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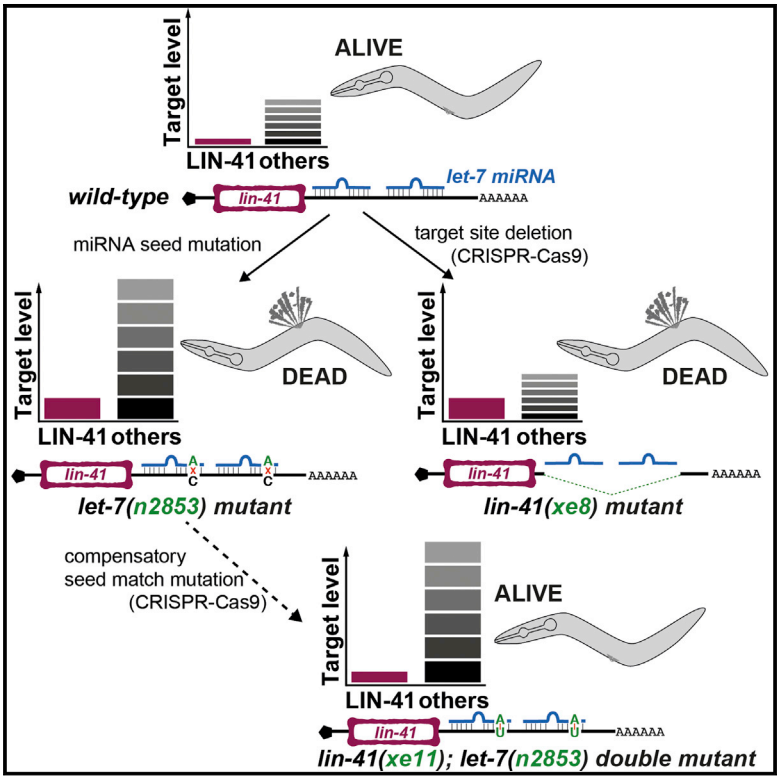
Developmental Cell

The *let-7* microRNA Directs Vulval Development through a Single Target

Graphical Abstract



Highlights

- *C. elegans* viability requires *let-7* miRNA activity in the vulval-uterine system
- This function of *let-7* is mediated by regulation of a single target: LIN-41/TRIM71
- Regulation of all other *let-7* targets, including LET-60/RAS, is dispensable
- *let-7* and LIN-41 direct vulval morphogenesis, not cell proliferation or specification

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In Brief

MicroRNAs are frequently thought to function through coordinated but modest repression of numerous targets. Using an elegant genome-editing approach, Ecsedi et al. show that the *let-7* miRNA ensures vulval integrity and *C. elegans* viability through regulation of one primary target, LIN-41/TRIM71.

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The *let-7* microRNA Directs Vulval Development through a Single Target

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SUMMARY

The *let-7* microRNA (miRNA) regulates stemness in animals ranging from worms to humans. However, the cause of the dramatic vulval rupturing phenotype of *let-7* mutant *C. elegans* has remained unknown. Consistent with the notion that miRNAs function by coordinately tuning the expression of many targets, bursting may result from joint dysregulation of several targets, possibly in the epidermis. Alternatively, overexpression of LET-60/RAS, a key vulva development gene and a phylogenetically conserved target of *let-7*, may be responsible. Here, we show that *let-7* functions in the vulval-uterine system to ensure vulval integrity but that regulation of most targets of *let-7*, including LET-60/RAS, is dispensable. Using CRISPR-Cas9 to edit endogenous *let-7* target sites, we found that regulation of LIN-41/TRIM71 alone is necessary and sufficient to prevent vulval rupturing. Hence, *let-7* does not function to reduce gene expression noise broadly, but to direct vulval development through extensive regulation of a single, defined target.

INTRODUCTION

The *lethal-7* (*let-7*) microRNA (miRNA) is essential for viability in *C. elegans*, with *let-7* mutant hermaphrodites dying by exploding through the vulva (Reinhart et al., 2000; Slack et al., 2000). Modulation of this phenotype has been used extensively and productively to identify and validate *let-7* targets, temporal patterning genes, as well as more general miRNA pathway factors (e.g., Andachi, 2008; Banerjee et al., 2010; Ding et al., 2008; Großhans et al., 2005; Hunter et al., 2013; Johnson et al., 2005; Lin et al., 2003; Parry et al., 2007; Slack et al., 2000). However, its basis has remained obscure.

Strikingly, individual depletion of several of the known targets of *let-7* suffices to prevent vulval bursting and restore viability (Andachi, 2008; Großhans et al., 2005; Hunter et al., 2013; Johnson et al., 2005; Slack et al., 2000). As miRNAs might primarily function to counter gene expression noise (Bartel, 2009; Ebert and Sharp, 2012), ensuring optimal expression levels of some genes and promoting complete repression, to inconsequential activity, of other genes, vulval rupturing thus might be a consequence of joint dysregulation of several targets.

Not only the identity and number of targets that *let-7* needs to regulate to ensure vulval integrity, but also *let-7*'s general biological function in this process remain unclear. Thus, although *let-7* miRNA functions as an ancient and fundamental regulator of stemness in animals (Büssing et al., 2008), it is not known whether and how this accounts for vulval bursting. Specifically, *C. elegans let-7* promotes differentiation and blocks proliferation of the epidermal seam cells at the transition from fourth larval (L4) to the adult stage (Reinhart et al., 2000; Slack et al., 2000). It does so, at least in part, by regulation of the TRIM-NHL (*tri*partite *m*otif-*N*CL-1, *H*T2A2, and *L*IN-41 domain) protein LIN-41/TRIM71, itself a key regulator of pluripotency and proliferation (reviewed in Ecsedi and Großhans, 2013). Genetic interactions further suggest that *let-7* functions through the transcription factor LIN-29, which may itself be a direct target of LIN-41 (Slack et al., 2000). As loss of *lin-29* expression in seam cells causes vulval rupturing (Bettinger et al., 1997), possibly by impairing attachment of the vulva to the seam, vulval rupturing of *let-7* mutant animals may similarly result from *let-7* dysfunction in the seam, rather than the vulva (Roush and Slack, 2008).

On the other hand, known targets of *let-7* include a key vulval development gene, *let-60/ras* (Großhans et al., 2005; Johnson et al., 2005), which is required for specification of vulval precursor cell (VPC) fates (Beitel et al., 1990; Han et al., 1990; Han and Sternberg, 1990). Conservation of RAS regulation by *let-7* in mammals (Johnson et al., 2005) implies a particularly important function of this small GTPase as a *let-7* target, possibly in the vulva. However, regulation has thus far only been demonstrated in seam cells (Johnson et al., 2005), and its physiological relevance is unknown for any tissue.

