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LIN-12/Notch Activation Leads to MicroRNA-Mediated Down-Regulation of Vav in *C. elegans*

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Abstract

Cell-cell interactions and cross-talk between signaling pathways specify *Caenorhabditis elegans* vulval precursor cells (VPCs) to adopt a spatial pattern: a central "1°" VPC, in which epidermal growth factor receptor (EGFR)–mitogen-activated protein kinase (MAPK) activity is high and LIN-12/Notch activity is low, flanked by two "2°" VPCs, in which LIN-12/Notch activity is high and EGFR-MAPK activity is low. Here, we identify a microRNA gene, *mir-61*, as a direct transcriptional target of LIN-12 and show that expression of *mir-61* promotes the 2° fate. We also identify *vav-1*, the ortholog of the Vav oncogene, as a target of *mir-61*, and show that down-regulation of VAV-1 promotes *lin-12* activity in specifying the 2° fate. Our results suggest that *lin-12*, *mir-61*, and *vav-1* form a feedback loop that helps maximize *lin-12* activity in the presumptive 2° VPCs.

Six multipotential VPCs, numbered P3.p to P8.p, adopt an invariant pattern of fates termed 3°-3°-2°-1°-2°-3° (Fig. 1A). Two signaling events specify this pattern: "inductive" signaling, mediated by an EGFR-Ras-MAPK pathway, and "lateral" signaling, mediated by LIN-12 (Fig. 1A) (1). The inductive signal from the gonad activates an EGFR-Ras-MAPK cascade in a graded fashion in the underlying VPCs, P5.p, P6.p, and P7.p. The centralmost VPC, P6.p, has the highest level of EGFR-Ras-MAPK activation and becomes the presumptive 1° VPC; it produces the lateral signal, which activates LIN-12 in P5.p and P7.p. When LIN-12 is activated, proteolysis releases its intracellular domain, which translocates to the nucleus and forms a transcriptional activation complex with the DNA binding protein LAG-1 (2). Transcriptional targets of LIN-12 in P5.p and P7.p can be identified by the presence of LAG-1 binding sites (LBSs) in their 5′ flanking regions and include genes that encode negative regulators of EGFR-Ras-MAPK activity in P5.p and P7.p, which inhibit the expression of 1° fate features in these cells (3).

Short regulatory microRNAs (miRNAs), first identified in *C. elegans* (4), mediate posttranscriptional down-regulation of target genes. The profound and pervasive roles that miRNAs play as critical regulators of developmental gene expression are only now becoming fully appreciated. We obtained an indication that a miRNA may be involved in

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Supporting Online Material www.sciencemag.org/cgi/content/full/1119481/DC1 Materials and Methods SOM Text Figs. S1 to S5 References and Notes

lateral signaling after observing a lateral signaling defect when a miRNA-processing gene was depleted (5). We then computationally identified *mir-61* as a potential miRNA that is transcribed when the lateral signal from P6.p activates LIN-12 in P5.p and P7.p (5). A *mir-61* transcriptional reporter is specifically expressed in P5.p and P7.p and their daughters (Fig. 1B) (6), consistent with a function for *mir-61* as a direct target of LIN-12 during lateral signaling. This inference was confirmed by mutating the LBSs in the *mir-61* promoter and finding that expression in P5.p and P7.p was lost (Fig. 1C) (7).

Ectopic expression has been a successful approach to elucidating the role of miRNAs for which null alleles are not available and obviates potential problems that may be posed by functional redundancy (8-10). Expression of *mir-61* ectopically in P6.p, the presumptive 1° VPC, causes expression of the canonical 2° fate marker *lin-11::gfp*, whereas expression of an unrelated miRNA does not (Fig. 2). These observations suggest that *mir-61* activity promotes the 2° fate.

miRNAs bind to sites in 3′ untranslated regions (UTRs) of target mRNAs and inhibit their translation (8). Thus, we hypothesized that *mir-61* is expressed in presumptive 2° VPCs in order to down-regulate potential target gene products that would interfere with specification of the 2° fate. We identified potential target genes of *mir-61* computationally (5), requiring that 3′ UTRs have at least seven bases of perfect complementarity to the 5′ end of *mir-61* and that the binding sites be conserved in *C. briggsae* orthologs. This analysis yielded three candidates, *vav-1*, *inx-1*, and *egl-46* (Fig. 3A) (5).

