A Negative Regulatory Loop between MicroRNA and Hox Gene Controls Posterior Identities in Caenorhabditis elegans

Zhongying Zhao1*, Thomas J. Boyle1, Zongzhi Liu2*, John I. Murray1, William B. Wood2, Robert H. Waterston1

1 Department of Genome Sciences, University of Washington, Seattle, Washington, United States of America, 2 Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, Colorado, United States of America

Abstract

MicroRNAs (miRNAs) have been found to regulate gene expression across eukaryotic species, but the function of most miRNA genes remains unknown. Here we describe how the analysis of the expression patterns of a well-conserved miRNA gene, mir-57, at cellular resolution for every minute during early development of Caenorhabditis elegans provided key insights in understanding its function. Remarkably, mir-57 expression shows strong positional bias but little tissue specificity, a pattern reminiscent of Hox gene function. Despite the minor defects produced by a loss of function mutation, overexpression of mir-57 causes dramatic posterior defects, which also mimic the phenotypes of mutant alleles of a posterior Hox gene, nob-1, an Abd homolog. More importantly, nob-1 expression is found in the same two posterior AB sublineages as those expressing mir-57 but with an earlier onset. Intriguingly, nob-1 functions as an activator for mir-57 expression; it is also a direct target of mir-57. In agreement with this, loss of mir-57 function partially rescues the nob-1 allele defects, indicating a negative feedback regulatory loop between the miRNA and Hox gene to provide positional cues. Given the conservation of the miRNA and Hox gene, the regulatory mechanism might be broadly used across species. The strategy used here to explore mir-57 function provides a path to dissect the regulatory relationship between genes.

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* E-mail: zyzhao@u.washington.edu

Current address: Department of Pathology Informatics, Yale University School of Medicine, New Haven, Connecticut, United States of America

Introduction

miRNAs are small endogenous RNA molecules found in most eukaryotic species that are involved in post-transcriptional regulation of genes required for cell fate determination, metabolism and carcinogenesis among other processes [1]. Like protein coding genes, miRNA genes are transcribed by RNA polymerase II [2,3], suggesting a transcriptional regulatory mechanism similar to that of messenger RNAs. Over 155 miRNAs have been identified in C. elegans through a combination of molecular and bioinformatics methods [4–8]. Many of these are well conserved across eukaryotes, but only a few have been functionally characterized. For example in C. elegans, the founding members of miRNAs lin-4 and let-7 [9,10] regulate lin-14 and hbl-1 respectively and are involved in controlling timing of development. In addition, the fts-6 and mir-273 miRNAs function sequentially and asymmetrically to control chemosensory neuronal developmental [11,12]. mir-67 has been shown to be a direct transcriptional target of LIN-12, and is involved in down regulation of vab-1, which in turn promotes LIN-12 activity in presumptive 2’ VPCs [13]. let-7 and its paralog mir-84 act synergistically to direct cessation of molting via the conserved nuclear hormone receptors NHR-25 and NHR-25 [14]. However, most miRNA genes in C. elegans show no or only very subtle phenotypic effects when the gene is deleted from the genome [15], making their function elusive by classical genetic assays.

One approach to determining the function of these miRNAs would be to use their detailed expression patterns to find genes with which they might interact. We have recently developed technology that allows automated determination of embryonic expression patterns of individual cells with one minute time resolution [16,17]. To apply the system to miRNAs and to see how the information might yield insights into their function, we examined the expression patterns of several miRNA genes of unknown function and found that mir-57, a miRNA gene conserved from nematodes to mammals (where it is named miR-10[7]), produced a particularly intriguing expression pattern. Strains carrying the mir-57 promoter fused to a red fluorescent reporter, mCherry, showed the gene is exclusively expressed in the posterior sublineages of ABp(l/r)(a/p)p and a variety of sublineages of the C founder cell. These lineages produce a variety of cell types but have in common a posterior location in the embryo. Genetic analysis of a deletion mutant and overexpressing lines of mir-57 supported its role in the development of the tail of the animal. This apparent position rather than tissue/cell dependent expression pattern and impact led us to examine its interactions...
Author Summary

miRNAs are small RNAs found in many multi-cellular species that inhibit gene expression. Many of them play important roles in cancer and cell fate determination, but the function of most miRNAs is uncertain. Using live cell imaging and automated expression analysis, we found a miRNA gene, *mir-57*, is expressed in a position rather than tissue dependent way. Hox genes also regulate cell fate patterning along anterior-posterior (a-p) axis across different tissues. By investigating interactions between genes of these classes expressed in *mir-57* expressing cells, we demonstrated by both genetic analysis and gene expression assays that a negative feedback loop between a posterior Hox gene, *nob-1*, and *mir-57* regulates posterior cell fate determination in *C. elegans*. On the one hand, the Hox gene is required for normal activation of *mir-57* expression, and on the other, the Hox gene functions as a direct target of and is repressed by the miRNA. Given the conservation of the two genes, a negative feedback loop between Hox and miRNA genes might be broadly used across species to regulate cell fate along the a-p axis. Detailed expression analysis may provide a general way to dissect the regulatory role of miRNAs.

Results

*mir-57* is expressed in posterior cells across tissue types

With the expectation that detailed expression analysis might suggest possible targets for *mir-57*, we began our investigation of the gene by determining its embryonic expression pattern with cellular resolution at one-minute time intervals [16,17]. Stably integrated lines with a 2.26 kb fragment upstream of the *mir-57* mature sequence fused with a fluorescent reporter mCherry [18] showed expression in the posterior cells of the embryo in a variety of tissues (Figure 1). Automated analysis of 3D time-lapse movies followed by manual editing revealed that the reporter was expressed in a bilaterally symmetric pattern in the posterior of the animal with only minimal levels detectable in the tails (Figure 1F, Figure S1). Inspection of the movies beyond the spike, rectal cells, the P11/12 cells and even body wall muscle cells (Figure 1F, Figure S1). Inspection of the movies beyond the comma stage also showed expression in the intestinal cells after elongation (data not shown).

Examination of larvae and adults showed that the reporter expression remained confined to the posterior of the animal with the exception of weak expression in more anterior intestinal cells (Figure 1E). Signal appears to increase through the L2 stage, after which it decreases with only minimal detectable in the tails of adults in hermaphrodites (data not shown). By contrast in males the adult tail shows higher levels of expression (Figure S2).

To confirm that the expression patterns of the promoter-reporter fusion reflect those of the native *mir-57* expression, we performed *in situ* hybridization on whole mounted embryos using an LNA-modified probe. This method lacks the cellular resolution obtained through the automated lineaging system, but staining was clearly most pronounced in the posterior of the embryo in a pattern consistent with the results of the reporter assay (Figure 1G), while no apparent staining was observed in the *mir-57* deletion strain (Figure 1D).

*mir-57* regulates posterior patterning

To gain insight into the possible functional roles of *mir-57*, we examined animals homozygous for a presumptive null allele, *gk175*, a 414 bp deletion that removes the entire stem loop structure of the miRNA (Figure 2A). The mature sequences of *mir-57* are identical between *C. elegans* and *C. briggsae* (Figure 2B and 2C). In agreement with previous results [15], at lower temperatures (15° and 20°C) we observed no obvious phenotype associated with other genes known to be involved in posterior fate specification, including *nob-1*, *vab-7*, and members of the *Notch* and *Wnt* pathways, *pop-1* and *lag-1*. Interactions of *mir-57* and *nob-1* mutants along with the presence of a putative *mir-57* binding site in the 3’ UTR of a *nob-1* isoform suggested that *mir-57* might directly regulate *nob-1* expression. To test this we examined the effects of the *nob-1* 3’ UTR reporter expression. Our combined results from functional assays provide support for a negative regulatory loop between the miRNA and Hox gene, giving *mir-57* an important role in posterior fate determination.