Here, we report that *let-7* activity in the seam alone does not suffice to ensure vulval integrity, and that *let-7* is needed in the vulval-uterine system to prevent vulval bursting. Nonetheless, VPC fates are specified correctly in the absence of *let-7*, and vulval integrity depends neither on regulation of LET-60/RAS nor broad repression of gene expression noise. Instead, it requires regulation of one *let-7* target alone, LIN-41, with uncoupling of all other targets from *let-7* being inconsequential for viability. Moreover, although both LIN-41/TRIM71 and *let-7* are known regulators of self-renewal, vulval bursting appears to be a consequence of morphogenesis, not cell proliferation defects. Our results demonstrate that genome-editing approaches can be utilized for direct and unequivocal target validation, reveal that regulation of a single target suffices to explain a major

biological function of a miRNA, and indicate that *let-7* and LIN-41 may function as a versatile regulatory module that can be integrated into distinct functional pathways.

RESULTS

Quantitative Imaging Reveals Repression of *let-60* by *let-7* in the L4 Stage

To obtain insight into potential *let-7* functions in the vulva, we sought to test if and to what extent *let-60* was regulated by *let-7*. To this end, we made use of a quantitative two-color fluorescent reporter system (Figure 1A) that we recently established and that will be described in more detail elsewhere (M.E. and H.G., unpublished data). Briefly, a ubiquitously and constitutively active *dpy-30* promoter drives expression of a destabilized nuclear GFP (GFP/PEST/H2B, green). The transgene further contains either the unregulated *unc-54* 3′UTR (yielding the gfp_unc-54 reporter) or the *let-60* 3′UTR (gfp_let-60). Integration of the transgenes in a defined genomic locus (Frøkjær-Jensen et al., 2008) and in single copy permits standardized and physiological transgene expression levels, which we surveyed in different tissues through confocal imaging. Finally, a second transgene, similarly integrated in the genome in single copy but in a distinct location, uses the same *dpy-30* promoter and an unregulated artificial 3′ UTR to express mCherry/H2B (red), permitting identification and digital segmentation of distinct cells as well as correction for biases arising in the imaging process.

The *let-60* 3′UTR was previously shown to confer *let-7*-dependent repression on a *lacZ* reporter in the epidermal seam cells (Johnson et al., 2005), and we confirmed repression of gfp_let-60 in this tissue (Figure 1B, arrow) as well as an additional epidermal compartment, the large syncytial hyp7 cell (Figure 1B, arrowhead). In both cell types, repression depended on both *let-7* and the 3′UTR, i.e., it was relieved by the *let-7*(*n2853*) loss-of-function mutation or substitution of the *let-60* 3′UTR through the *unc-54* 3′UTR (Figure 1B). To quantify the extent of silencing, we computed repression of the gfp_let-60 reporter relative to the gfp_unc-54 reporter at the L4 stage (Experimental Procedures). The results of this analysis confirmed *let-7*-dependent repression of gfp_let-60 in the epidermis (Figure 1C). By contrast, *let-7* repressed gfp_let-60 very modestly in the vulva (Figures 1B and 1C).

The extent of regulation of an mRNA may not be a good predictor of its relevance as an miRNA target if a gene is expressed at levels very close to its activity threshold (Bartel, 2009). However, as detailed below, LET-60 functions in the vulva to specify VPC fates during the L3 stage (Sternberg, 2005), and repression of gfp_let-60 was undetectable prior to the L4 stage in both the vulva and the epidermis (Figure 1C). The timing of repression is consistent with accumulation of bulk *let-7* during the L4 stage, and suggests that the dynamics of *let-7* accumulation in whole worm RNA are also representative of *let-7* accumulation in the vulva. However, it argues against a role of *let-7*-mediated repression of *let-60* in VPC specification, which occurs during the L3 stage.

let-7 Is Dispensable for VPC Specification by LET-60

Despite the use of a short-lived reporter fluorophore (Frand et al., 2005), it remained formally possible that the kinetics of repression of endogenous *let-60* differed from those revealed by the target reporter. Therefore, we examined VPC specification directly. In this process (reviewed in Sternberg, 2005), epidermal growth factor signaling from the anchor cell specifies the primary (1°) fate in its closest epidermal neighbor, P6.p, by activating LET-60 signaling. This cell then expresses an inhibitory lateral Notch signal, which suppresses LET-60 activity in the adjacent P5.p and P7.p VPCs so that these adopt the 2° fate. Conversely, elevated LET-60 activity results in ectopic induction of the 1° cell fate in P5.p and P7.p, which can be visualized through expression of the 1° cell fate marker *egl-17::cfp* (Inoue et al., 2002). Consistent with unaltered *let-60* expression in the L3 stage, *let-7* mutant animals do not exhibit any ectopic induction of the 1° cell fate in the descendants of P5.p and P7.p (Figure 1D). Indeed, these cells express a 2° cell fate reporter, *lin-11::gfp* (Gupta and Sternberg, 2002), at the same time and in the same pattern as wild-type animals, confirming their proper specification (Figure 1E). These results are reflected by proper formation of a morphologically normal vulva observed in the L4 stage (see below) and lack of vulvaless and multivulva phenotypes in *let-7* mutant animals ($n > 250$). Moreover, as we show below, uncoupling of *let-60* from *let-7*-mediated silencing fails to invoke vulva bursting. In sum, although the *let-60* 3′UTR confers some repression by *let-7* at the L4 stage, particularly in the epidermis, *let-7* and its regulation of *let-60* are dispensable for early VPC fate specification.

Loss of *let-7* Activity Leads to Vulva Morphogenesis Defects

Since VPC specification appeared unaffected in *let-7* mutant animals, we examined subsequent stages of vulva development and found the vulva of *let-7*(*n2853*) worms to be morphologically normal until the late L4 stage (Figures 2A–2C; Movie S1 available online). Specifically, the vulva includes the normal number of 22 cells forming seven ring-like structures (toroids), and the anchor cell invades the vulva as in wild-type, forming an utse (uterine-seam) cell with a thin cytoplasm over the vulva lumen ($n > 250$; Figure 2A, arrow). Vulval eversion is also executed properly, resulting in a closed, compacted vulva at the transition to adulthood. However, at a variable time point in the young-adult stage, just before bursting, the middle portion of the vulva starts protruding from the plane of the worm and an empty space between the vulva, uterus, and intestinal tube is created (Figure 2D). Subsequently, the intestine herniates through the vulva leading to the death of the animals (Movie S1).

Notably, there is neither loss of vulva toroids nor herniation between the vulva and the epidermis. Instead, the *let-7* mutant animals burst through the lumen of an apparently normal vulva. This suggests that the connection between the ventralmost vulva toroid, vulA, and the epidermis is unaffected, and AJM/mCherry, a marker of cell-cell contacts, does in fact accumulate strongly at the site between vulA and hyp7 (Figures 2B and 2C, arrowhead). We also clearly observed a connection between the dorsalmost toroid, vulF, and utse (Figures 2B and 2C, arrow). Finally, the utse cell has a wild-type morphology (Figure 2A). With much of vulva development in *let-7* mutant animals thus occurring normally, bursting appears to be a consequence of subtle defects in morphogenesis rather than gross developmental aberrations.