To assess candidate genes in vivo, we developed a simple heterologous assay to circumvent potential detection problems due to weak or transient *mir-61* expression in the VPCs. This assay may be used to test whether any miRNA can target the 3′ UTR of any candidate target gene. We expressed *mir-61* in coelomocytes, distinctive cells for which strong promoters are available (11,12). On a second transgene, we expressed two reporters in coelomocytes: one a yellow fluorescent protein (YFP) reporter with the 3′ UTR of the putative target gene and the other a cyan fluorescent protein (CFP) reporter with the *unc-54* 3′ UTR, which does not contain any *mir-61*–binding sites (13). If a candidate is a bona fide target, then we would expect to see coelomocytes displaying CFP expression and down-regulation of YFP expression. Using this assay, we obtained evidence that *mir-61* can regulate the expression of the three candidate genes identified by using the criteria described above (Fig. 3A) (5).

mir-61 is also expressed in cells other than the VPCs, and *lin-12* activity specifies many other cell fate decisions (14), so even bona fide targets of *mir-61* may not be relevant to lateral signaling. The desired target genes should be transcribed in the VPCs but posttranscriptionally down-regulated by way of their 3′ UTRs in P5.p and P7.p and their daughters. We fused the 5′ upstream sequence of *vav-1*, *inx-1*, or *egl-46* to *yfp::unc-54 3′UTR* and found that only *vav-1* is expressed in the VPCs and their daughters (Fig. 3B). When we replaced the *unc-54* 3′UTR with the *vav-1* 3′UTR, creating a sensor construct, *vav-1* expression was lost in P5.p and P7.p in a significant proportion of hermaphrodites; this loss depends on an intact *mir-61* target site (Fig. 3B). These observations indicate that *vav-1* is posttranscriptionally regulated in P5.p and P7.p, consistent with regulation by endogenous *mir-61*, and suggest that VAV-1 may be down-regulated in presumptive 2° VPCs to promote *lin-12* activity.

VAV-1 is an ortholog of the Vav oncoprotein, which has guanine nucleotide exchange factor (GEF) activity and additional domains that mediate interactions with other proteins (15); thus, there are many different potential mechanisms by which Vav proteins may modulate the activity of signaling pathways in presumptive 2° VPCs. In mammalian cells, in some contexts, Vav appears to be a positive regulator of MAPK signaling, but in others, it

has no effect (15). Loss of *vav-1* activity does not prevent vulval induction, which indicates that *vav-1* is not required for EGFR-MAPK signaling in the cellular context of VPCs (16) and that down-regulation of VAV-1 by *mir-61* may not specifically attenuate EGFR-MAPK signaling in presumptive 2° VPCs in response to the low level of inductive signal (3).

Alternatively, VAV-1 may be a negative regulator of LIN-12. If so, then down-regulation of VAV-1 by *mir-61* would increase *lin-12* activity in P5.p and P7.p, independent of any input from the inductive signaling pathway. We therefore looked at whether loss of *vav-1* activity enhances *lin-12* activity under conditions where inductive signaling does not occur. The alleles *lin-12(n379)* and *lin-12(n676)* result in mild constitutive activity: Hermaphrodites lack an anchor cell, and all VPCs generally adopt the 3° fate, as there is no inductive signal to specify a 1° fate and insufficient constitutive *lin-12* activity to promote anchor cell–independent 2° fates (14). In such backgrounds, loss of some negative regulators of LIN-12 increases LIN-12 stability or activity, which causes all six VPCs to adopt the 2° fate and to generate multiple pseudovulvae (17). Negative regulators that behave in this manner include SEL-10/Fbw7, which promotes ubiquitin-mediated turnover of LIN-12/Notch (18), and SEL-9, which functions in secretory protein quality control (19). We found that *vav-1(RNAi)* significantly enhances the constitutive activity of *lin-12(n379)* and *lin-12(n676)*, which increases the number of hermaphrodites with multiple pseudovulvae (Fig. 4A). These results suggest that *vav-1* is a negative regulator of *lin-12* activity.