Figure 1. The *mir-57* gene is expressed in posterior sublineages in the posterior regions of the animal. (A) A 3-D projection of a confocal z-stack of a comma stage embryo with a stably integrated mCherry reporter driven by a *mir-57* promoter segment (p*mir-57*::HIS-24::mCherry). The *mir-57* reporter (red, or yellow when co-localized with green) is detected only in the posterior cells of the embryo. The embryo is shown in a ventral view with anterior to the left. The embryo is also ubiquitously labeled with GFP for lineaging. (B) A space filling model of the same embryo with expressing cells color coded by their lineage origins (see panel F for color code). Non-expressing cells are gray and rendered partially transparent. Note the *mir-57* expressing cells are clustered within the posterior region of embryo. (C) *In situ* hybridization of a one-and-a-half fold wild type (N2) embryo using a DIG labeled anti-sense LNA *mir-57* probe (See Materials and Methods). The anti-DIG staining is apparent as darker regions over the tail (arrowhead). (D) The same *in situ* staining for *mir-57*(gk175) deletion embryos. No apparent staining was seen in the tail region (arrowhead). (E) A 3-D projection of an L1 larva expressing both *mir-57* (red) and the lineaging marker (green). Most expressing cells are seen in the tail of the animal except for a few intestinal cells. (F) Embryonic expression of *mir-57* within cell lineages. Expressing cells were detected in the four AB sublineages and the C sublineages beginning from the 200 cell stage. The fates of the expressing cells are color coded below the leaves. The color coding of bars under each sublineage correspond to those used in the 3-D space filling model in (B). By convention, anterior daughters are placed on the left branches. Cell deaths are indicated with “X”.

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with the homozygous mutant and similar or only slightly increased rates of embryonic or L1 larval arrest and adult sterility compared to wild type hermaphrodite animals (Table 1). However, at 26°C the mutants exhibited significantly increased rates of arrest and sterility (p < 0.05, Student’s T test). Many animals arrested as embryos before elongation (data not shown). Those arrested as larvae often had abnormal tails (23 of 47 examined). Most commonly, the tail contained a preanal bulge and vacuolated regions (Figure 3A and 3B). In about 5% of the arrested larvae, we observed a forked tail spike, something we have never observed in wild type (Figure 3B). Because we had also observed expression of mir-57 in the adult male tail, we wondered if the male tail morphology might be a more sensitive assay for mir-57 activity.

However, of mir-57 null males that develop into adulthood and were recognizably male, we found no defects in their tail structures compared to wild type at either room and elevated temperature (26°C, n = 38). In summary, the spatial correlation observed between mir-57 expressing cells in embryos and the defects associated with its loss of function suggests a likely role of mir-57 in regulating posterior cell fate specification in development.

Because the loss-of-function allele for mir-57 only produced moderate defects in embryos and the larval tail, we reasoned that the effect of the allele is possibly masked by other miRNAs or factors that function redundantly with mir-57. An alternative approach to defining the roles of functionally redundant genes is to overexpress individual genes. Therefore, we examined the phenotypic effects of mir-57 overexpression. In contrast to the low copy, integrated transgenes generated by bombardment that were used to assay expression, we used microinjection to create extrachromosomal arrays, which are expected to have many copies of the transgenes. Arrays containing the intact structural gene along with the upstream region (−2,260 to +234) produced pronounced defects exclusively

Table 1. Characterization of mir-57(gk175) phenotypes.

| Genotype   | mir-57(gk175) | N2 |
|------------|---------------|----|
|            | Emb(%) | Lav(%) | Ste (%) |            | Emb(%) | Lav(%) | Ste (%) |
| 15°C       | 1.6(514) | 0.5(325) | 2.7(339) | 1.2(277) | 0.8(363) | 0(710) |
| 20°C       | 1.3(840) | 2.0(359) | 5.3(472) | 0.7(647) | 0.5(451) | 1.2(389) |
| 26°C       | 6.7(892) | 5.6(841) | 32.1(978) | 1.3(360) | 1.5(399) | 5.7(451) |

Number of worms scored is indicated in parentheses. doi:10.1371/journal.pgen.1001089.t001

Figure 2. The mir-57 locus and its predicted stem loop sequences. (A) mir-57 locus. The 414 deletion, gk175, shown as a red bar, removes the entire structural gene. Regions of conservation with homologous regions of C. briggsae (WABA alignments) are indicated below (arrowheads). The region containing the mir-57 gene is expanded below, showing the mature mirRNA (green) within the predicted stem-loop region (black bar). A predicted LAG-1 binding site 55 bp upstream of the mir-57 mature RNA is shown as a solid black circle. (B,C) predicted stem-loops for C. elegans mir-57 (B) or its equivalent in C. briggsae (C). The mature mir-57 sequences are highlighted in red. The predicted stem-loops are modified from MirBase. Note the mir-57 mature sequences are identical between the two species.

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Figure 3. Phenotypes associated with mir-57 loss of function and misexpression. Homozygous mir-57(gk175) animals placed at 26°C occasionally had preanal bulges (arrowhead in A) or vacuolated cells (arrow in B) in the tail. The animal in B also exhibited a forked tail spike (arrowhead). mir-57 overexpression from extrachromosomal arrays resulted in Nob (C,D) and Vab-like (E) tail phenotypes as indicated by arrowheads. A wild-type larva (F) is shown for comparison. Males carrying the mir-57 extrachromosomal arrays produced abnormal tails (G) compared to wild type (H). The anus is indicated by arrowheads and tail rays by arrows. Scale bars indicate 20 μm in all pictures.

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in posterior region that resembled those seen in mutations of a posterior Hox gene, nob-1, which produces Vab (Varially Abnormal in tail) and Nob (NO-Back end) animals (Figure 3C–3F and see below). These phenotypes were typically more severe and affected a higher proportion of the adults at 20 °C than were seen in the null allele at 26 °C (Table 1 and Table 2). mir-57(gk175) animals injected with the same fragment also produced a comparable frequency of Vab progeny (Table 2). Adult male worms carrying the array often failed to develop proper tail rays (Figure 5G and 5H) and were unable to mate successfully to produce progeny (n = 34). The average ray number of each side of a male tail in the array containing animals is 3.2±1.2 (n = 30) compared to the 0.6±1.2 (n = 57) in wide type animals. The array containing males also produce only a few sperm (Figure S8A) compared to wild type (Figure S8B). Both the tail defects and the paucity of sperm likely contribute to the inability of mir-57 overexpressing males to produce progeny. No defects were seen in the anterior body of either hermaphrodites (112) or males (n = 320) that carry the arrays, consistent with the expression patterns of mir-57.

