptional repressor, and UBC-18–ARI-1, a complex involved in ubiquitin-mediated proteolysis, negatively regulate SUP-35 abundance through distinct mechanisms. We also show that HCF-1, a C. elegans homolog of host cell factor 1, functionally antagonizes LIN-35 in the regulation of sup-35. Our cumulative findings piece together the components of a novel regulatory network that includes LIN-35/Rb, which functions to control organ morphogenesis. Our results also shed light on general mechanisms that may underlie developmental genetic redundancies as well as principles that may govern complex disease traits.

Introduction

Genetic redundancy describes the phenomenon in which the combined inactivation of two distinct genes produces a phenotype that is not observed in either single mutant. One of the current challenges facing geneticists and developmental biologists alike is to understand the underlying bases of genetic redundancy at the molecular level. This may in many cases prove to be a difficult undertaking given the complexity of regulatory networks and the many difficulties associated with establishing clear connections between seemingly disparate genes. Nonetheless, redundancy is an issue of great biological importance, as evidenced in C. elegans, where most genes fail to show obvious or highly penetrant phenotypes following inhibition or inactivation [1–3].

To date, the most intensively studied case of genetic redundancy in C. elegans involves the Synthetic Multivulval (SynMuv) genes (for a review, see [4]). The SynMuv genes can in most cases be divided into two principal non-overlapping groups, termed class A and class B [5]. Inhibition of individual class A or class B genes does not typically alter normal patterns of vulval cell induction in hermaphrodites. In contrast, the combined loss in activity of any class A–class B gene pair leads to the ectopic induction of vulval tissue (the Muv phenotype). In addition, a class C group of SynMuv genes has recently been identified; mutations in class C genes are synthetic with mutations in both class A and class B SynMuv genes [6].

Extensive work has shed considerable light on the role of SynMuv genes in vulval development. Namely, most class A and B genes act within the hypodermis, a multi-nucleate epidermal tissue that lies adjacent to the developing vulval precursor cells (VPCs), where they redundantly inhibit the expression of the EGF-like ligand LIN-3 [7]. Secreted LIN-3 induces vulval cell development through activation of a conserved EGFR–Ras–Map kinase pathway in the VPCs [8]. Thus, in the absence of both class A and class B SynMuv activity, abnormally high levels of LIN-3, secreted by the hypodermis, leads to the hyperinduction of vulval cell fates.

Based on studies in C. elegans, Drosophila, and mammals, the large majority of proteins encoded by the class B SynMuv gene family function within a conserved set of structurally related transcriptional repressor complexes that include DRM (Dp, Rb and MuvB) and NuRD (nucleosome remodeling and histone deacetylase;
Mechanism of Coordinated Pharyngeal Development

Author Summary

One of the more puzzling aspects of genetics is that the inactivation of many genes fails to produce strong deleterious effects on the organisms that carry those genes. In some cases, however, the combined inactivation of two or more such genes can lead to the expression of robust abnormal phenotypes. These types of synthetic genetic interactions are thought to reflect the presence of functional overlap or redundancy between the involved genes. The root mechanisms that underlie synthetic interactions are thought to be complex and are in most cases poorly understood. Our work here focuses on one case study where we have uncovered the molecular basis underlying a complex set of genetic redundancies in C. elegans. More specifically, we have discovered a novel regulatory network that connects eight genes controlling embryonic for gut development in the nematode C. elegans. By solving mechanisms of this nature, our analysis provides a means for understanding more generally the principles that govern genetic redundancies. Our work also provides insight into the bases of complex disease traits, where the combined interactions of multiple genetic factors leads to outcomes that determine health or disease.

Through an analysis of the suppressor mutation sup-35, we demonstrate that SUP-35 acts as an inhibitor of pha-1 transcription. Furthermore, we show that LIN-35 and UBC-18 act through distinct mechanisms to negatively regulate SUP-35 expression. Thus, the simultaneous loss of lin-35 and ubc-18 leads to increased levels of SUP-35, which in turn trigger a reduction in the levels of PHA-1. These findings provide a straightforward explanation for the observed genetic interactions between these genes and more generally provide further insight into the nature of mechanisms that can underlie genetic redundancies.

Results

sup-35 encodes a Zn-finger protein with homology to RMD family members

As described in the Introduction, lin-35 mutations are strongly synthetic with hypomorphic mutations that affect the pha-1 locus, leading to strong pharyngeal morphogenesis defects [29]. In addition, recessive mutations in three genetic loci (sup-35, sup-36, and sup-37) were demonstrated to strongly suppress the embryonic- and larval-lethal phenotype of strong loss-of-function pha-1 mutants [31]. We have previously shown that mutations in sup-36 and sup-37 efficiently suppress the synthetic lethality of lin-35; pha-1 and lin-35; ubc-18 double mutants [29]. As described below, these and other related synthetic genotypes were also suppressed by mutations in sup-35. Thus, to learn more about the interplay between these various factors and their roles in pharyngeal development, we sought to identify the sup-35 locus.

Previous mapping data had placed sup-35 on LGIII, ~0.1 CM to the left of the pha-1 locus [31]. To identify the gene encoding sup-35, we carried out RNAi feeding of 384 clones corresponding to genes in the region proximal to pha-1. Two clones, which target the highly related genes Y48A6C.1 and Y48A6C.3, were identified that strongly suppress the embryonic lethality of pha-1(e2123ts) mutants (referred to hereafter as pha-1(ts)) at the non-permissive temperature of 25°C (Table 1). These RNAi clones also suppress the less severe L1 larval-arrest phenotype of pha-1(ts) mutants at intermediate temperature of 20°C (data not shown). Because Y48A6C.1 and Y48A6C.3 share extensive sequence homology (an 878-bp segment present in both genes is 99% identical), each RNAi construct is expected to inhibit both gene products through off-target effects; no additional off targets for these RNAi constructs are predicted. These results suggest that sup-35 may be encoded by either Y48A6C.1 or Y48A6C.3. However, an additional RNAi construct that is expected to target Y48A6C.1, but not Y48A6C.3, failed to suppress pha-1(ts) mutants at 25°C, suggesting that Y48A6C.3 is the relevant locus (data not shown).