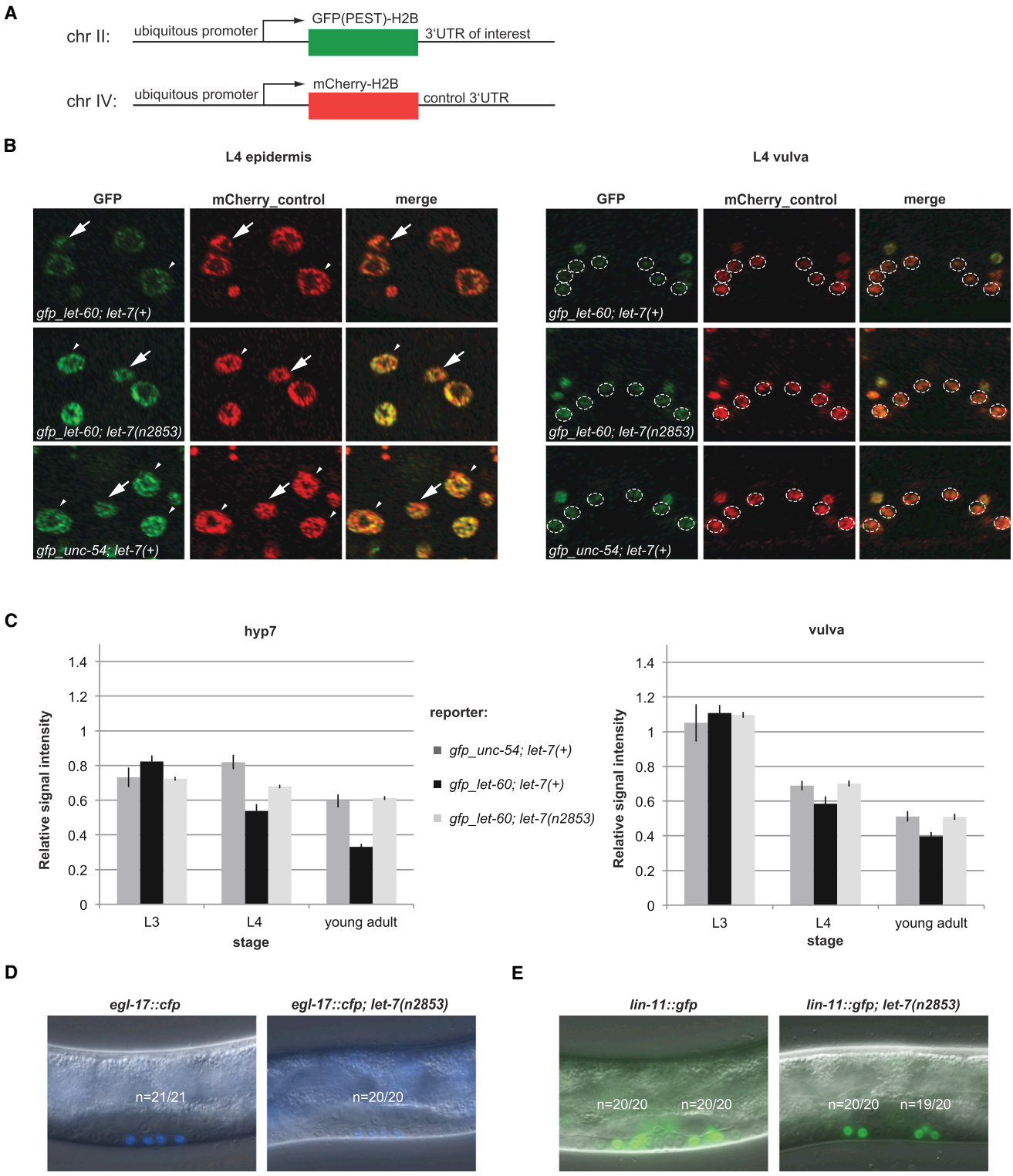


Figure 1. *let-7* and Its Regulation of *let-60* Are Dispensable for VPC Specification

(A) Schematic depiction of a dual-color miRNA target reporter system. Chr II and chr IV indicate the respective chromosomes into which the transgenes were integrated.

(B and C) Reporter assays reveal that the *let-60* 3′UTR confers *let-7*-dependent repression mostly in the epidermis (arrowhead, hyp7; arrow, seam cell; encircled, vulval cells) and from L4 stage on. The unregulated *unc-54* 3′UTR does not confer repression. Error bars (C), SEM.

(D and E) Expression of the 1° and 2° fate reporter *egl-17* and *lin-11*, respectively, is unaffected in *let-7* mutant animals. Fraction of animals with expression is indicated.