To examine the cell patterning defects in the tail, we injected the mir-57 promoter (a 2260 bp fragment upstream from the miRNA mature sequence, see below) into a strain expressing hypodermal marker, ajm-1::GFP. As expected, the injection produced Vab and Nob animals as did in N2 animals (Table 2). The marker showed that the hypodermal cells are severely disorganized in the tails of Nob/Vab animals as opposed to those in uninjected control animals (Figure 4), supporting a role of mir-57 in patterning posterior cells.

An intact mir-57 structural gene is required for producing Nob/Vab phenotypes

The tail defects associated with the mir-57 arrays could derive either from elevated expression of the transgene or from the action of other sequences/elements in the 2.26 kb construct, which contains several regions conserved in C. briggsae (Figure 2). To test the first possibility, we drove mir-57 expression by a vab-7 promoter. The vab-7 gene, an even-skipped homolog, is expressed in the posterior of the embryo in regions overlapping with those expressing mir-57, as determined by automated lineage-based expression analysis of a vab-7 promoter::mCherry fusion integrated transgene (Figure 4, See Text S1). Extrachromosomal arrays of the prab-7::mir-57 construct produced Vab/Nob animals, albeit at a lower rate than the native mir-57 promoter. The transgenic animals also yielded other phenotypes, including Dpy (12%, n = 262) and molting defects (3%, n = 340) (Figure S5). Importantly, a control construct with a mutated mature sequence of mir-57 produced no Vab/Nob animals (n = 347) (Table 2), indicating that presence of mir-57 structural gene on the array is essential for the development of tail defects. Therefore, the tail defects can arise either from ectopic expression of mir-57 driven by vab-7 promoter or its overexpression produced by the action of other sequences/elements in the 2.26 kb mir-57 promoter. We speculate that the difference in the details of the phenotypes results from the differences in vab-7 expression pattern from that of the mir-57 promoter.

To test the effects of the putative cis-elements within the upstream region, we created constructs containing the upstream region separate from the structural gene. Surprisingly, injection of fragments (~2260 to ~63) lacking the transcribed portion of mir-57 and a predicted upstream LAG-1 binding site yielded a comparable fraction of animals with abnormal tails (Table 2). We postulated that the high copy number of the mir-57 promoter fragment could have disrupted normal regulation of the genomic copy of mir-57, possibly titrating out an important inhibitory factor. Therefore, we introduced both the full length and truncated arrays into a background containing the genomic mir-57 deletion allele, gk175. The full promoter-gene construct produced abnormal tails at rate similar to that seen in the wild type background. However, the absence of the genomic mir-57 gene completely abolished the Vab and Nob phenotypes produced by the mir-57 promoter fragment (Table 2), showing these effects required the presence of an intact copy of mir-57 either in the genome or on the array. Thus, the abnormal tail phenotypes produced by the mir-57 promoter fragment require at least one functioning copy of the mir-57 structural gene.

![Figure 4. Hypodermal defects associated with mir-57 overexpression.](image)

**Figure 4. Hypodermal defects associated with mir-57 overexpression.** (A) A Nob animal produced by injection of the mir-57 promoter segment shows disorganized hypodermal cells in the tail as labeled with ajm-1::GFP. (B) Uninjected control animal shows well organized hypodermal cells in the tail. Anterior is to the left. A 10 μm scale bar is indicated in each picture.

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| Table 2. Phenotypes of mir-57 transgenic arrays. |
|-----------------------------------------|---------|---------|---------|---------|---------|
| **Promoter** | **miRNA** | **Position** | **Lines** | **Vab/Nob%** | **Background** |
| mir-57 | mir-57 | −2260 to 234 | 8 | 29.3(625) | wild type |
| mir-57 | none | −2260 to −63 | 5 | 33.9(373) | wild type |
| mir-57 | mir-57 | −2260 to 234 | 2 | 36.4(522) | mir-57(gk175) |
| mir-57 | none | −2260 to −63 | 6 | 0.583 | mir-57(gk175) |
| vab-7 | mir-57 | −3664 to 36 | 3 | 11.3(362) | wild type |
| vab-7 | mir-57* | −3664 to 36 | 7 | 0(347) | wild type |
| vab-7 | none | −3664 to 36 | 5 | 0.831 | wild type |

*Mutated mir-57 mature sequence (see Materials and Methods).

*Coordinates in base pairs relative to the start of the mature mir-57 sequence.

*Coordinates in base pairs relative to the translational start of vab-7 sequence.

*The sequences are fused with mir-57 genomic region (~50 to 234 bp relative to the start of mature mir-57 sequence).

*Number of animals scored.

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to detect the miRNA expression directly. As expected, both wild type and mir-57 mutant animals injected with the full genomic region of mir-57 (−2260 to +234) showed much higher levels of the miRNA transcripts, i.e., about 5 times higher than that of the uninjected controls. No signals were detected for the mir-57 deletion strain (Figure 5). In addition, injection of the regulatory region alone (−2260 to −63) into the wild type animals also produced high levels of the miRNA transcripts, comparable to that seen for injections with the full mir-57 region (Figure 5, Table 2). Given the partial transmission of the extrachromosomal arrays, the overexpression level for the array-containing animals is likely to be even higher than the increases measured for the populations of worms as a whole. These results strongly support the hypothesis that the array of the extra cis-regulatory fragments titrates out repressors controlling the endogenous expression of mir-57, thus increasing mir-57 expression. Dissecting the molecular identities of the repressors is beyond the scope of this paper.

Next, to observe directly the effects of the mir-57 extrachromosomal arrays on the activity of a genomic copy of the mir-57 promoter, we introduced the mir-57 promoter array into a background containing the integrated mir-57::mCherry reporter construct. Because of the instability of the promoter array, we did not carry out automated lineage-based expression analysis. However, examination of many individual expressing animals (n = 122) showed a fraction of animals (consistent with the inheritance of the array) with much earlier, more intense and more anterior expression of the reporter (Figure 6). Taken together these results show that overexpression of mir-57 either from the arrays directly or from extra copies of the promoter region leading to the overexpression of the endogenous mir-57 gene can perturb posterior development and thus produce Vab/Nob phenotypes.

### Interactions of mir-57 with other factors involved in posterior patterning

Because these results implicate mir-57 in posterior development, we examined its relationship to other genes with a known role in posterior embryonic development in *C. elegans*. These include genes in the Notch and Wnt pathways that provide signals that differentiate anterior daughters from posterior daughters as well as homeobox and Hox related genes involved in posterior patterning [19–24]. We used RNAi against genes of the Notch and Wnt pathways that specify cell lineage fates to look more broadly for interactions between these pathways and mir-57. RNAi against pop-1, a gene involved in Wnt signaling and required for anterior-posterior lineage fate polarity [19] converts ABpl/r/pp lineages to ABpl/r/pp lineages, as judged by both lineage fate and expression patterns (Figure S7). The RNAi is quite effective as evidenced by the complete homeotic lineage fate transformation from MS to E. These results indicate that the correct expression of mir-57 is dependent on the lineage fate specified by the Wnt signaling pathway. Similarly, RNAi against lag-1, a gene involved in Notch signaling, converts ABplap and ABplpp to ABalap and ABarpp fates respectively with concomitant loss of mir-57 expression in these altered lineages (Figure S7). In the C lineage, mir-57 expression is dependent on the cell fates specified by PAL-1 as RNAi against the gene completely abolished the mir-57 expression with concomitant cell fate changes as judged by the loss of asymmetry of cell cycle timing between CxA and Cxp (Figure S8). Thus, in all these cases, mir-57 expression is dependent on the lineage fates and thus likely downstream of these decisions.