Additional support for Y48A6C.3 as the affected locus was provided by sequencing both Y48A6C.1 and Y48A6C.3 in sup-35(e2223) pha-1(ts) double mutants. We detected a T-to-A transversion at nucleotide position 19 of the Y48A6C.3 open reading frame, resulting in the conversion of a cysteine to a serine at amino acid position seven. In contrast, we failed to identify any differences in the Y48A6C.1 locus between the published wild-type (N2) and sup-35(e2223) mutant sequences. Furthermore, we identified sequence alterations in Y48A6C.3 in five previously isolated alleles of sup-33 [31] as well as in 14 additional alleles identified by our laboratory. A summary of our sequence analysis is shown in Figure 1A. Two alleles, fd35 and fd42, contained a single nucleotide insertion and deletion, respectively, leading to frameshifts within exon 5 of Y48A6C.3. All other sup-35 alleles contained either large deletions or insertions within Y48A6C.3, and are presumed to be null alleles (Figure 1A and data not shown). Taken together, our findings strongly indicate that sup-35...
is encoded by Y48A6C.3. Furthermore, given that the majority of these alleles were identified as spontaneous revertants (31) and this work), the sup-35 genomic region would appear to be unusually unstable and subject to recombination events that lead to gross alterations of the locus.

Based on the WormBase predicted gene model, as well as an ORFeome-generated full-length cDNA, sup-35 encodes a 332-amino-acid protein containing two N-terminal C2H2-type Zn-finger domains along with two tetratrico peptide repeats (TPR) at its C terminus. The molecular lesion identified in sup-35(e2223) is predicted to disrupt the first Zn finger, indicating that this domain is likely to be essential for SUP-35 function. The presence of the Zn-finger motif suggests a potential role for SUP-35 in transcriptional regulation. Alternatively, the Zn-fingers may be involved in protein-RNA, protein-protein, or protein-lipid interactions.

Interestingly, other than its close paralog Y48A6C.1, SUP-35 is most similar to an evolutionarily conserved family of RMD (regulators of microtubule dynamics) proteins (Figure 1B; 32). Of the six RMD family members in C. elegans, SUP-35 is most similar to RMD-2; the C-terminal 215 amino acids of SUP-35 are 52% identical to a corresponding region in RMD-2, which in turn shares greater homology with SUP-35 than with other C. elegans RMD proteins (Figure 1B and data not shown). Interestingly, RMD-2, along with RMD-1 and RMD-3, can physically associate with microtubules in vitro 32). Consistent with the RMD-like domain of SUP-35 having an important functional role is the observation that two alleles of sup-33, fd35 and fd42, may specifically affect this region of the protein. Nevertheless, SUP-35 differs from other C. elegans RMD family members, as well as RMD proteins in other organisms, by the presence of its unique N-terminal Zn-finger domains. The TPR domains in SUP-35 suggest a possible role in protein-protein interactions [33].

SUP-35 shows a dynamic pattern of expression during embryogenesis

To assess the pattern of SUP-35 expression during development, multiple independent transgenic strains were generated expressing full-length SUP-35 fused to GFP under the control of the native sup-35 promoter/enhancer region (also see Materials and Methods). For reasons described below, the SUP-35::GFP expression analysis was performed in sup-36 and sup-37 mutant backgrounds, both of which gave identical results.

SUP-35::GFP expression was first observed in embryos at around the 50- to 100-cell stage. Expression of SUP-35::GFP was ubiquitous throughout the proliferative phase of embryogenesis and was strongly enriched in the cytoplasm (Figure 2A and 2B). Commensurate with the onset of visible morphogenesis (~400 minutes), SUP-35::GFP localization became pronounced in nuclei, most notably in cells comprising the pharyngeal primordium (Figure 2C and 2D). Pharyngeal cells also maintained nuclear SUP-35::GFP expression throughout larval stages and into adulthood (data not shown). In addition, weaker SUP-35::GFP could be detected in the nuclei of several non-pharyngeal cells in the posterior.

Mutations in sup-35 suppress synthetic pharyngeal defects

Mutations in either sup-36 or sup-37 are capable of suppressing all pair-wise combinations of mutations in lin-35, ubc-18, and pha-1 [29]. Consistent with this, the same constellation of synthetic-lethal mutations was efficiently suppressed by loss of sup-35 (Table 1). This includes suppression by the canonical allele of sup-33, e2223; a consortium-generated deletion allele, tm1810; and by sup-35(RNAi). Suppression by sup-35(tm1810) also further confirms the molecular identity of this locus.