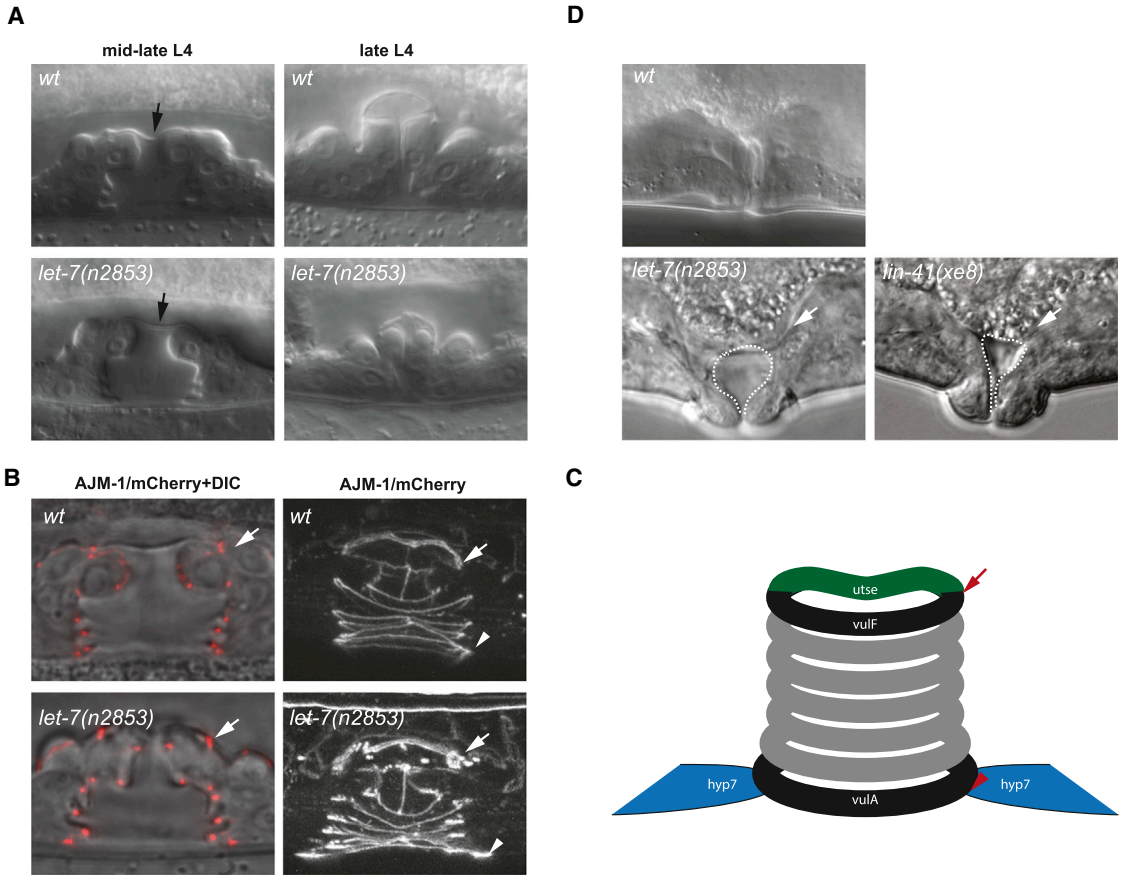


Figure 2. Loss of *let-7* Leads to Vulva Morphogenesis Defects at the Young-Adult Stage

(A) Differential interference contrast images of the developing vulva at the L4 stage show no evident abnormalities in *let-7* mutant worms. Arrows, utse cell process.

(B and C) Vulval toroids and the vulval-uterine connection are formed properly in *let-7*(*n2853*) animals. Arrowheads point to the vulA-hyp7 and arrows point to the vulF-utse connection, respectively, as (B) highlighted by AJM-1/mCherry accumulation and (C) shown in a schematic representation of an L4 stage vulva. In (C), relevant vulval toroids and nonvulval cells are indicated. For simplicity, toroids are shown as continuous rings, although they typically consist of unfused cells at this stage.

(D) Characteristic vulva defects of *let-7*(*n2853*) and *lin-41*(*xe8*) worms at the young-adult stage immediately before bursting. See Figure 4 for details on *lin-41*(*xe8*). WT, wild-type N2.

See also Movie S1.

In the seam cells, the LIN-29 transcription factor is an important, albeit indirect effector of *let-7*, which is regulated, directly or indirectly, by the *let-7* target LIN-41 (Slack et al., 2000). However, although *lin-29* is expressed in the vulva, *let-7* mutant worms do not exhibit the uterine and anchor cell defects characteristic for *lin-29* mutants. Thus, the anchor cell invades normally and fuses to form a wild-type utse in *let-7* (Figure 2A), but not *lin-29* mutant (Newman et al., 2000) worms. Additionally, the uterine π-cell fate is specified in *let-7* mutant worms just as in wild-type, as assessed by a *lin-11::gfp* reporter (data not shown). Moreover, and in contrast to the reported effect of *lin-29* loss on gene expression in the L4 vulva (Inoue et al., 2005), we could not detect any abnormality in the vulval expression of the *lin-11::gfp* or *egl-17::cfp* reporters at the L4 or young-adult stage in *let-7*(*n2853*) worms (data not shown). We conclude that the vulva defects caused by loss of *let-7* and *lin-29* are fundamentally different, suggesting that LIN-29 is not the key effector of *let-7* in the vulva.

let-7 Activity beyond the Epidermis Is Required to Prevent Vulval Bursting

Although we found the putative *let-7* promoter to be active in the vulva (Figure S1A), as previously reported by others (Esquela-Kerscher et al., 2005; Kai et al., 2013), the extensive posttranscriptional regulation known to act on miRNAs generally and *let-7* specifically (Krol et al., 2010) left open the possibility that there were only small amounts of active *let-7* in the vulva. This would explain both the modest repression of gfp_let-60 in the vulva and the incongruence of *let-7* and *lin-29* mutant vulva phenotypes. Hence, to test whether *let-7* function was entirely dispensable in the vulva, we sought to uncouple vulval and epidermal functions by expressing *let-7* from heterologous promoters in a tissue-specific manner (Figure S1). As a control, ubiquitous and constitutive expression of *pri-let-7* from the *tbb-1* promoter restored epidermal differentiation, assayed by formation of cuticular alae, and prevented bursting of *let-7*(*mn112*)-null mutants (Figure S1). By contrast, expression of