These results suggest that mir-57 might be a direct target of one or more of these early regulators. Computational analysis revealed the presence of a putative LAG-1 binding motif 55 bp upstream of the mir-57 mature sequence (Figure 2A), suggesting that lag-1 might directly regulate mir-57 expression. To test the hypothesis, we produced a modified mir-57::HIS-24::mCherry fusion construct with the LAG-1 site removed by site-directed mutagenesis and used it to generate transgenic lines by microinjection. Arrays without the LAG-1 site also yielded high fractions of Vab/Nob progeny (data not shown), consistent with our earlier observations with the promoter region and indicating that this site is not responsible for the observed Vab/Nob phenotypes. However, arrays lacking the LAG-1 site failed to express the reporter in the tail, whereas the wild type promoter yielded strong expression, indicating that mir-57 is likely a direct target of LAG-1.

**Figure 5. Northern blot results for mir-57 overexpression.** (A) 20 μg total RNA from wild type (wt) or mir-57 mutant (Δ) 4 worms with (+) or without (−) injection of genomic DNA containing both regulatory and structural sequences of mir-57 or with the injection of mir-57 promoter only were separated on a 15% urea denaturing gel, blotted and hybridized using an antisense mir-57 LNA probe and an antisense U6 DNA probe (See Materials and Methods). In order the lanes show wild type; the mir-57 deletion strain; animals with transgenic arrays of the mir-57 genomic fragment in the wild type background; animals with transgenic arrays of the with mir-57 genomic fragment in the mir-57 deletion background; and finally animals with arrays of the mir-57 promoter in a wild type background. Arrays of the mir-57 genomic fragment into both wild type and the mir-57 deletion mutant resulted in elevated levels of the mir-57 miRNA. Arrays of mir-57 promoter alone in the wild type background produced mir-57 miRNA levels similar to that seen using the intact mir-57 region. No signal was detected for mir-57 deletion strain. (B) Quantification of expression changes measured by the intensities of the mir-57 signal from lanes in panel A and normalized against U6 (See Materials and Methods). doi:10.1371/journal.pgen.1001089.g005

**nob-1** is co-expressed with mir-57 spatially but with an earlier onset

In addition to the Notch and Wnt pathways, the nob-1 gene, an ABd-B Hox gene homolog, is known to be involved in posterior pattern specification, with loss-of-function alleles resulting in embryonic lethality and larval arrest [22]. A hypomorphic allele, ct230, produces both Nob and Vab phenotypes quite similar to those that result from mir-57 overexpression, suggesting that the mir-57 and nob-1 might function in the same pathways. To explore this possibility, we profiled the expression of nob-1 with resolution comparable to that of mir-57 by automated lineage-based expression analysis using an integrated NOB-1::GFP protein fusion transgene (see Materials and Methods). The reporter was detectable from roughly the 100-cell stage in posterior progeny of ABpp[Δ]/r/pp and ABpl[Δ]/r/ap, the same sublineages in which mir-57 is also expressed (Figure 6). These results confirmed and refined the nob-1 expression pattern determined independently using a similar rescuing NOB-1::GFP construct (E. Kress, L. Edgar and W. B. Wood unpublished). Despite the striking overlap of
expression between mir-57 and nob-1 in the AB sublineages, their temporal expression patterns are quite different. The nob-1 reporter appears at about the 100-cell stage with a fairly uniform time of onset in different cells whereas mir-57 expression mostly appears after the 200-cell stage, although the more posterior the cells are, the earlier onset is detected. Expression of nob-1 was either very weak or not observed in the C lineage but was seen in the posterior E lineage by the 100-cell stage (Figure 6). As described above, expression of mir-57 was clearly present in the C lineage and only expressed in the E lineage after elongation (data not shown) so that spatial expression of nob-1 and mir-57 are not totally overlapping in these lineages.

nob-1 RNAi downregulates mir-57 expression

Given their similar expression patterns in the AB sublineages and the earlier onset of nob-1 expression compared to that of mir-57, we reasoned that nob-1 might be required for mir-57 expression. To test this hypothesis, we depleted nob-1 activity by RNAi and observed its effects on the mir-57 reporter expression with time using automated lineaging. The RNAi produced Vab/Nob progeny at rates comparable to the wild type and that overexpression of mir-57 might inhibit nob-1 expression, and thus the altered expression of mir-57 after RNAi against nob-1 is similarly plotted (red). Vertical blue lines mark cell divisions. The vertical black line indicates the time point for the measurement in B (below). (B) mir-57 expression in all the progeny of ABplpp at the indicated time point (215, the vertical black line in (A)) between the RNAi treated (red) and untreated animals (green). For simplicity, cell names include only the extension from ABplpp. The expression intensities were derived from the average values of six RNAi treated and six untreated image series and standard deviations are shown as vertical bars (See Materials and Methods).

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Figure 6. Embryonic lineage-based expression of nob-1. Shown are the superimposed lineage based expression trees between NOB-1::GFP (green) and mir-57::His-24::mCherry (red). The branch lengths (division timing) of nob-1 and mir-57 expressing trees are normalized against Sulston’s lineage trees and the expression values are interpolated accordingly. In lineages expressing both genes, such as the ABplpp, nob-1 signal appears a full cell cycle before the onset of mir-57 expression. Cell deaths are indicated with “X”. doi:10.1371/journal.pgen.1001089.g006

Figure 7. Effect of nob-1 RNAi on the expression of mir-57. (A) expression intensity of mir-57 (black) and nob-1 (green) in ABplpppppppp and its ancestors is plotted against developmental time in minutes at 20°C. The altered expression of mir-57 after RNAi against nob-1 is similarly plotted (red). Vertical blue lines mark cell divisions. The vertical black line indicates the time point for the measurement in B (below). (B) mir-57 expression in all the progeny of ABplpp at the indicated time point (215, the vertical black line in (A)) between the RNAi treated (red) and untreated animals (green). For simplicity, cell names include only the extension from ABplpp. The expression intensities were derived from the average values of six RNAi treated and six untreated image series and standard deviations are shown as vertical bars (See Materials and Methods).

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nob-1 is a direct target of mir-57

Given that miRNAs generally act by reducing gene expression and that overexpression of mir-57 produced tail defects similar to those of nob-1 reduction-of-function mutations, we reasoned that overexpression of mir-57 might inhibit nob-1 expression, and thus produce the observed Nob/Vab phenotypes. Consistent with this, the nob-1b 3’ UTR, one of the two alternative nob-1 3’ UTRs, contains a predicted mir-57 binding site [7] (Figure 8A and 8B). The putative binding site for mir-57 has the highest score among all the predicted mRNA binding sites within the nob-1 3’ UTR [7]. To explore experimentally the functional role of the mir-57 binding site, we used mir-57 promoter driven reporter constructs followed by nob-1 3’ UTRs to induce strong overexpression of the endogenous mir-57 in order to produce a robust effect. We made three constructs: two of them using the 3’ UTRs from the different splice isoforms, nob-1a and nob-1b and a third construct using the 3’

other factors may be involved in the control of mir-57 expression in this sublineage (Figure S4 and Figure S8).
bars are coding exons and black lines introns. A putative 12077884, WS205). The mature alternative forms of binding site on the reporter expression. Shown are boxplots of tail Putative seed sequences are enclosed in the rectangle. (C) Effect of the site within the putative mir-57 functional target of the wild type background, the arrays, because they contain the which were then crossed into the background by microinjection to create extrachromosomal arrays, ods). These constructs were introduced into the wild type N2 background significantly decrease its transcript level, roughly four