Previous studies from our laboratory have implicated the RING finger-domain protein, ARH-1, as the primary co-partner of UBC-18 in the regulation of pharyngeal development [34]. Consistent with this, a consortium-generated deletion allele of arh-1, tm2549, showed strong synthetic interactions with pha-1(ts), and this lethality was suppressed by sup-35(RNAi) (Table 1). Taken together, these findings suggest that sup-35 functions within a regulatory network that includes pha-1, lin-35, ubc-18, and arh-1 to control pharyngeal development.

sup-35 suppression of pha-1 mutations requires residual PHA-1 activity

Extragenic suppression in C. elegans arises through a number of distinct mechanisms [35]. Such mechanisms can, in some cases, be distinguished based on whether or not suppression occurs in the presence of a null allele. For this reason, we first sought to determine whether the strongest characterized allele of pha-1, e2123ts, retains activity at the non-permissive temperature of 25°C; e2123ts is a missense mutation that leads to a conversion of cysteine to tyrosine at amino acid position 169 of PHA-1 [36]. We thus generated high-copy extrachromosomal arrays carrying the pha-1(ts) variant in mutant animals that were already chromosomally homozygous for the pha-1(ts) mutation. We then assayed for the ability of pha-1(ts) high-copy overexpression to rescue the lethal phenotype of pha-1(ts) mutants at 25°C. If the protein product of pha-1(ts) were to retain residual activity at 25°C, we...
would expect to see some suppression of $pha-1(ts)$ temperature sensitivity. As shown in Table 2, overexpression of $pha-1(ts)$ efficiently rescued defects associated with genomic $pha-1(ts)$ loss of function, indicating that, at 25°C, $pha-1(e2123ts)$ does not behave as a null allele.

Given the absence of a well-characterized null allele of $pha-1$, we decided to make use of a regional deficiency on chromosome III, tDf2, which removes both the $pha-1$ and $sup-35$ loci, as well as 46–72 additional genes (Figure 3A). Previous analysis, along with our current work, indicates that homozygous tDf2/tDf2 mutants arrest as embryos that display a phenotype closely resembling $pha-1$ strong loss-of-function mutations, suggesting that $pha-1$ may be the earliest-acting zygotic gene within the region deleted by the deficiency [31]. If so, then the apparent lack of suppression observed in tDf2/tDf2 embryos, where both $sup-35$ and $pha-1$ are deleted, would suggest that loss of $sup-35$ cannot suppress the $pha-1$ null phenotype. Alternatively, another early-acting gene within the deficiency, one that is not suppressed by loss of $sup-35$, could be responsible for the $pha-1$-like phenotype observed in tDf2/tDf2 homozygotes.

To distinguish between these two possibilities, we introduced an extrachromosomal array containing wild-type copies of $pha-1$ into a balanced strain that carries the tDf2 deficiency (tDf2/qC1 dpy-19 glp-1). In the absence of any array, this strain segregates 25%...
tDf2 progeny that arrest as dead embryos with morphological defects similar to those observed for pha-1(ts) mutants at 25°C (Figure 3B). Strikingly, in the presence of pha-1 rescuing arrays, we observed a substantial decrease in the frequency of embryonic lethality (Figure 3B). This effect was observed using multiple independently generated arrays, with the extent of embryonic rescue corresponding closely to the transmission frequencies of the individual arrays (Figure 3B and data not shown). Furthermore, we observed a proportional increase in the percentage of array-positive larval-lethal animals (Figure 3B), indicating that some other gene within the deficiency is required for progression through larval development. Taken together, these results demonstrate that pha-1 is the earliest-acting zygotic gene within tDf2 and, most importantly, that loss of sup-35 cannot suppress the pha-1 null genotype. These findings are also consistent with the observation that sup-35 pha-1(e2123)/tDf2 animals, which carry only a single copy of the pha-1 hypomorphic allele, display much weaker suppression than that of sup-35 pha-1(e2123) animals, which retain two copies of this allele (data not shown; [31]).

As an additional test, we made use of two recently generated deletion alleles of pha-1 (tm3671 and tm3569; gift of National Bioresource Project). tm3671 is a 203-bp deletion that removes part of the second exon of pha-1, creating a premature stop codon after 30 amino acids and is a presumed null allele. tm3569 contains an in-frame 568-bp deletion extending from exon 2 through exon 4, which removes 149 amino acids of PHA-1 (isoform Y48A6C.5a). Both pha-1(tm3671)/+ and pha-1(tm3569)/+ heterozygous hermaphrodites produce ~25% embryonic-lethal F1

Table 2. pha-1(e2123ts) retains partial activity at non-permissive temperatures.

| Genotype              | Total Embryos | % Embryonic lethality at 25°C | % Larval lethality at 25°C |
|-----------------------|---------------|------------------------------|----------------------------|
| pha-1(e2123ts)        | 384           | 94.2                         | 5.7                        |
| pha-1(e2123ts); fdEx51| 128 (GFP+)    | 0                            | 0                          |
|                       | 199 (GFP−)    | 94.9                         | 5.0                        |
| pha-1(e2123ts); fdEx53| 141 (GFP+)    | 0                            | 0                          |
|                       | 255 (GFP−)    | 94.5                         | 5.4                        |

The independently derived extrachromosomal arrays fdEx51 and fdEx53 carry multiple copies of the pha-1(e2123ts) allele in addition to the sur-5::GFP marker.

*Both embryonic- and larval-lethal animals exhibited the Pun phenotype.

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progeny that phenocopy pha-1(ts) embryos (at 25°C). Consistent with our deficiency analysis, growth of pha-1(tm3671)/+ and pha-1(tm3569)/+ heterozygotes on sup-35(RNAi) failed to decrease the percentage of embryonic-arrested progeny, further indicating that reduction of sup-35 activity cannot suppress complete loss of function of pha-1 (data not shown). In contrast, sup-35(RNAi) efficiently suppressed the lethality of pha-1(ts) mutants (at 25°C), as well as all tested synthetic phenotypes (Table 1).