We have shown that *mir-61* is a direct transcriptional target of the LIN-12/Notch pathway and that *vav-1* is a target of *mir-61* in the VPCs. We have also shown that ectopic *mir-61* promotes the 2° fate and that VAV-1 is a negative regulator of *lin-12* activity. We propose that activation of *mir-61* transcription by LIN-12 and the consequent down-regulation of VAV-1 constitute a positive-feedback loop that promotes LIN-12 activity in presumptive 2° VPCs (Fig. 4B).

Although there are many possible molecular mechanisms that may underlie this positive-feedback loop, it is notable that Vav has many domains that could couple it to receptors or to mediators of endocytosis (15). Indeed, Vav proteins have recently been shown to affect endocytosis of the activated Ephrin receptor (20). Perhaps down-regulation of VAV-1 in presumptive 2° VPCs decreases the rate of internalization, promotes endocytic recycling of LIN-12 or required proteases, or alters another aspect of trafficking that favors ectodomain shedding or transmembrane cleavage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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5. Supporting Online Material is available on *Science* Online.

6. All transcriptional reporters were made by polymerase chain reaction (PCR) fusion, from the predicted start to the next predicted upstream gene, and extrachromosomal arrays were marked with *pha-1(+)*. Expression of *mir-61* transcription was studied by using the *arIs107* integrant, which displayed the same pattern as extrachromosomal arrays.
7. *mir-61* is also expressed in cells of the somatic gonad in which LIN-12 is active, and this expression is also lost when the LBSs are mutated (Fig. 1, B and C). In contrast, expression in other tissues where we have no evidence that *lin-12* activity is functionally relevant, such as intestinal cells, is unaffected. These observations suggest that the loss of expression in P5.p and P7.p reflects lack of response to *lin-12* and not loss of a general enhancer.
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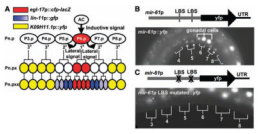

Fig. 1.

mir-61 is a direct target of LIN-12 in presumptive 2° VPCs. (**A**) An inductive signal from the anchor cell (AC) of the gonad activates EGFR-MAPK signaling primarily in P6.p, and a lateral signal from P6.p activates LIN-12 in P5.p and P7.p. The descendants of the 1° and 2° VPCs form the vulva, and the progeny of 3° VPCs fuse with the hypodermal syncytium. We used the 1° fate marker *arIs92[egl-17*p::*cfp-lacZ]* (3), the 2° fate marker *nIs106[lin-11*p::*gfp]* (21), and the 3° fate marker *arIs101[K09H11.1*p*::yfp]* (22). (**B**) *mir-61* is expressed in P5.p and P7.p. The *mir-61* promoter contains two LBSs that are conserved in the *C. briggsae* ortholog of *mir-61* (5). A reporter containing 1 kb upstream of *mir-61* fused to YFP is expressed in P5.p and P7.p (22) and their daughters (shown here). Prominent expression in cells of the gonad in which LIN-12 is active is also seen. (**C**) LBSs are required for *mir-61* expression in P5.p and P7.p. Two LBSs (YRTGRGAA) (3,23) that are conserved in *C. briggsae* were mutated to YRAGRGAA; a third nonconserved sequence, RTGGGAA, was also mutated to RAGGGAA. In three individual lines analyzed, expression of YFP in P5.p and P7.p disappeared. Expression in cells in which *lin-12* is not known to play a role in cell fate specification was normal, but gonadal expression was also abolished.