Figure 8. Validation of nob-1 as a target of mir-57. (A) Two alternative forms of nob-1 3’ UTRs (grey bars) are shown in scale. Blue bars are coding exons and black lines introns. A putative mir-57 binding site within the nob-1b UTR is shown as a white star. (B) Sequence of a putative mir-57 binding site within the nob-1b 3’ UTR (chrIII:12077862-12077884, WS205). The mature -mir-57 sequence is highlighted in red. Putative seed sequences are enclosed in the rectangle. (C) Effect of the binding site on the reporter expression. Shown are boxplots of tail expression intensities derived from three different 3’ UTRs: nob-1b, nob-1bm (nob-1b UTR with site-directed removal of the binding site), nob-1a injected into wild type (wt, first three columns) or mir-57 mutant (Δ last three columns) animals as indicated (See Materials and Methods). (D) Micrographs of reporter expression in wild type (a and b) or mir-57 mutant animals (c). Animals were injected with constructs carrying wild type (a) or mutated nob-1b UTR (b). The same array as that used in (a) was crossed into mir-57 mutant animals (c). d, e, f are DIC pictures of a, b and c respectively. Vab tails are caused by mir-57 promoter array. doi:10.1371/journal.pgen.1001089.g008

UTR from nob-1 in which the candidate mir-57 binding site was removed by site-directed mutagenesis (See Materials and Methods). These constructs were introduced into the wild type N2 background by microinjection to create extrachromosomal arrays, which were then crossed into the mir-57 deletion background. In the wild type background, the arrays, because they contain the mir-57 promoter region, should result in overexpression of mir-57 from the genomic copy. As predicted, expression of the reporter gene is significantly reduced by the presence of the binding site in nob-1b (p<0.01, Student’s two-tailed t test, Figure 8C and 8D). The reduction in reporter construct expression was not observed in the absence of a functional mir-57 gene, indicating that nob-1 is a direct functional target of mir-57. A caveat for these experiments, however, is that all DNA constructs contained a mir-57 promoter introduced by microinjection, which as we described above leads to overexpression of mir-57. Thus, these results may exaggerate the effects of a more physiological level of mir-57 expression, but the results clearly show that the binding site is functional in vivo.

We looked genetically for further support that nob-1 is a target of mir-57. By this hypothesis, mir-57 loss of function mutations might lead to increased expression of nob-1 although in contrast to the overexpression experiments, such an effect might be attenuated by other, redundant regulators of nob-1. In agreement with this, overexpression of nob-1 from an extrachromosomal array partially mimics the phenotypes of mir-57 loss of function albeit at a lower level (Table S1, n = 3). However, we did not observe male tail defects in nob-1 overexpressing strains, suggesting that mir-57 may regulate male tail development independent of nob-1. The lower penetrance might reflect partial transmission of extrachromosomal array, alternatively, other targets of mir-57 may also contribute to the observed phenotypes. Further, using a reporter to assay nob-1 expression, the loss of function mutation in mir-57 only produced modest effects on the nob-1 expression (Figure S9, See Text S1), suggesting the miRNA functions redundantly with other miRNAs or pathways.

To look for biochemical evidence that nob-1 is a target of mir-57, we measured the endogenous levels of the two alternative nob-1 transcripts, nob-1a and nob-1b in the presence or absence of the genomic mir-57 copy using real-time PCR assay. Deletion of mir-57 significantly increases the nob-1b transcript level (p<0.01, Student’s t test, Figure 9A) and mir-57 promoter arrays in the N2 background significantly decrease its transcript level, roughly four

Figure 9. Genetic interactions between nob-1 and mir-57. (A) Real time RT-PCR assay of nob-1a (white) and nob-1b (grey) transcripts in the presence (wt) or absence (Δ) of genomic mir-57. Deletion of genomic mir-57 increases nob-1b transcripts about two-fold while no effects were observed for nob-1a transcript level. The nob-1b transcript level is roughly four-fold lower in mir-57 promoter-array wild type strain (+) than that in wild type only (−). (B) The percentage of Vab animals is plotted for mutants or RNAi of nob-1 in the presence or absence of genomic mir-57. The left two of experiments contrast the phenotypes of nob-1(ct230) and nob-1(ct230; mir-57(gk175)), whereas the right two contrast nob-1(RNAi); and nob-1(RNAi); mir-57(gk175). The fraction of nob-1 mutant animals exhibiting abnormal tail phenotypes is reduced in both cases by the removal of the genomic mir-57. doi:10.1371/journal.pgen.1001089.g009
mir-57 had no effect on the 200-cell stage, which in turn dampens the expression of reduction in the fraction of progeny exhibiting the Nob phenotype. Thus, in normal development, our data suggests that mir-57 activity might ameliorate the effects of the hypomorphic allele.

Discussion

The detailed analysis of the expression pattern of mir-57 and nob-1, coupled with the phenotypes observed in the absence and overexpression of mir-57 demonstrate that the miRNA gene plays a role in combination with the posterior Hox gene to specify posterior identity. The expression patterns reported here generally agree with previously described patterns [25] but with much higher temporal and spatial resolution, allowing us to infer and test the detailed functional relationships with other genes operating in related pathways. For example, both mir-57 and nob-1 are expressed in the same AB sublineages, ABp(r)ap and ABp(r)pp, which give rise to a variety of tissue/organ types in the posterior region, the region where the abnormal phenotypes were observed. Based on the detailed expression map as well as their similar phenotypes, we examined the interaction between mir-57 and other genes implicated in posterior development in C. elegans including nob-1. The expression of mir-57 in the posterior sublineages of AB founder cells was dependent upon their proper specification through the Notch and Wnt pathways. In addition the expression of the mir-57 transgene required the presence of a binding site within its promoter sequence for the Notch pathway factor LAG-1, suggesting that the gene might be directly regulated by the pathway. More dramatically, reduced function of nob-1 delayed the onset and reduced the level of mir-57 expression and in turn, nob-1 is to be a direct target of and repressed by mir-57. This would constitute a negative regulatory loop between the miRNA and the Hox gene in providing positional cues for posterior development. The results suggest that the Nob/Vab phenotypes produced by overexpression of mir-57 are likely caused at least in part by down regulation of nob-1 activities within the posterior region, mimicking those of hypomorphic or null nob-1 mutants. This is supported by the evidence that mir-57 promoter injection produced a significant decrease of nob-1b, but not nob-1a, transcripts (Figure 9A). Similarly, the Emb phenotype observed for the mir-57 null mutant at 26°C could be partially reproduced by nob-1 overexpression. Further support for mir-57 regulation of nob-1 comes from the fact that the mir-57 mutation partially alleviated the phenotypes of nob-1(ct230), a hypomorphic allele, presumably resulting from partial release of the repression of nob-1. Thus, in normal development, our data suggests that nob-1 expression begins by the 100-cell stage, activates mir-57 by the 200-cell stage, which in turn dampens the expression of nob-1, perhaps in conjunction with other miRNAs. This down regulation might function to reinforce the transcriptional silencing of nob-1 activity in late embryonic stages when it may no longer be required to provide positional cues for posterior fate specifications. Such reciprocal regulation between the Hox gene and the miRNA might provide more robust control over the regional identity than the Hox gene alone. It should be noted that mir-57 expression is very likely subject to control of positional cues other than nob-1 because expression onset of nob-1 seems synchronized but that for mir-57 are not (Figure 6).