SUP-35 is a transcriptional repressor of pha-1

Given that loss of sup-35 cannot suppress the pha-1 null genotype, we hypothesized that SUP-35 may function as a negative upstream regulator of pha-1. Furthermore, because SUP-35 contains C2H2-type Zn fingers that are critical for its activity (Figure 1A), we reasoned that SUP-35 may mediate repression of pha-1 at the level of transcription (Figure 4A). Consistent with this, qRT-PCR experiments revealed embryonic pha-1 mRNA levels to be 2- to 4-fold more abundant in sup-35(tm1810) mutants as compared with wild type using two independent internal normalization controls (Figure 4B). An even greater increase in pha-1 mRNA levels was observed in embryos from sup-35(tm2223) pha-1(ts) double mutants relative to pha-1(ts) single mutants (Figure 4C). This latter result is significant in that pha-1 mRNA levels were assessed in a genetic background in which PHA-1 activity is compromised. The observed difference in the degree to which pha-1 is upregulated in these strains could reflect a heightened sensitivity to SUP-35 levels in the pha-1 mutant background or could be due to differences between the two sup-35 alleles used in these studies.

As a second test, we made use of a previously described strain that expresses a functional full-length PHA-1::GFP fusion protein [29]. Because this fusion protein is regulated by sequences derived from the native pha-1 promoter, its expression should be sensitive to alterations in the activities of endogenous transcriptional regulators. Consistent with data obtained from qRT-PCR, PHA-1::GFP was upregulated at least 2-fold in sup-35(tm1810) mutants relative to wild-type embryos (Figure 4D and 4F–4I; Figure S1). These findings also indicate that changes in pha-1 mRNA levels lead to corresponding changes in the abundance of PHA-1 protein.

The above results indicate that SUP-35 may negatively regulate pha-1 at the level of transcription or mRNA stability. To distinguish between these possibilities, we assayed expression levels of a Ppha-1::GFP reporter [29] in wild-type and sup-35 mutants. Because this construct contains only the 5' upstream regulatory region of pha-1, effects on mRNA stability through the pha-1 3'UTR should not be observed. Using this reporter, we observed that Ppha-1::GFP is upregulated ~3-fold in sup-35 mutants versus wild-type embryos (Figure 4E; Figure S1). Taken together, these
SUP-35 negatively regulates *pha-1*. (A) Testable model for the regulation of PHA-1 by SUP-35. (B,C) Quantification of endogenous *pha-1* mRNA levels in embryos by qRT-PCR in *sup-35(tm1810)* single mutants (B) or *sup-35(e2223) pha-1(e2123)* double mutants (C) using *act-1* (black bar) and *ama-1* (gray bar) as loading controls. Fold changes were obtained after normalizing to wild type (B) or *pha-1(e2123)* single mutants (C). Error bars represent s.e.m. Means of the indicated groups were analyzed for significance using a two-tailed Student’s t-test (*p<0.0). Quantification of PHA-1::GFP (D) and P*pha-1::GFP* (E) fluorescence in wild-type and *sup-35(tm1810)* mutants. The average mean GFP intensity for each genotype was analyzed for significance using a two-tailed Student’s t-test (*p<0.0001). (F–I) Representative GFP (F,H) and corresponding DIC (G,I) images of PHA-1::GFP expression in wild type (F,G) and *sup-35(tm1810)* mutants (H,I). Digital camera exposure times were identical for all embryos assayed. Mean GFP intensities were determined as described in Materials and Methods. Scale bar in F, 10 μm for F–I.

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data provide strong evidence that SUP-35 normally functions to inhibit pha-1 at the level of transcription.

SUP-35 acts genetically upstream of sup-36 and sup-37 to inhibit pha-1

If SUP-35 negatively regulates pha-1, then sup-35 overexpression should cause a reduction in PHA-1 levels and therefore would be expected to phenocopy pha-1 loss-of-function mutations. Consistent with this, extensive attempts to revert the suppression of sup-35; pha-1 mutants through the expression of wild-type sup-35 via an extrachromosomal array failed to generate stable transgenic lines. This includes experiments in which sup-35 was engineered to be present at low copy numbers. In addition, sup-35 transgenic expression was also highly toxic to wild-type animals, as was expression of the SUP-35::GFP fusion protein. Given that SUP-35 may require the pha-1 suppressors SUP-36 and SUP-37 to mediate its activities, we hypothesized that SUP-35 overexpression may not be toxic in genetic backgrounds that remove either sup-36 or sup-37 activities. Consistent with this prediction, we encountered no difficulties in obtaining stable transgenic lines carrying wild-type sup-35 (or SUP-35::GFP) at high copy number in either the sup-36 or sup-37 mutant background (also see Materials and Methods). This finding indicates that SUP-36 and SUP-37 function genetically downstream of SUP-35. However, SUP-36 and SUP-37 could conceivably function upstream of SUP-35 if they are required for SUP-35 activation.

To determine directly the phenotypic effects of SUP-35 overexpression in a wild-type background, we performed a series of genetic crosses, an example of which is shown in Figure 5A. sup-35 overexpression was toxic to hermaphrodites that expressed both sup-36 and sup-37 zygotically, even if sup-36 or sup-37 were absent maternally. Surprisingly, sup-35 overexpression was not toxic in males that expressed both sup-36 and sup-37 zygotically, provided that either sup-36 or sup-37 maternal contributions were absent. In contrast, sup-35 overexpression was toxic to both males and hermaphrodites when both sup-36 and sup-37 were present maternally and zygotically. Most strikingly, non-viable sup-35-overexpressing embryos and larvae obtained through these crosses had a phenotype that was identical to strong pha-1 loss-of-function mutations (Figure 5B and 5C). Taken together, these results are consistent with our finding that SUP-35 functions as a negative regulator of pha-1 and further indicate that SUP-35 acts together with SUP-36 and SUP-37 to control pha-1 expression levels.