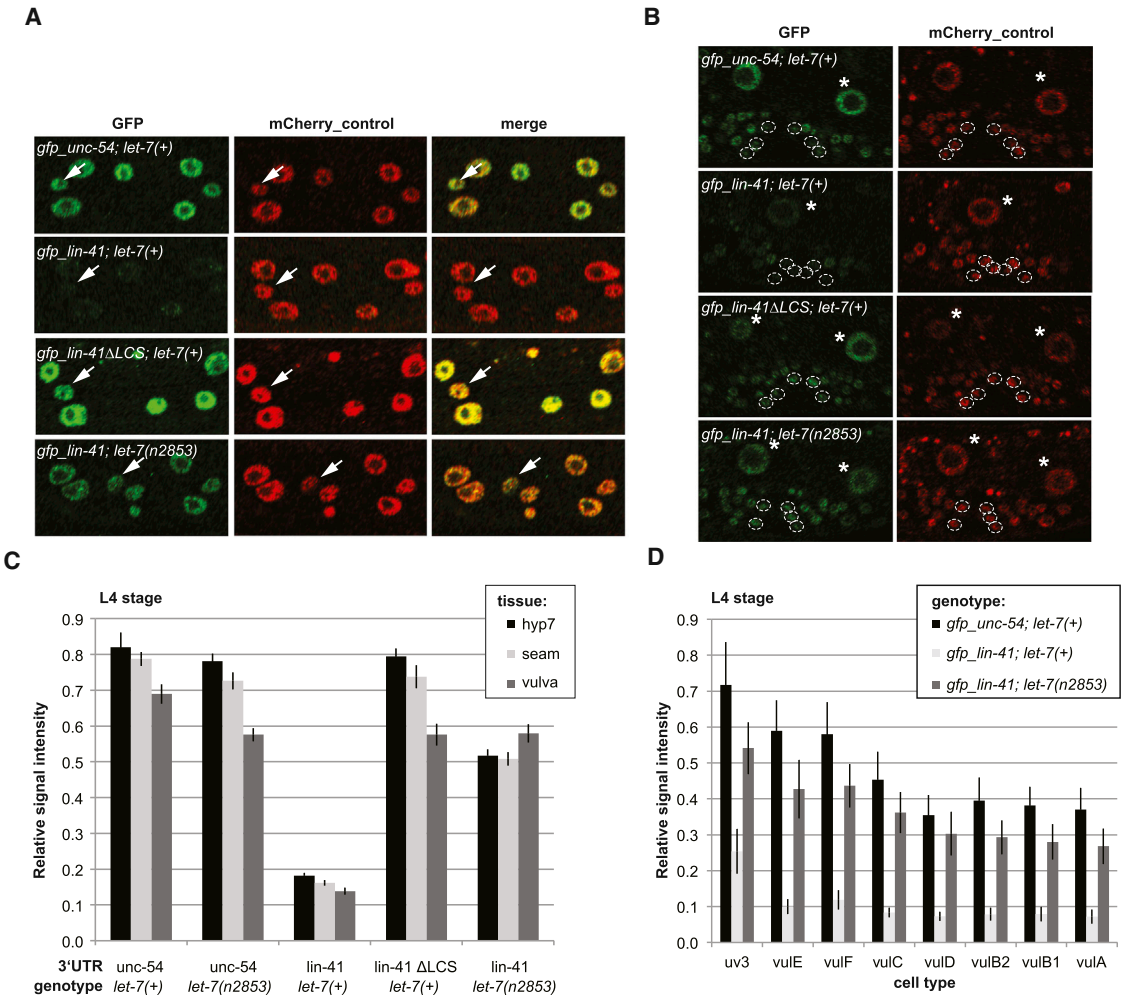


Figure 3. *let-7* Mediates Extensive Repression of *lin-41* in the Vulva

(A–C) A reporter system analogous to Figure 1A, but using a *lin-41* 3′UTR, reveals extensive *let-7* activity in the vulva; *lin-41 ΔLCS* denotes a variant lacking the two functional *let-7* complementary sites in the *lin-41* 3′UTR. In (A and B), vulval cells are encircled, arrows mark seam cells, and asterisks mark intestinal cells. Error bars (C), SEM. Data for the control *unc-54* reporter from Figure 1C is included for reference.

(D) *let-7* is active in all vulval and the uterine uv3 cells at the late L4 stage. Error bars, SEM.

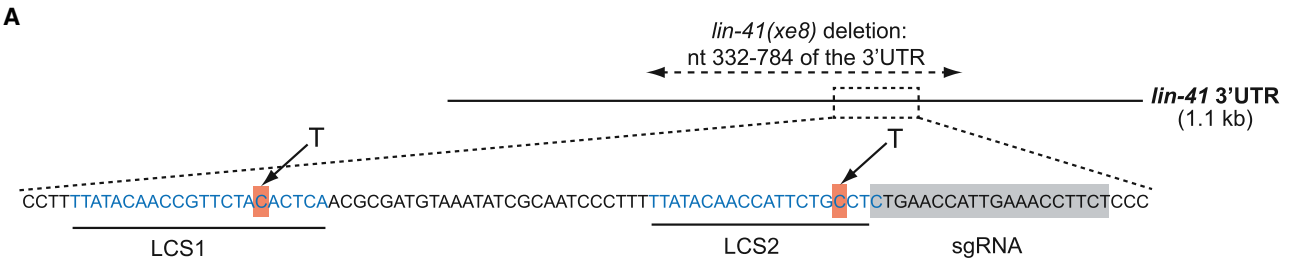
See also Figure S1.

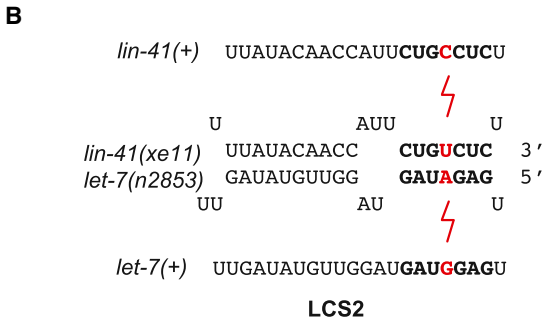
let-7 from the epidermis-specific *elt-3* promoter restored epidermal differentiation, but failed to suppress the bursting phenotype (Figure S1). Hence, *let-7* activity in other tissues, either in addition or alternatively to the epidermis, is needed to prevent vulva bursting.

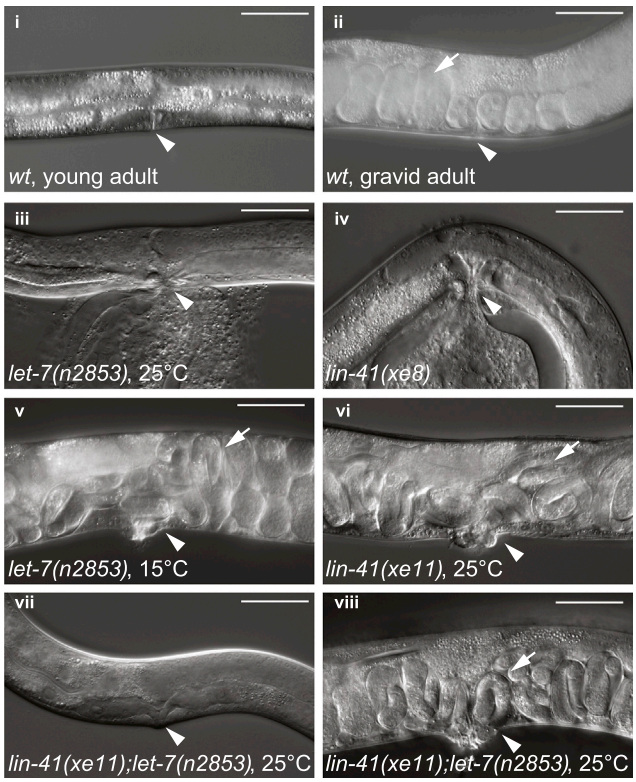
We were unable to find a promoter that drove *let-7* expression exclusively in the vulva (data not shown), either as a consequence of the shared developmental history of epidermis and vulva, or due to an epidermal enhancer element in the *pri-let-7* (Kai et al., 2013). This precluded direct demonstration that *let-7* activity in the vulva sufficed to prevent bursting. However, *let-7* expression in only the seam, uterus, and vulva from the *his-2* promoter restored both epidermal differentiation and vulva function (Figure S1). Hence, we conclude that epidermal differentiation defects are not, or not solely, responsible for vulva rupturing, and that *let-7* activity in the uterus and/or the vulva is required for vulval integrity.