In vertebrates, miRNA genes have also been found to have a role in Hox gene regulation, helping to reinforce posterior prevalence [26]. Notably, the closest homolog to mir-57 in other species is the broadly conserved gene mirR-10. mir-R-10 has been found to repress Hox gene activities in zebrafish spinal cord [27]. In mouse, mir-10a and another miRNA gene, mir-196a, are embedded in a Hox gene cluster and their expression is apparently overlapping with those of Hox genes [28]. In addition, mir-196a represses Hoxb8, indicating its restricted expression pattern likely reflects a role of microRNA in the patterning of the Hox complex. Interestingly, miR-126 has been shown to regulate Hox9a by binding to its target sites within the homeobox domain [29]. A negative regulatory loop between miRNA genes and other transcription factors has also been described in vertebrates [30,31]. By miRNA profiling using an E2F1-inducible Saos-1 cell line, miRNAs miR-449a/b were identified as direct transcriptional targets of E2F1 [30]. miR-449a/b negatively regulates E2F activity through a feedback loop mechanism by targeting oncogenic CDK6 and CDC25A, leading to dephosphorylation of pRb, which is required for activation of E2F-responsive genes to promote cell cycle progression. miR-133b is also involved in a feedback regulatory loop that includes a paired-like homeodomain transcription factor Pitx3 in mammalian midbrain dopaminergic neurons [31]. Thus, such a negative feedback regulation between miRNAs and homeodomain transcription factors may provide a conserved mechanism to control metazoan position specific patterning. Although mir-57 is not found within a Hox gene cluster, Hox genes in C. elegans are only relatively loosely clustered [32].

Like many miRNA genes, mir-57 most likely functions redundantly with other miRNA genes. Deletion of the gene had little effect on the worm’s fitness except at high temperatures and even here the defects were minor and penetrance incomplete. Overexpression of the gene produced more dramatic effects, but the overexpression phenotypes of miRNAs must be interpreted with caution since high miRNA levels may create off-target effects. However, the observed phenotypes were consistent with the expression pattern. A more systematic investigation of the high resolution expression patterns of the full set of miRNA genes would provide valuable insight into the likely partners, just as the detailed expression information here provided hints as to function.

The ability of the 2.26 kb sequence upstream of the mature mir-57 miRNA to produce Vab/Nob phenotypes is intriguing. Although no evidence of another gene in this region has been found in extensive RNA-seq studies [33], we cannot entirely rule out the presence of such a gene. Nonetheless, the dependence of the resultant phenotypes on the presence of a functional copy of the mir-57 gene, either in cis or in the genome, argues that the presence of the fragment in high copy number results in mir-57 misexpression. Our Northern blot results provide direct evidence that injection of either the mir-57 genomic region including its mature sequence and flanking sequences or the promoter region of mir-57 alone produced overexpression of the miRNA, which likely underlies the tail defects associated with these assays. The ability of
the pmab-7:mir-57 arrays to partially mimic the phenotypes also implies that overexpression of mir-57 is the primary cause for the observed defects. This would be consistent with the 2.2 kb fragment binding and titrating out a repressor of mir-57 but what that factor might be remains unknown.

Although the nob-1 gene seems to be one direct target of mir-57, there are undoubtedly many other direct or indirect ones both within the AB sublineages where both mir-57 and nob-1 are expressed and also in the C lineage where mir-57 is found in the absence of nob-1. MirBase lists a total of 492 candidate targets for mir-57 [7,34] but which of these might be functional targets in the C lineage is unclear. Again detailed expression patterns would greatly restrict the list of possibilities. Taken together, by using the automatic high-resolution gene expression technology, we were able to identify a negative regulatory loop between mir-57 and a Hox gene to control regional identity.

Materials and Methods

Strains and genetics

All the strains were maintained on NGM plates with OP50 E. coli at room temperature except for temperature sensitive assay of mir-57 mutant phenotypes at the indicated temperature. The following strains were used in the assay: N2; VC347, mir-57(gk175) II; RW10029, unc-119(ed3), stis1007[his-72::GFP, unc-119(+)], stis10025[pie-1::GFP::his-38, unc-119(+)]; RW20050, mir-57(gk175) II; BW1379, nob-1(ct320) III; RW20051, mir-57(gk175) II, nob-1(ct320) III; RW10044, unc-119(ed3), stis10044[pmir-57::HIS-24::mCherry, unc-119(+)]; RW10226, unc-119(ed3), stis10226[HIS-72::mCherry, unc-119(+)]; BW2020, ctIs57[NOB-1::GFP, rol-6 dm]; RW10044, unc-119(ed3), stis10044[pmir-57::HIS-24::mCherry, unc-119(+)]; stis10026[HIS-72::GFP]; RW10174, unc-119(ed3), stis10174[pmir-57::HIS-24::mCherry, unc-119(+)]; RW10199, unc-119(ed3), stis10199[pmir-57::HIS-24::mCherry, unc-119(+)]; RW10226, unc-119(ed3), ctIs57[NOB-1::GFP, rol-6 d], stis10226[HIS-72::mCherry]; PS1657, unc-119(ed3), slyIs78[ajm-1::GFP + unc-119(+)]; The -mir-57 (gk175) allele was backcrossed to N2 Bristol strain five times before phenotypic assay. The presence of -mir-57(gk175) allele was followed by single worm PCR. The following phenotypes were scored at 15°C, 20°C and 26°C respectively for N2 and RW20050 strains: Emb (embryonic lethality), Lva (larva arrest), Ste (sterility).

Transgenic constructs

A 2260 bp (~2260 to -1) fragment upstream of mir-57 was cloned into the restriction sites upstream of a HIS-24::mCherry cassette using AvrII and SmaI sites in the pJM20 vector [17] to give rise to pZZ1. mCherry is a worm optimized derivative of Cherry [18]. Histone HIS-24 was used to direct the reporter signal into nucleus. pJM20 also contains transgenic selection marker unc-119(+). The pZZ1 (Pmir-57::HIS-24::mCherry, unc-119+) construct was bombarded into unc-119(ed3) worms to generate integrated reporter expressing strains for expression profiling. A total of 12 independent lines were generated and all of them showed the similar expression patterns. A single line was backcrossed three times with N2 and the resultant reporter expressing strain was mated into the lineageing strain, RW10029 [16], which ubiquitously expresses nuclear localized GFP to generate a strain RW10048 that are homozygous for both GFP and RFP loci. Similar method was used to generate reporter-expressing strains using RW10174 and RW10199 for automatic profiling of pmab-7::mir-57 transcription respectively (See Text S1 for the primer used). To build a translational GFP fusion reporter strain for nob-1, an approximately 15 kb fragment spanning the entire coding region plus its upstream regulatory sequences was fused in frame with GFP followed by unc-54 3’ UTR. The construct was co-injected into N2 with pRF4(rol-6::dsu1006). The resulting transgenic strain was integrated into the genome by gamma irradiation to give rise to BW2020.