SUP-35 acts through pha-1 to suppress synthetic pharyngeal defects

Our above analyses strongly indicate that sup-35 suppression of partial loss-of-function mutations in pha-1 occurs through the upregulation of pha-1 mRNA, which in turn leads to increased PHA-1 protein levels (Figure 4). An extension of this model is that suppression of the synthetic pharyngeal phenotypes by sup-35 (Table 1) may occur through an identical mechanism. If that is the case, then an increase in PHA-1 levels, even in the presence of wild-type sup-35, should be sufficient to suppress the synthetic phenotype of lin-35; ucb-18 double mutants. To address this, we overexpressed wild-type PHA-1 from high-copy extrachromosomal arrays in lin-35; ucb-18 double mutants and assayed for rescue. Strong suppression of synthetic lethality was observed in three out of three independent transgenic lines, leading to the generation of viable double mutant strains that carried only the PHA-1-overexpression transgenic array. This finding is consistent with the hypothesis that sup-35-mediated suppression of pha-1(t) and the synthetic phenotypes occurs through the same mechanism.

A second prediction of the above model is that inhibition of pha-1 activity should revert the suppression observed in lin-35; sup-35(tm1810) ucb-18 triple mutants (Table 1). We therefore subjected triple mutants to pha-1(RNAi) feeding and assayed for loss of suppression. Whereas 100% (n = 255) of lin-35; sup-35 ucb-18 animals reached adulthood when grown on vector-RNAi control plates, only 12.9% (n = 200) of triple mutants grown on pha-1(RNAi) escaped embryonic or early-larval arrest. This finding further supports the model that sup-35-mediated suppression of both strong loss-of-function pha-1 mutants and the synthetic genotypes occurs through the common mechanism of increasing PHA-1 levels.

LIN-35, UBC-18–ARI-1, and HCF-1 function upstream of SUP-35 to regulate PHA-1 expression

In considering potential regulatory networks that could account for both the molecular and genetic data described above, we were able to construct a relatively straightforward model. In this scenario, LIN-35, functioning as a transcriptional repressor (Figure 6A), and UBC-18–ARI-1, acting as a complex to promote target protein degradation (Figure 7A), are negative regulators of SUP-35. Thus in lin-35; ucb-18 double mutants, increased levels of SUP-35 would lead to the inhibition of PHA-1 and associated defects in pharyngeal development.

We first tested this model by examining the role of LIN-35 in the expression of endogenous sup-35. Consistent with the model, embryonic levels of sup-35 mRNA are increased ~4-fold in lin-35 mutants as compared with wild type (Figure 6B). Correspondingly, SUP-35::GFP was upregulated 2- to 3-fold in embryos following lin-35(RNAi) treatment (Figure 6C; Figure S2), indicating that changes in sup-35 mRNA levels are further reflected by changes in the abundance of SUP-35 protein. Most importantly, we observed an ~5- to 10-fold reduction in the levels of endogenous pha-1 mRNA in embryos derived from lin-35 mutants versus those from wild type (Figure 6B). This latter result also provides an explanation for why mutations in lin-35 are strongly synthetic with hypomorphic mutations in pha-1 (also see Discussion).

We next examined the roles of UBC-18 and ARI-1 in the regulation of SUP-35 and PHA-1. In contrast to findings from lin-35 mutants, embryonic sup-35 mRNA levels in ucb-18 mutants were identical to those observed in wild type (Figure 7B). Nonetheless, embryonic SUP-35::GFP protein levels were substantially increased following RNAi inhibition of ubc-18 or ari-1 (Figure 7C; Figure S2). These results indicate that UBC-18–ARI-1 negatively regulates SUP-35 post-transcriptionally, possibly at the level of SUP-35 stability. Consistent with this, we find that SUP-35::GFP is a target for ubiquitination in cell extracts from whole worms (Figure 7D). Furthermore, we observed that the increase in SUP-35 levels in ucb-18 mutants correlates with a decrease in the expression levels of pha-1 mRNA (Figure 7B). These findings, in combination with other molecular and genetic data, strongly support the model that LIN-35 and UBC-18–ARI-1 promote pha-1 transcription by inhibiting SUP-35 expression and stability.

In previous studies, we have implicated the C. elegans E2F ortholog, EFL-1, as a regulatory partner of LIN-35 in the control of pharyngeal development [29], and have also defined the C. elegans E2F consensus binding motif [13]. Consistent with a role for E2F in the regulation of sup-35, we identified three candidate E2F binding sites within the first 700 bp of the sup-35 promoter region. One of these sites, located approximately 230 bp upstream of the predicted transcriptional start site (GATTGGCCGGCT), conforms to all published criteria, suggesting that E2F may potentially regulate sup-35 directly.
Studies in mammals have implicated HCF-1 (host cell factor 1), as an important physical and functional co-partner of E2F in the activation of E2F target genes [37,38]. For example, loss of HCF-1 activity in hamster cells leads to a reduction in the expression of E2F-regulated genes required for G1 entry resulting in arrest in G0 [39]. Interestingly, this G0 arrest can be bypassed through the inhibition of pRb family members, indicating that mammalian HCF-1 and pRb carry out opposing functions on E2F targets [40].

The presence of a structurally and functionally conserved ortholog of HCF-1 in *C. elegans* [41–43], led us to hypothesize that a similar regulatory relationship may exist in *C. elegans* (Figure 8A). To test this, we assayed levels of sup-35 mRNA in *lin-35* mutants subjected to hcf-1(RNAi) by qRT-PCR. Notably, we observed an ~2-fold reduction in the levels of sup-35 mRNA in *lin-35; hcf-1(RNAi)* embryos as compared with *lin-35* mutants treated with a control RNAi (Figure 8B).