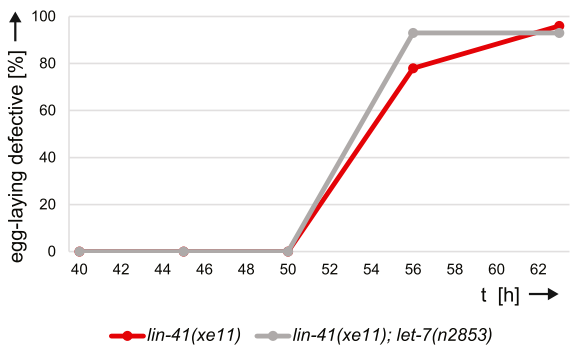
let-7 Is Highly Active against *lin-41* in the Vulva

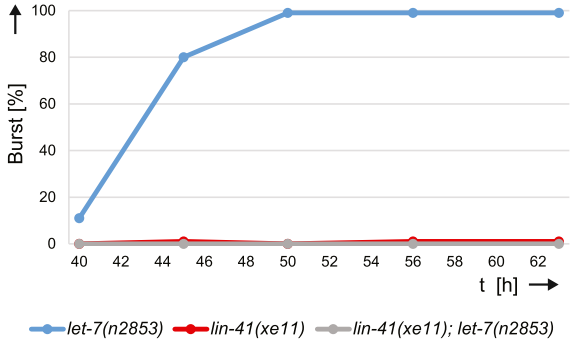
The above results suggested that *let-7* was functional in the vulva but argued against LET-60 as a relevant target. Hence, we sought to establish other targets. We focused on LIN-41 because of its important developmental functions and the fact that its regulation by *let-7* is highly conserved among animals. As expected, a gfp_lin-41 reporter was extensively (≥4-fold) silenced in the epidermis at the late L4 stage (Figures 3A and 3C). Deletion of the two functional *let-7* complementary sites (LCSs) (Vella et al., 2004) abolished this regulation (gfp_lin-41ΔLCS, Figures 3A and 3C). Extensive silencing of gfp_lin-41 also occurred in the vulva, and was again relieved for the gfp_lin-41ΔLCS reporter (Figures 3B and 3C). The *let-7(n2853)* mutation similarly desilenced gfp_lin-41. Finally, and consistent with *let-7* promoter activity, we found *let-7*-mediated repression of *lin-41* to occur in all vulval cells, as well as the uterine uv3 cell (Figure 3D). We conclude that *let-7* displays robust activity in the











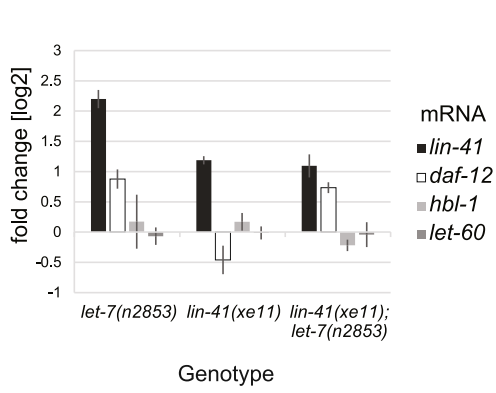


Figure 4. *lin-41* Is the Key *let-7* Target

(A and B) 3′UTR mutant *lin-41* alleles created by genome editing. (B) illustrates how gene conversion in LCS2 restores complementarity to the *let-7(n2853)* mutant miRNA. Note that *xe11* carries the corresponding double mutation in LCS1 and LCS2, restoring activity of *let-7(n2853)* to both sites; for simplicity, only LCS2 is shown.

(C) The *let-7(n2853ts)* animals are viable but egg-laying defective (Egl), causing internal hatching of progeny (Bag) when reared at 15°C; *lin-41(xe11)* seed-match point mutations cause similar Egl and Bag phenotypes at all temperatures tested. Inactivation of *let-7* by growth of *let-7(n2853ts)* at 25°C leads to vulva bursting,

(legend continued on next page)

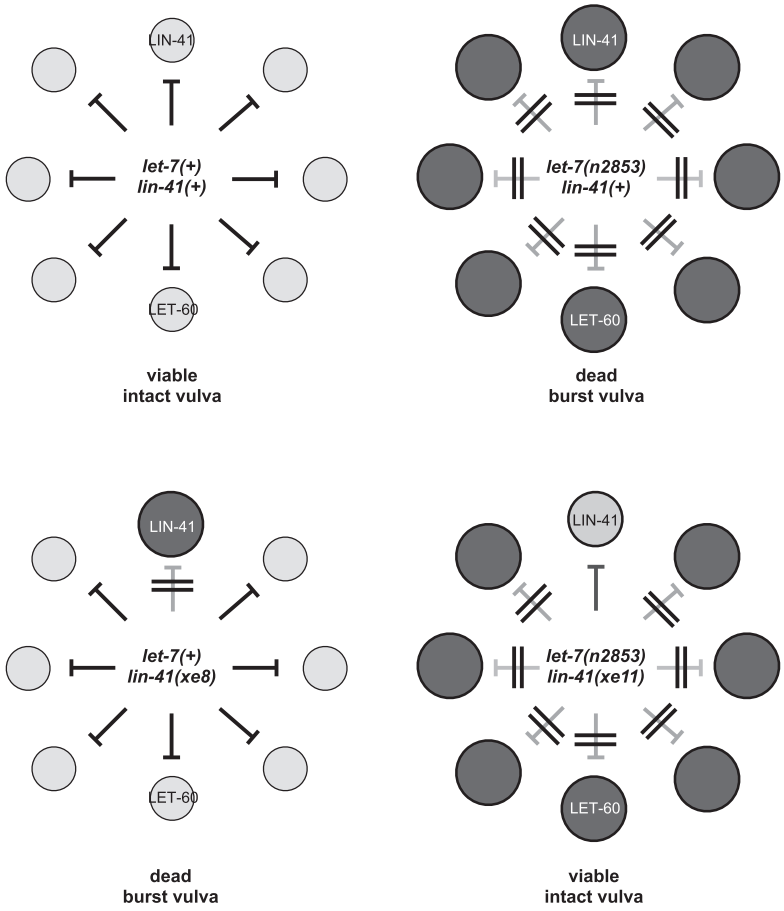


Figure 5. Schematic Depiction of the Effects of *let-7* and *lin-41* Alleles on *let-7* Target Expression and Phenotypes

Spheres represent individual targets with larger sphere size and darker shades of gray symbolizing higher expression levels. The number of actual or predicted *let-7* targets may differ and, for clarity, only LIN-41 and LET-60 are labeled. See main text for details.

vulva. Repression in this organ is likely to reflect physiological regulation of *lin-41*, because the *lin-41* promoter is active in the vulva (Slack et al., 2000) and yields GFP accumulation levels comparable to that achieved with the *dpy-30* promoter that we used to express reporter genes (data not shown).

Dysregulation of *lin-41* Is Necessary and Sufficient for *let-7* Mutant Phenotypes

To test to what extent dysregulation of *lin-41* contributed to *let-7* mutant phenotypes, we sought to uncouple *lin-41* from *let-7* regulation. We used targeted genome modification by CRISPR-Cas9 to modify the endogenous *lin-41* 3′UTR (Figure 4A). Strikingly, a partial 3′UTR deletion, *lin-41*(*xe8* [*ΔI:9,335,206:9,335,654*]), which eliminated a sequence stretch of ~450 nt from the *lin-41* 3′UTR that includes the two LCSs, sufficed to phenocopy loss of *let-7*, causing penetrant vulva bursting (Figure 4C, i–iv). By contrast, expression of a functional *let-60* transgene, which contained the unregulated *unc-54* 3′UTR in place of the *let-60* 3′UTR, failed to invoke bursting even in the presence of the two endogenous, wild-type *let-60* alleles (n = 100).