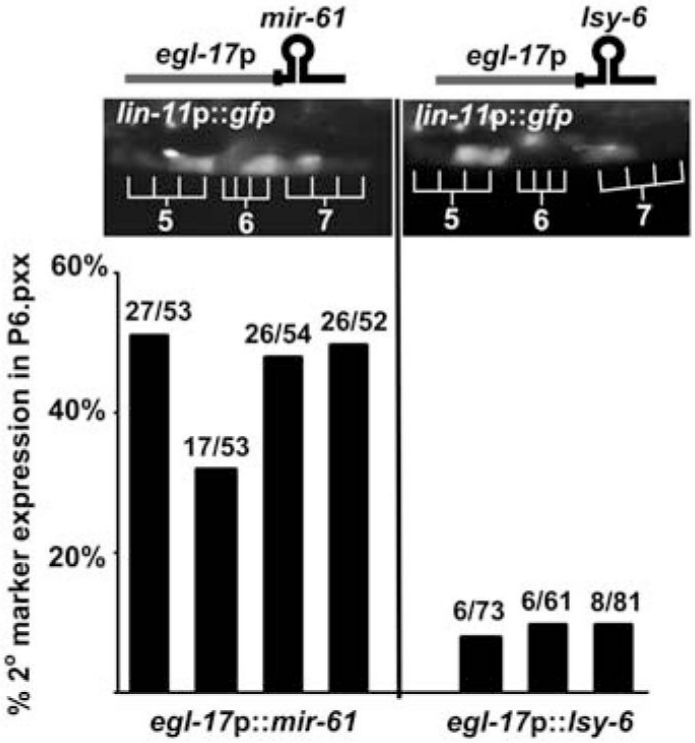

Fig. 2.

Ectopic expression of *mir-61* in P6.p confers 2° fate characteristics. Expression of the 2° fate marker *nIs106[lin-11*p::*gfp]* (see Fig. 1A) was assessed when *egl-17*p (3,24) was used for ectopic expression of *mir-61* or the unrelated miRNA *lsy-6* (25) in P6.p and its descendants. Ectopic *lin11*p*::gfp* expression in P6.p descendants was observed only when *mir-61* was expressed. Each bar represents an independent transgenic line. The number of individuals expressing the 2° marker out of the total is given above each bar.

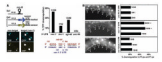

Fig. 3.

vav-1 is a target of *mir-61*. (**A**) Rapid assay to validate predicted miRNA targets. Components are expressed in coelomocytes to determine whether a miRNA causes down-regulation of YFP in a sensor construct containing a test UTR without affecting CFP in a marker construct with the neutral *unc-54* 3′ UTR (26). Here, an array carrying *unc-122*p*::mir-61* was combined with an array carrying the *unc-122*p*::cfp::unc-54 3 ′UTR* marker and *hlh-8*p*::yfp::vav-1 3 ′UTR* sensor (right) or an array carrying *hlh-8*p*::yfp::unc-54 3 ′UTR* in lieu of the sensor (left) (6). The presence of the *mir-61*–expressing array does not affect expression of YFP produced from a sensor construct that contains a nontarget UTR (in this case, the *unc-54* UTR) (triangles). In contrast, YFP expressed from a sensor construct that contains a target UTR (shown here, the *vav-1* UTR) is not seen, whereas the CFP marker shows that the array is present and expressed (arrows). The graphs indicate the percentage of worms that show the down-regulation of YFP signal. The alignment shows predicted configuration of *mir-61* (in red) binding to its target site in the 3′ UTR of *vav-1* (in blue). (**B**) VAV-1 is posttranscriptionally down-regulated in P5.p and P7.p. The 8.4-kb upstream region of *vav-1* drives expression of YFP in all VPCs and their daughters. When the *unc-54* 3′ UTR is replaced by *vav-1* 3′ UTR, down-regulation of YFP expression in P5.px and P7.px is evident. Mutation of the *mir-61*–complementary sequence in the *vav-1* 3′UTR from TAGTCA to GTCGAC causes persistent YFP expression. In the graph, each bar represents an individual line. ***P* < 0.01 by Fisher's exact test. We minimized the potential lack of expression in P5.px or P7.px due to genetic mosaicism by including data only for animals in which expression could be seen in P3.px, P4.px, and P8.px.