To see if the observed reduction in sup-35 mRNA levels by hcf-1(RNAi) has a functional consequence in *lin-35; ubc-18* and *lin-35; pha-1* double mutants, we carried out hcf-1(RNAi) in these backgrounds and assayed for suppression of larval arrest, leading to the generation of fertile adults. Notably, reduction of hcf-1 activity led to pronounced suppression of arrest in both *lin-35; ubc-18* and *lin-35; pha-1* mutant backgrounds (Figure 8C). We note that the partial phenotypic suppression of the synthetic mutants by hcf-1(RNAi) is consistent with the incomplete correction of sup-35 overexpression in *lin-35; hcf-1(RNAi)* embryos (Figure 8B).

In addition, hcf-1(RNAi) resulted in suppression of *pha-1(ts)* mutants at the intermediate temperature of 20°C, leading to a marked

**Figure 5. SUP-35 overexpression phenocopies pha-1 loss of function.** (A) Representative genetic strategy applied to assay the effects of SUP-35 overexpression in *pha-1* and *sup-36* mutant backgrounds. Wild-type males carrying an integrated myo-2::GFP reporter (mIs11) were crossed into *pha-1*; sup-36 hermaphrodites carrying a sup-35-overexpressing extrachromosomal array (fdEx59). Although this mating failed to produce viable F1 cross-progeny hermaphrodites, fertile cross-progeny males were generated, which were identified by expression of the myo-2::GFP reporter. F1 cross-progeny males were then mated to wild-type or *sup-36* hermaphrodites, and cross-progeny were identified based on the myo-2::GFP reporter. Mating into the N2 strain failed to produce viable cross-progeny males or hermaphrodites, whereas mating to *sup-36* generated both viable and non-viable male and hermaphrodite F2 cross-progeny. Non-viable F2 cross-progeny from both matings displayed a Pun (Pharynx unattached) phenotype, and these animals uniformly carried the fdEx59 array. Identical results were also obtained for *sup-37* mutants using the above strategy, and similar findings were obtained for both *sup-36* and *sup-37* using additional genetic approaches (see Materials and Methods). (B,C) DIC images of a typical non-viable embryo (B) and larva (C) obtained through the above mating. Note the Pun phenotype. Black and white arrowheads indicate the anterior and posterior pharyngeal boundaries, respectively. Scale bar in C, 1 μm for B,C.

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A model for the redundant regulation of PHA-1

In previous work, we have shown that LIN-35, a transcriptional repressor, and UBC-18-ARI-1, an E2 ubiquitin ligase complex, redundantly regulate pharyngeal morphogenesis [21,34]. In addition, mutations in lin-35, ubc-18, and arl-1 strongly enhance the pharyngeal morphogenetic defects of partial loss-of-function mutations in pha-1 [29,34]. In our current study, we provide both molecular and genetic evidence that LIN-35 and UBC-18-ARI-1 function as negative regulators of SUP-35, which in turn functions as a transcriptional repressor of pha-1. Thus, in our model, both LIN-35 and UBC-18-ARI-1 are positive, albeit indirect, regulators of PHA-1 through the inhibition of SUP-35 (Figure 9).

Evidence to support this model includes the findings that pha-1 overexpression efficiently rescued the synthetic lethality of lin-35; ubc-18 double mutants and that the suppression observed in lin-35; ubc-18 sup-35 triple mutants was reversed by pha-1(RNAi). Furthermore, sup-35 overexpression in a wild-type background phenocopied pha-1 loss of function (Figure 5). Consistent with the genetic data, qRT-PCR and GFP reporters indicate that sup-35 mRNA and protein levels were upregulated in embryos where lin-35 activity had been compromised, whereas ubc-18 and arl-1 specifically affected SUP-35 protein levels (Figure 6). Additionally, endogenous pha-1 mRNA levels were decreased in lin-35 and ubc-18 mutants, whereas pha-1 mRNA and protein levels were increased in sup-35 mutants (Figure 6, Figure 7). This model accounts for both the synthetic lethality of lin-35; ubc-18 double mutants as well as the genetic interactions observed between pha-1 and lin-35, ubc-18, and arl-1, as pha-1 hypomorphic mutations would be expected to be hypersensitive to conditions that further reduce pha-1 mRNA levels.

An additional prediction of this model is that strong loss-of-function pha-1 mutants should minimally phenocopy the defects observed in lin-35; ubc-18 and lin-35; pha-1 mutants. Specifically, lin-35; ubc-18 and lin-35; pha-1 mutants show early-stage defects in the re-orientation of anterior epithelial cells within the pharyngeal primordium [21,29]. Surprisingly, however, we had previously failed to observe re-orientation defects in pha-1(ts) embryos grown at 25°C [29], even though these mutants show severe pharyngeal morphogenesis defects at later stages [29,30]. We have subsequently repeated these experiments and, consistent with our earlier study, find little or no evidence for early-stage morphogenesis defects in pha-1(ts) embryos grown at the non-permissive temperature on either NGM or vector-RNAi control plates (data...
In contrast, pha-1(ts) mutants grown at 16°C on pha-1(RNAi) plates did display early-stage pharyngeal morphogenesis defects, demonstrating that a specific reduction in pha-1 activity can phenocopy the early-stage defects observed in the synthetic mutants (data not shown). Moreover, the frequency and severity of pha-1(ts); pha-1(RNAi) morphogenesis defects were similar to those observed for pha-1(ts); lin-35(RNAi) and pha-1(ts); ubc-18(RNAi) embryos grown at 16°C (data not shown). These observations indicate that early-stage defects in pha-1(ts) mutants are suppressed by growth at 25°C, suggesting an effect of temperature on the underlying process of cell re-orientation. Most importantly, these findings are internally consistent with our model, in which PHA-1 levels are positively regulated by LIN-35 and UBC-18 through the inhibition of SUP-35 (Figure 9).