To dissect further the relevance of *lin-41* regulation by *let-7*, we made more specific mutations, introducing one C-to-U point mutation in each of the endogenous LCS1 and LCS2. Although merely replacing a canonical Watson-Crick base pair in the miRNA:target duplex with a G-U wobble (Figure 4B), this not only caused a partial derepression of *lin-41* (Figure 4F), but also sufficed to phenocopy hypomorphic *let-7* mutations: Similarly to *let-7*(*n2853ts*) animals reared at lower temperatures that are permissible for viability, *lin-41*(*xe11* [*I:C9,335,211T, I:C9,335,260T*]) displayed egg-laying defects (Egl) and subsequent internal hatching of progeny (bag of worms, Bag) (Figure 4C, v and vi). Vulval dysfunction was highly penetrant with >95% of *lin-41*(*xe11*) mutant animals exhibiting the Egl phenotype (n > 100, Figure 4D).

We introduced these specific mutations into the *lin-41*(*xe11*) strain, because they are compensatory to the G-to-A change in the seed of the *let-7*(*n2853*) mutant miRNA (Figure 4B). This permitted us to engineer a situation where all *let-7* targets except for *lin-41* were dysregulated by generating *lin-41*(*xe11*);*let-7*(*n2853*) double-mutant animals (Figure 5). Strikingly, whereas 99% of *let-7*(*n2853*) single-mutant animals succumbed to vulval bursting at 25°C, 0% of *lin-41*(*xe11*);*let-7*(*n2853*) animals did (Figure 4C, iii and vii; Figure 4E, n = 96 each). Thus, restored regulation of this single target is fully sufficient to suppress *let-7* mutant lethality (Figure 5).

Quantitative real-time PCR confirmed that *lin-41* mRNA levels are reduced in *lin-41*(*xe11*);*let-7*(*n2853*) double-mutant relative to *let-7*(*n2853*) single-mutant animals (Figure 4F). By contrast, the levels of *daf-12*, *hbl-1*, and *let-60* were comparable between the single- and double-mutant animals (Figure 4F). However, consistent with the fact that older *lin-41*(*xe11*);*let-7*(*n2853*) animals develop the Egl phenotype characteristic of *lin-41*(*xe11*) single-mutant animals (Figure 4C,viii; Figure 4D), *lin-41* mRNA levels were not completely restored to wild-type levels

as does loss of LCSs in the *lin-41* 3′UTR (*lin-41*(*xe8*)). The *lin-41*(*xe11*) point mutations suppress bursting when present in *let-7*(*n2853*) animals at 25°C. Older, gravid animals continue to exhibit Egl and Bag phenotypes. Wild-type (WT) N2 animals are shown for comparison. Arrows, embryos; arrowheads, vulvae. Scale bar, 50 μm.

(D and E) Egl and bursting phenotypes were scored for the indicated mutant animals at the indicated time of growth after hatching at 25°C. Note that *let-7*(*2853*) mutant animals are dead by 50 h and thus fail to develop an Egl phenotype. Egl phenotypes develop progressively as egg production only starts at the adult stage.

(F) Quantification by quantitative real-time PCR confirms reduced *lin-41* levels in *lin-41*(*xe11*);*let-7*(*n2853*) double- relative to *let-7*(*n2853*) single-mutant animals. Shown are the fold changes of the indicated mRNAs in the indicated mutant relative to wild-type N2 strains in late L4-stage animals (n = 3; error bars, SEM).

(Figure 4F), presumably because *let-7* miRNA levels are reduced in the *let-7*(*2853*) mutant relative to wild-type worms (Chatterjee and Großhans, 2009; Reinhart et al., 2000), and/or because the thermodynamically less favorable A-U base pair may not fully substitute for the original G-C base pair.

Taken together, these data reveal that *lin-41*(*xe11*) phenotypes are due to uncoupling from regulation by *let-7*, and demonstrate that *lin-41* is the key target of *let-7* in the vulva.

DISCUSSION

Although vulval bursting is the most prominent phenotype that *let-7* mutant worms exhibit, its basis has remained unknown. Here, we have tested and refuted two possible models, namely that vulval bursting is simply a consequence of *let-7* dysfunction in the epidermis or that it is a result of defects in VPC fate determination due to dysregulation of LET-60. Instead, we find that vulval integrity requires *let-7* activity in the vulval-uterine system and regulation of LIN-41, but not LET-60. Indeed, LIN-41 is the single key target for *let-7* in this process, with regulation of all other targets being dispensable (Figure 5).

A detailed understanding of how LIN-41 promotes vulval integrity may require further insight into the process of vulval morphogenesis itself, which is currently not well understood. However, we note that, intriguingly, the fly LIN-41 homolog *dappled/wech* has been shown to mediate muscle attachment to the body wall by linking integrins and the cytoskeleton (Löer et al., 2008). Thus, it will be interesting to determine in future research whether LIN-41 directs vulval integrity by contributing directly to structural integrity of the vulva, or whether its preferred mode of action involves posttranscriptional and/or posttranslational regulation of specific target genes (Ecsedi and Großhans, 2013). Indeed, one may speculate that it is the diverse molecular activities of LIN-41 that provide the versatility of the *let-7*/LIN-41 regulatory module, which regulates tissue integrity in the vulva (this study), but self-renewal and differentiation in the *C. elegans* epidermis as well as many other contexts (Ecsedi and Großhans, 2013; Büssing et al., 2008).

It remains well possible that targets distinct from LIN-41 could mediate other functions of *let-7*, be it in other tissues or when examining animals grown in more challenging environments. Nonetheless, that regulation of LIN-41 alone is central to *let-7*'s function in vulva development surprised us. It contrasts not only with the general notion that miRNAs typically function by coordinately regulating a large number of targets in a given cell (Bartel, 2009; Ebert and Sharp, 2012), but, more specifically, also with the fact that depletion of numerous other target genes can suppress vulval bursting of *let-7* mutants (Andachi, 2008; Großhans et al., 2005; Hunter et al., 2013; Johnson et al., 2005).

An explanation of why depletion of these *let-7*-regulated genes prevents vulval bursting is currently elusive. In one scenario, *let-7* targets might be part of a complex regulatory network where targets regulate one another in a coherent manner. Thus, depletion or overexpression of any one target would cause codepletion and co-overexpression, respectively, of all other targets. However, we found that the expression of a *let-60* transgene uncoupled from *let-7* regulation fails to yield vulval bursting. This was true even when present in addition to the two endogenous *let-60* alleles, leading to a >2-fold increase in *let-60* mRNA levels. Hence, we can rule out *let-60* as part of such a network. Moreover, the reduction of *lin-41* mRNA levels in the *lin-41*(*xe11*);*let-7(n2853)* double-mutant relative to the *let-7*(*n2853*) single-mutant animals did not lead to a codepletion of *hbl-1*, *daf-12*, or *let-60* mRNAs. Similarly, none of these mRNAs were increased in the *lin-41*(*xe11*) mutant relative to wild-type animals, despite an increase in *lin-41* mRNA levels. Indeed, further testing revealed that depletion of *let-60* and *hbl-1* mRNA by RNAi also failed to invoke a codepletion of *lin-41* mRNA (M.R. and H.G., unpublished data). Only in the case of *daf-12*(*RNAi*) did we see a decrease of *lin-41* mRNA levels, albeit to a highly variable degree (5%–87% decrease relative to a mock RNAi control; M.R. and H.G., unpublished data). Hence, although the formal possibility remains that some *let-7* targets cross-regulate one another in a coherent manner, we can exclude this as a general principle. In particular, there is no evidence for *lin-41* regulating any of the other targets.