The constructs for site-directed removal of the LAG binding site and the mutated mir-57 as well as for the hybrid construct between mir-57 and rol-7 promoter, were built by the fusion PCR technique as described below [35] (See Text S1 for the primers used). To build the construct with site directed removal of LAG-1 site within the mir-57 promoter, the fragments flanking LAG-1 site were PCR amplified from pZZ1 and fused together by PCR. The resulting construct contains the full mir-57 promoter (~2260 to -1) except for the LAG-1 site (See Text S1 for the primer sequences). To build mutagenized mir-57 driven by rol-7 promoter, two pairs of primers were designed so that they overlap on the mature mir-57 sequence. The mir-57 mature sequence was mutated into an unrelated sequence (See Text S1 for the primers used). To build the hybrid construct between mir-57 and rol-7 promoter, the 3400 bp rol-7 promoter was fused with 284 bp mir-57 stem-loop plus its flanking sequences. The mir-57 overexpression construct is a 2494 bp PCR product that includes 2260 bp promoter sequences and the 234 bp stem-loop and its downstream sequence. The mir-57 promoter only construct is the PCR product that is -2260 to -63 bp from the mir-57 mature sequence. These PCR products were co-injected with pRF4(mtl-6 d) with concentrations of 20 and 100 ng/μl respectively. The mir-57 promoter only PCR product (~2260 to -63) was also injected into both wild type and mir-57 deletion animals in the same concentrations. The overexpression phenotypes for hermaphrodite were scored from three independent transgenic lines synchronized at L2 stage.

Embryonic expression profiling

Embryonic lineage analysis and expression profiling of mir-57 was performed with strain RW10048 using StarryNite and AceTree as described [17,36] with modifications. Strain RW10048 was used for profiling of mir-57 expression. Given the relatively late stage of mir-57 expression during embryogenesis, we traced the relevant embryonic lineage until the last round of cell division in both “forward” and “backward” directions. In the “forward” direction, we traced the lineage using the standard method for those up to 350-cell stage. For the “backward” direction, we started with the reporter expressing cells at a late stage of embryogenesis, for example, comma stage, and traced the cells backward until the progenitors could be reliably linked to the nuclei identified by the forward tracking method. In this way we captured the expressing lineages at the late period of embryogenesis up to comma stage. To profile nob-1 expression, we made a strain RW10226 that ubiquitously expresses histone::mCherry in somatic nuclei and crossed it into BW2020, a NOB-1::GFP expressing strain for lineageing using mCherry labeled nuclei. Due to the lack of the germline expression of mCherry, cell lineage from one to about 70 celled embryo were manually traced using DIC images. After the embryonic cell lineage was produced for both mir-57 and nob-1 expressing strains, pixel densities from GFP (nob-1) or RFP (mir-57) channel were extracted and aligned against the lineage tree branch for each time point and all nuclei. To compare the expression from different experiments, division timing was normalized against those derived from Sulston lineage tree [37] and expression values interpolated accordingly. A total of six and four embryos were profiled for mir-57 and nob-1 expressing embryos respectively. To assign the expression values of reporter expressing cells, we did the background subtraction (blot) to eliminate marginally expressing cells and thus increase the
replicates and the presence of the roller or male animals were put in the same plate with 10 N2 hermaphrodite animals in 3 To test the fertility of the array containing males, 10 males were 57 promoter array containing animals (n = 38). For male progeny was examined. About six-hours. 450-cell stage for the selected sublineages. To plot expression of ABplpp_ppppp with time, we used both raw and background subtracted expression values and the two data sets agree well with one another in terms of relative intensity (data not shown).

In situ hybridization

In situ hybridization was performed as described [38,39] with following modifications. DIG labeled LNA-modified probe complementary to the mature mir-57 was made by Exiqon with the sequence ACACAGACGTGATCTACAGGTTA. The mix-staged embryos were immobilized on poly-lysine coated slides followed by methanol fixation. The hybridization and wash were performed at 37°C (20°C below the probe melting temperature) as suggested elsewhere [39].

Northern blot

Worm populations synchronized at the L4 stage were incubated with acid phenol (pH 4.5) at 65°C for one hour followed by centrifugation. The top layer was transferred to Phase Lock GelHeavy tubes (Sigma-Aldrich) and centrifuged for 1 minute. The aqueous layers were re-extracted with phenol and chloroform followed by precipitation and washing. A total of 20 µg total RNA was loaded onto 15% polyacrylamide denaturing (urea) gel for each sample and transferred to Nylon (+) membrane by electroblotting in 1 X TBE buffer. The mir-57 antisense LNA probe and U-6 antisense probe (GTCATTCCTTGCGGAGGGCCATGAATGTTCCTCCTGTATTGTCGATA) were 5′ labeled with γ-32P ATP. The two probes were mixed and hybridized to the membranes at 50°C overnight in the hybridization buffer (5 X Denhardt’s, 2 X SSC, 0.1% SDS) and washed twice at 30°C in the wash solutions (2 X SSC, 0.1% SDS). The blots were visualized and signals quantified using Amersham Biosciences phosphorimagier according to the manufacturer’s instructions.

RNAi

All RNAi experiments were done by microinjection as described [40]. The primers used for PCR amplification of genomic fragments for nob-1, pal-1, pop-1 and lag-1 were derived from Ahringer’s oligonucleotides [41] flanked with a T3 or T7 promoter at each end. The single-stranded transcript resulted from T3 and T7 RNA polymerase were pooled and annealed at 68°C for 10 minutes and 37°C for 30 minutes. The concentration for the injected dsRNA is 200 ng/µl in ddH2O. Two- or four-celled embryos were retrieved from adult worms after 16-24 hours of after injection by cutting the uterus and mounted for imaging for about six-hours.

Characterization of male tails

The number of the tail rays was counted only on one side of the male tails from both N2 (n = 57), mir-57(gk175) (n = 32) and the mir-57 promoter array containing animals (n = 38). For mir-57 animals, the ray number was counted on both room temperature and 26°C. To test the fertility of the array containing males, 10 males were put in the same plate with 10 N2 hermaphrodite animals in 3 replicates and the presence of the roller or male animals were examined for the successful crossing. The fertility of the mir-57 males was examined in a similar fashion but only the presence of male progeny was examined.

Microscopy and imaging

All of the post-embryonic images were taken with a ZEISS Axioplan 2 compound microscope equipped with AxioCamHR camera using a 63X objective lens. The fluorescent images for embryos were taken with a ZEISS LSM510 confocal microscope. Four-D imaging for lineage analysis and gene expression profiling was conducted essentially as described previously [17].