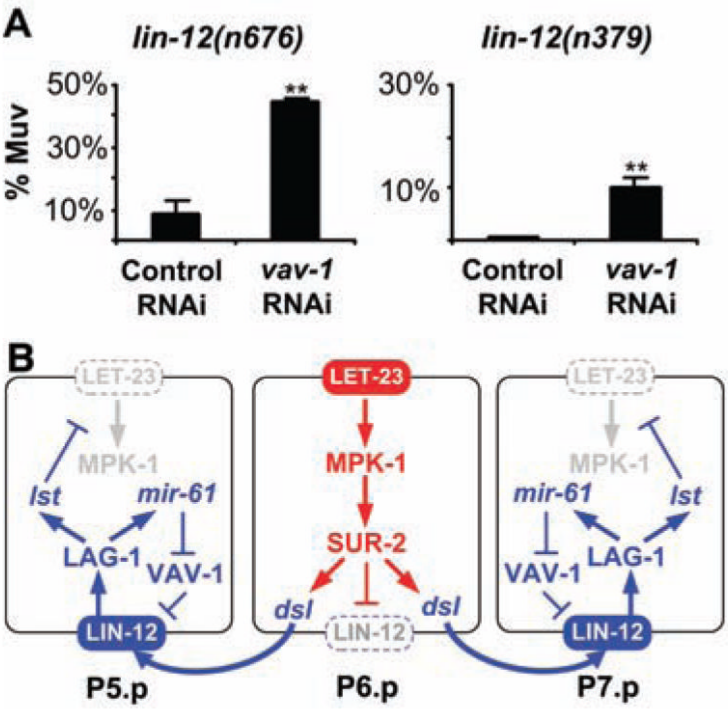

Fig. 4.

vav-1 is a negative regulator of *lin-12* activity in the VPCs. (**A**) *vav-1(RNAi)* enhances *lin-12* activity in VPCs. The average percentage of worms with a Multivulva (Muv) phenotype (three or more pseudovulvae) on three independent plates is shown. For *lin-12(n676); vav-1(RNAi)*, the number of Muv hermaphrodites out of the total per plate was 50 out of 109, 49 out of 112, and 54 out of 125, with the control *lin-12(n676); gfp(RNAi)* values of 8 out of 157, 17 out of 168, and 18 out of 152. For *lin-12(n379); vav-1(RNAi)*, the numbers were 20 out of 187, 25 out of 208, and 13 out of 167, with *lin-12(n379); gfp(RNAi*) values of 1 out of 204, 1 out of 228, and 2 out of 225. Error bar indicates SD. $^{**}P < 0.01$ by Student's *t* test. (**B**) *mir-61*, *vav-1*, and the circuitry underlying specification of the 2° VPC fate. Activation of the EGFR-MAPK pathway in P6.p has two consequences for lateral signaling: transcription of the three Delta/Serrate/LAG-2 (DSL) ligands that constitute the lateral signal, which activates LIN-12/Notch in P5.p and P7.p (27), and internalization of LIN-12, which is necessary for lateral signal activity (28). Activation of LIN-12 in P5.p and P7.p activates a set of *lst* genes that counteract the EGFR-MAPK pathway (3), and *mir-61*, which posttranscriptionally down-regulates VAV-1 to promote *lin-12* activity.