Our observation that mutations in sup-36 and sup-37 abolish SUP-35-mediated toxicity indicate that sup-36 and sup-37 act genetically downstream of SUP-35. Thus, SUP-35 and SUP-37 may potentially function downstream of SUP-35 in a linear pathway to control pha-1 expression. Alternatively, SUP-36 and SUP-37 may act in a complex with SUP-35, or in a parallel pathway that is required for SUP-35 activation (Figure 9).

We also find that inhibition of hcf-1 by RNAi leads to a partial, though significant, suppression of larval arrest in lin-35; ubc-18 and lin-35; pha-1(ts) mutants as well as the substantive suppression of both the L1 arrest and Pun (Pharynx unattached) phenotypes of pha-1(ts) mutants at 20°C. This genetic suppression correlates well with the observed decrease in sup-33 mRNA levels in lin-35; hcf-1(RNAi) embryos. These results are consistent with our current model as
well as previously published findings on mammalian HCF-1 [29,44], and append our model with the addition of a phylogenetically-conserved component of the E2F network (Figure 9). Our finding also indicates that additional novel suppressors may be identified through the use of sensitized strains.

Elucidating the mechanistic bases of synthetic genetic interactions will continue to be a major challenge for the field of developmental genetics. These types of interactions will also likely be critical to our understanding of complex disease traits in humans. For example, a recent commentary in the New England Journal of Medicine states that “many, rather than few, variant risk alleles are responsible for the majority of the inherited risk of each common disease” [45].

Our current analysis provides a straightforward model to account for the genetic redundancies observed in an additional case study. Although understanding different sets of genetic
interactions will undoubtedly require unique solutions, we contend that certain patterns of redundancy are likely to emerge. In this case, we have shown that a redundancy between a transcriptional regulator, LIN-35, and a mediator of protein stability, UBC-18–ARI-1 can be explained through the negative regulation of a common target, SUP-35. Similarly, we have previously shown that LIN-35 and FZR-1, a substrate-specificity component of the APC (anaphase-promoting complex) E3 ligase, mutually inhibit the expression levels of G1 cyclins [14]. Thus, a potential theme to emerge from our studies is the redundant control of common targets through distinct mechanisms of negative regulation. Additional studies into synthetic phenotypes in C. elegans and other systems should further elucidate general themes that may govern genetic redundancy.

Materials and Methods

Strains and maintenance

C. elegans were maintained using standard procedures [46]. Strains used in our analysis include GE24 [pha-1(e2123)], GE348 [dpy-18 sup-35(e2223) pha-1(e2123)], WY83 [lin-35; ubc-18; kuEx119 (lin-35+; sur-5::GFP), WY119 [lin-35;pha-1(fd1); kuEx119], sup-35(tm1810), WY477 [dpy-18 pha-1(e2123); ar-1(tm2349)], WY482 [sup-35(tm1810); SM469 (PHA-1::GFP; pRF4 rol-6)], WY527–528, [lin-35 ubc-18; kuEx119; fdEx72–73 (pBX rol-6(su1006)]), WY529–530 [lin-35; ubc-18; fdEx72–73], GE2158 [Dpy-19(e1259) glp-1(q339)], WY539–542 [unc-13 lin-35; dpy-17 ubc-18 sup-35(tm1810)], GE348 [dpy-18 sup-35(e2223) pha-1(e2123)], GE551 [vab-7(e1562) sup-35(t1013) pha-1(e2123)], GE552 [vab-7(e1562) sup-35(t1014) pha-1(e2123)], GE913 [vab-7(e1562) sup-35(t1016) pha-1(e2123)], GE557 [vab-7(e1562) sup-35(t1015) pha-1(e2123)], GE915 [vab-7(e1562) sup-35(t1017) pha-1(e2123)], and WY453–466 [sup-35 (fd33–46) pha-1(e2123)]. SM35 [PHA-1::GFP], SM36 [Ppha-1::GFP].

To analyze SUP-35 overexpression and toxicity, the following strains were generated using either a sup-35 genomic fragment or a cloned sup-35::GFP construct: WY512–513 [pha-1(e2123); sup-36(e2217); fdEx57–58 (sup-35::GFP; rol-6)], WY514–517 [pha-1(e2123); sup-36(e2217); fdEx59–62 (sup-35 genomic fragment; sur-5::GFP)], WY518 [pha-1(e2123); sup-37(e2215); fdEx63 (sup-35::GFP; rol-6)], WY519–520 [pha-1(e2123); sup-37(e2215); fdEx64–65 (sup-35 genomic fragment; sur-5::GFP)], WY523–524 [dpy-
11 sup-3; fdEx68–69 (sup-35 genomic fragment;sur-5::GFP)]; WY525–
526, [dyf-11 sup-3; fdEx70–71 (sup-35::GFP; rol-6)].

Strains used for rescue analysis of pha-1(e2123ts) and the chromosomal deficiency td12 included WY506–511 [pha-
1(e2123ts); fdEx51–56(pBX/e2123; sur-5::GFP)] and WY531–534
([df2/qc1 dpy-19(e2123) gfp-1(q339); fdEx74–77pBX; sur-5::GFP]).

Construction of plasmids

Fluorescence microscopy was performed using a Nikon Eclipse microscope. Quantification of the GFP fluorescence in embryos was carried out using Open Lab Software Version 5.0.2. All images were captured using identical exposure times, and all embryos used in our analysis were of similar developmental stages (~200–300 cells). An average of the mean fluorescence was calculated to compare GFP expression levels. P values were determined using a Student’s t-test.