Quantification of mir-57 expression

To test the effect of the predicted mir-57 target site within the nob-1 3′ UTR on the reporter expression, the pZZ1 construct was modified so that the let-858 3′ UTR was replaced with the 3′ UTR either from nob-1a or nob-1b by the following methods. The fragment containing Pmir-37::HHS-24::mCherry was PCR amplified from pZZ1. The 3′ UTRs from nob-1a or nob-1b, termed as nob-1a or nob-1b respectively were amplified from N2 genomic DNA (See Text S1 for the primers used). The UTRs were fused with the above reporter at the 3′ end by fusion PCR. The resulting fusion products were cut with BanHI and ApaI and ligated with pZZ1 cut with the same restriction enzymes to give rise to pZZ43 (nob-1a UTR) and pZZ44 (nob-1b UTR) respectively. Sliced directed removal of a putative mir-57 binding site within the nob-1b 3′ UTR was performed in the similar way as that used for LAG-1 site-directed mutation described above and was termed as nob-1 bm hereafter. The three UTR constructs for nob-1a, nob-1b and nob-1bm were co-injected with pRF4 (rol-6d) into wild type (N2) with concentrations 20 (UTR constructs) and 100 (pRF4) µg/ul respectively. Three independent lines were obtained for each injection. The extrachromosomal arrays of the transgenic animals were crossed into the mutant mir-57(gk175) background and genotyped by single worm PCR. The three independent transgenic lines from each injection and crossing were individually synchronized by bleaching and eggs were put on unseeded plates overnight at room temperature. The hatched worms were transferred to seeded NGM plates and allowed to grow for 24 hours. The microphotographs for tail expression were taken using AxioCamHR camera using a 63X object with 919 ms exposure for each independent lines. A total of 39 animals from each independent line were used for taking micrographic photos. The RFP intensities in tail regions (measure from anus to tail end, if anus not available, measured from the end of intestine to the end of tail) were quantified using ImageJ and the data were averaged for box plotting with R package.

Quantification of nob-1 transcript

To quantify the change of nob-1 transcript in the presence and absence of -genomic mir-57, we performed Real-time RT-PCR using LightCycler (Roche) with QuantiTect SYBR Green Kit (Cat # 204143) and QuantiTect Reverse Transcription Kit (Cat # 205311). Four strains were used for total RNA extraction using RNAeasy Fibrous Tissue Mini Kit (Cat # 74704): N2, VC347(mir-57/+) into wild type (N2) background and genotyped by single worm PCR. The three independent transgenic lines from each injection and crossing were individually synchronized by bleaching and eggs were put on unseeded plates overnight at room temperature. The hatched worms were transferred to seeded NGM plates and allowed to grow for 24 hours. The microphotographs for tail expression were taken using AxioCamHR camera using a 63X object with 919 ms exposure for each independent lines. A total of 39 animals from each independent line were used for taking micrographic photos. The RFP intensities in tail regions (measure from anus to tail end, if anus not available, measured from the end of intestine to the end of tail) were quantified using ImageJ and the data were averaged for box plotting with R package.
GTGACGCAGTTCTTCTTG. Expression levels are normalized against gpd-1 before statistical analysis.

**Genetic interaction between nob-1 and mir-57**

nob-1(ct230) and mir-57(gk175) double mutant was made by crossing and genotyped by single worm PCR. RNAi by injection against nob-1 was performed as described previously for N2 and mir-57 mutant animals. Vab or Nob (data not shown) phenotypes were scored for nob-1(ct230), nob-1(ct230) and mir-57(gk175) double, nob-1(RNAi), nob-1(RNAi) and mir-57(gk175) double animals. 10 young adults were plated on a single plate for each genotypes and allowed to lay eggs for 6 hours and picked off. The phenotypes were scored for three successive days at room temperature. Each experiment was performed in three replicates.

**Supporting Information**

**Figure S1** mir-57 shows expression in a variety of cell types in the posterior sublineages. The figure is complementary to the Figure 1 but indicates the terminal cell fates of the expressing sublineages. Vertical bars denote scaled red intensity represented as arbitrary Boyle Unit.

Found at: doi:10.1371/journal.pgen.1001089.s001 (7.26 MB TIF)

**Figure S2** mir-57 expression in adult male tail. (A) DIC; (B) RFP; (C) merged.

Found at: doi:10.1371/journal.pgen.1001089.s002 (2.00 MB TIF)

**Figure S3** Adult male phenotypes associated with mir-57 overexpression. Note, mir-57 overexpressing animals develop few tail rays and produced only a few sperms (B) compared to N2 (A) animals as indicated by arrow head.

Found at: doi:10.1371/journal.pgen.1001089.s003 (1.54 MB TIF)

**Figure S4** Lineage expression of mCherry reporter driven by vab-7 and pal-1 promoter. (A) vab-7 showed expression in C lineage except for the anterior half of hypodermal sublineages, i.e., Caaa and Cpaaa. (B) pal-1 showed expression in all P2 sublineage except for germline precursor Z2 and Z4 (not traced as far as other sublineages). Note that all of the reporter-expressing cells are located within the posterior part of embryo (data not shown).

Found at: doi:10.1371/journal.pgen.1001089.s004 (1.74 MB TIF)

**Figure S5** Other posterior defects associated with injection of fusion between vab-7 promoter and mir-57 stem loop sequences. Shown are molting defects (A) and other tail abnormalities (B-D) as well as Dpy (E).

Found at: doi:10.1371/journal.pgen.1001089.s005 (4.30 MB TIF)

**Figure S6** Injection of mir-57 promoters caused earlier onset and ectopic expression (more anterior) of mir-57 in the embryo. An approximately 350 celled embryo was photographed for mir-57 expression in both wild type background animals (A-C) and those carrying the mir-57 promoter array (D-F). The array containing animals were verified by following their tail phenotypes after hatching.

Found at: doi:10.1371/journal.pgen.1001089.s006 (4.20 MB TIF)

**Figure S7** mir-57 expression is dependent on the lineage fate. RNAi against pop-1 produced homeotic lineage fate transformation, i.e., from ABplap into ABplpp (A,F) and MS into E like lineage (C, data not shown) (the later transformation serves as a reference for the RNAi effectiveness) while mir-57 expression and lineage fate remained unchanged in ABplpp (B,G). Note: mir-57 expression in ABplap becomes characteristic of that of ABplpp (A,F,G). RNAi against lag-1 transformed the posterior lineage fates of ABplap and ABplpp into those of anterior ones, i.e., ABalap (D) and ABArpp (E) respectively. mir-57 expression is abolished in both lineages.

Found at: doi:10.1371/journal.pgen.1001089.s007 (1.59 MB TIF)

**Figure S8** RNAi against pal-1 altered the mir-57 expression in C lineage. Compared to wild type (A), the treatment abolished the fate asymmetry between C derived hypodermis and body wall muscle as well as mir-57 expression (B).

Found at: doi:10.1371/journal.pgen.1001089.s008 (3.77 MB TIF)

**Figure S9** Effects of mir-57 loss of function on the NOB-1 expression. Shown are the notched boxplots of NOB-1 expression in the presence and absence of mir-57. NOB-1 expression in N2 background is shown as green and the expression in mir-57 deletion background (VC347) as red.

Found at: doi:10.1371/journal.pgen.1001089.s009 (0.81 MB TIF)

**Table S1** Phenotypes of NOB-1 overexpression.

Found at: doi:10.1371/journal.pgen.1001089.s010 (0.03 MB DOC)

**Text S1** Supporting Materials and Methods.

Found at: doi:10.1371/journal.pgen.1001089.s011 (0.04 MB DOC)

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**Author Contributions**

Conceived and designed the experiments: ZZ, RHW. Performed the experiments: ZZ, ZL, JIM. Analyzed the data: ZZ, TJB, RHW. Contributed reagents/materials/analysis tools: ZZ, TJB, ZL, JIM, WBW, RHW. Wrote the paper: ZZ, RHW.

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