Whereas complex cross-regulation among *let-7* targets thus appears unlikely, we note that the previous experiments that showed suppression of vulval bursting involved depletion of candidate target genes by RNAi or constitutive inactivation throughout development, almost inevitably resulting in different kinetics and/or extents of target silencing relative to the physiological regulation by *let-7*. This might put the affected cells and tissues on a different developmental trajectory, a concern that seems particularly relevant for genes such as *lin-14*, *lin-28*, or *daf-12* that are known to specify temporal cell fates.

Irrespective of the mechanisms by which knockdown of additional *let-7*-regulated genes prevents vulval bursting, our findings clearly illustrate the pitfalls of functional miRNA target validation through circumstantial evidence, and highlight the utility of genome editing to obtain more direct evidence for a physiologically relevant interaction. Indeed, by combining this approach with genetic interaction studies as we have done here, it becomes feasible to dissect the extent to which individual targets contribute to particular functions of a specific miRNA. This will then not only provide insight into the biological functions of miRNAs and their targets, but it may also facilitate the development of targeted therapeutic approaches through modulation of miRNA activity.

EXPERIMENTAL PROCEDURES

Worm Handling and Strains

Worms were grown using standard methods, and experiments were performed at 25°C unless indicated otherwise. The genotypes of the strains investigated are listed in the Supplemental Experimental Procedures.

miRNA Target Reporters

Reporter constructs were generated as described in the Supplemental Experimental Procedures and integrated in single copy in defined genomic locations via MosSCI (Frøkjær-Jensen et al., 2008, 2012). Integrant worms were outcrossed at least three times. To examine transgene expression, z stacks of 0.4 μm thickness were acquired in green, red, and transmitted light channels at 40× magnification (63× for analysis of different vulva cells) on a Zeiss LSM 700 confocal microscope coupled to Zeiss Zen 2010 software equipped with a multiposition tile scan macro (Life Imaging Centre). The z stacks were stitched together and compiled into a single image using XUVtools software (Emmenlauer et al., 2009). Worms were staged based on gonad length and vulva morphology. Cells of interest were selected in the red channel in the cell counter macro in Image Fiji. Images were segmented around these seed

points using a k-means segmentation algorithm in MATLAB (MathWorks). Signal intensity in the green channel was divided by the red signal intensity for each cell, and relative signal intensities were averaged for each tissue in each worm. Finally, the mean signal intensity per group of worms (or group of cells) and the corresponding SEM were calculated. To quantify regulation of target reporters in different tissues (Figures 1C and 3C), at least 20 worms per condition (genotype, stage) were analyzed; to quantify target reporters in different vulva cell types (Figure 3D), 30 worms per condition were analyzed.

AJM-1/mCherry Imaging and 3D Reconstruction

AJM-1/mCherry worms in wild-type and *let-7*(*n2853*) animals were imaged on a Zeiss LSM 700 confocal microscope at 63× magnification in red and transmitted light channels; z stacks of 0.4 μm thickness were acquired. Maximum intensity projections were generated using Bitplane Imaris and MATLAB software.

Time-Lapse Imaging

Worms were immobilized on a 3% agarose pad in 10 mM levamisole. Images were acquired on a Zeiss Z1 microscope with a motorized stage and coupled to ZEN blue software. Pictures were taken every 2 min in several focal planes. Pictures taken at different time points were compiled together in a movie using Image Fiji software.

Tissue-Specific *let-7* Rescue

Plasmids with a tissue-specific promoter, *let-7* rescue fragment (X chromosome: 14743506-14744528) and operon linker_gfp-h2b (Merritt et al., 2008) were recombined in a MosSCI-compatible Gateway destination vector and integrated into the *C. elegans* genome in position ttTi5605 as a single copy (Frøkjær-Jensen et al., 2008). Following backcrossing, the worm lines obtained were crossed into the *let*(*mn112*)-null mutant balanced with an extrachromosomal *let-7* rescue array, and the progeny without the array was used for experiments. See also Figure S1B.

Targeted Genome Editing using Cas9-CRISPR

Worms were injected with an injection mix containing 200 ng/μl pIK82 [*peft-3::Cas9::2xNLS::tbb-2*], a derivative of pIK86 (Katic and Großhans, 2013); 200 ng/μl *pU6::lin-41sgRNA*, a derivative of *pU6::unc-119sgRNA* (Friedland et al., 2013); 100 ng/μl *lin-41* 3′UTR repair template (*pENTR_R2-L3_lin-41*(*n2853*) 3′UTR); and 5 ng/μl pCFJ104 (*pmyo-2::mCherry*M) (Frøkjær-Jensen et al., 2008) as a coinjection marker. Single F1 worms carrying the coinjection marker were picked to individual plates. In the progeny, potential mutants were identified by vulva phenotypes, analyzed by DNA sequencing, and, upon loss of the coinjection marker, backcrossed three times.

let-60::unc-54$_{3'UTR}$

To uncouple *let-60* from regulation by *let-7*, we created a transgene, in which the *let-60* 3′UTR was replaced with that of *unc-54*, and integrated it in single copy in chromosome (chr) II (Frøkjær-Jensen et al., 2008). The transgene was functional as it was capable of restoring viability of *let-60*(*ok1932*) mutant animals. When tested in wild-type animals, i.e., in the presence of two endogenous *let-60* alleles, a 2.3-fold increase in *let-60* mRNA levels resulted as determined by quantitative real-time PCR on RNA collected from L4-stage animals (data not shown). Irrespective of the status of the endogenous *let-60* locus, presence of the transgene failed to cause the vulval rupturing phenotype characteristic of *let-7* loss of function and *lin-41* gain of function, respectively.

RNA Isolation and Quantitative Real-Time PCR

RNA was isolated from worm pellets using TRI Reagent (Molecular Research Center) following the manufacturer's instructions after a freeze-thaw process. cDNA was generated from 500 ng of total RNA per sample using ImProm-II Reverse Transcription System (Promega) and random hexamers according to the manufacturer's protocol. Quantitative real-time PCR was performed on a StepOnePlus Real-time PCR System using SYBR Green PCR Master Mix (Applied Biosystems) following the supplier's protocol in a 25 μl reaction containing 6 μl 1:480 diluted cDNA. Transcript levels of *pgk-1* or *act-1* were used for normalization. Oligonucleotide primer sequences are provided in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, one figure, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2014.12.018.

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