SUP-35 overexpression and toxicity

Because multicopy transgene expression of SUP-35 and SUP-
35::GFP was toxic in wild-type backgrounds, arrays were initially generated in sup-36 and sup-37 mutants. To determine the effect of SUP-35 and SUP-35::GFP overexpression in wild-type animals, males of genotype +/++; me311 (myo-2::GFP) were crossed to pha-
1(e2123ts); sup-36; fdEx59 hermaphrodites. fdEx59 expresses wild-
type sup-35 and the co-injection marker sur-5::GFP. Such crosses resulted in the generation of fdEx59+ F1 males only, which were identified by the presence of both sup-35::GFP and myo-2::GFP. F1 males were then mated to either N2 hermaphrodites or homozygous sup-36 hermaphrodites. When the F1 males were crossed to sup-36 hermaphrodites, non-viable Pun and viable cross-progeny animals were obtained, whereas all the cross-
progeny from the N2 hermaphrodite matings were non-viable and exhibited the Pun phenotype. These results were reproduced using three independently generated extrachromosomal arrays in both sup-36 and sup-37 mutant backgrounds. Similar results were also obtained for the SUP-35::GFP construct co-injected with pRF4.

As an alternative approach, males of the genotype dpy-13 unc-24/+ were crossed to pha-1(e2123ts); sup-36; fdEx59 hermaph-
rodites. F1 hermaphrodites were placed on individual plates and allowed to self; cross-progeny were determined by the presence of viable Dpy Unc animals. In the event that SUP-35 overexpression was non-toxic, half of the cross-progeny F1 [pha-1(e2123ts)/+; sup-36/+
+; dpy-13 unc-24; fdEx59] should have segregated one-sixteenth of the F2 animals with a genotype of +/+; dpy-13 unc-24; fdEx59. Although our cross results in a high frequency of F1 cross-progeny males, they failed to produce F1 hermaphrodites that segregated Dpy Unc F2 animals. To extend these results, F1 cross-
progeny males were subsequently crossed to N2 hermaphrodites. This cross resulted in fdEx59+ animals that arrested uniformly as arrested embryos or larvae that exhibited the Pun phenotype. Again, these results were reproducible with other independently generated arrays and when analogous crosses were performed in the sup-37 mutant background.

RNAi

RNAi feeding was carried out using standard protocols, and
plates were cultured at 25°C to score for transgene [49]. The
RNAi constructs fjaV406A6C3, fjaV406A6C5, and fjaR010112 had been used to target sup-35, pha-1, and ubc-18 gene products, respectively. RNAi constructs used to target lin-33 and avi-1 were previously described [14,34]. hcf-1(RNAi) feeding was carried out using construct fjaC46A5.9, corresponding to exons 2–4. RNAi injection of hcf-1 was carried out by gonadal injection of dsRNA (~1.0 mg/ml) corresponding to exons 5 and 6.

Fluorescence microscopy and measurements

Mechanism of Coordinated Pharyngeal Development

Plasmodi pBX, which contains a rescuing segment
526, [dyf-11 sup-3; fdEx51–56(pBX/e2123; sur-5::GFP)] and WY531–534
([df2/qc1 dpy-19(e2123) gfp-1(q339); fdEx74–77pBX; sur-5::GFP]).
using the BioRad SYBR green supermix with the following reaction conditions: initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 30 seconds and a combined annealing and extension step at 60°C for 30 seconds. After the final amplification cycle, a melt curve analysis was performed to examine the specificity of the reaction. The fold-change of the mRNA levels was calculated by the delta-delta Ct method. For each qRT-PCR experiment, amplification was done in triplicate for both the test and the normalization genes, and the results were checked for reproducibility using at least one biological duplicate. In addition, all data were reproduced using at least two biological replicates. P values were determined using a Student’s t-test.

Immunoprecipitation and western blotting

Mixed-stage worms from 10 large NGM-OP50 plates were pooled and washed with M9 and distilled water and resuspended in 500 μl of homogenization buffer (20 mM Tris-HCl pH 7.5, 100 mM NaCl, 5 mM MgCl2, 1 mM EDTA, 1 mM DTT, 1% TritonX-100, protease inhibitors). Worms were then sonicated, incubated on ice, and lysates were cleared of large particles by centrifugation. To immunoprecipitate SUP-35::GFP, precleared worm lysate was incubated with 5 μg of polyclonal anti-GFP antibody (Santa Cruz) at 4°C overnight at 4°C. The resulting immune complex was pulled down using 30 μl of proteinA-sepharose beads (Invitrogen) by over-night incubation at 4°C. Beads were washed 3 x with cold homogenization buffer and subjected to SDS-PAGE and western blot analysis. Westerns to detect ubiquitinated products were carried out using either 2 μg of monoclonal anti-ubiquitin antibody (Santa Cruz) or 2 μg of monoclonal anti-GFP primary antibody (Invitrogen). Visualization was carried out using HRP-conjugated goat anti-mouse secondary antibodies (Santa Cruz) at 1:5000 and peroxidase activity was detected by the enhanced chemiluminescence assay (Pierce). LLnL-treated Jurkat cell lysate (Santa Cruz) was used as a positive control for ubiquitination.

Supporting Information

Figure S1 Quantification of PHA-1::GFP (A and B) and Pbga-7::GFP (C and D) fluorescence intensities in individual embryos in N2 (A and C) and sup-35(tm1810) mutant backgrounds (B and D). Found at: doi:10.1371/journal.pgen.1000510.s001 (0.20 MB TIF)

Figure S2 Quantification of SUP-35::GFP fluorescence intensities in individual embryos following treatment of strains with vector RNAi (A), lin-35(RNAi) (B), ubc-18(RNAi) (C), and arit-1(RNAi) (D). Found at: doi:10.1371/journal.pgen.1000510.s002 (0.23 MB TIF)

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Author Contributions

Conceived and designed the experiments: KM DSF. Performed the experiments: KM. Analyzed the data: KM DSF. Wrote the paper: KM DSF.

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