

## NAR Breakthrough Article

# An interplay of miRNA abundance and target site architecture determines miRNA activity and specificity

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### ABSTRACT

MicroRNAs often occur in families whose members share an identical 5' terminal 'seed' sequence. The seed is a major determinant of miRNA activity, and family members are thought to act redundantly on target mRNAs with perfect seed matches, i.e. sequences complementary to the seed. However, recently sequences outside the seed were reported to promote silencing by individual miRNA family members. Here, we examine this concept and the importance of miRNA specificity for the robustness of developmental gene control. Using the *let-7* miRNA family in *Caenorhabditis elegans*, we find that seed match imperfections can increase specificity by requiring extensive pairing outside the miRNA seed region for efficient silencing and that such specificity is needed for faithful worm development. In addition, for some target site architectures, elevated miRNA levels can compensate for a lack of complementarity outside the seed. Thus, some target sites require higher miRNA concentration for silencing than others, contrasting with a traditional binary distinction between functional and non-functional sites. We conclude that changing miRNA concentrations can alter cellular miRNA target repertoires. This diversifies possible biological outcomes of miRNA-mediated gene regulation and stresses the importance of target validation under physiological conditions to understand miRNA functions *in vivo*.

### INTRODUCTION

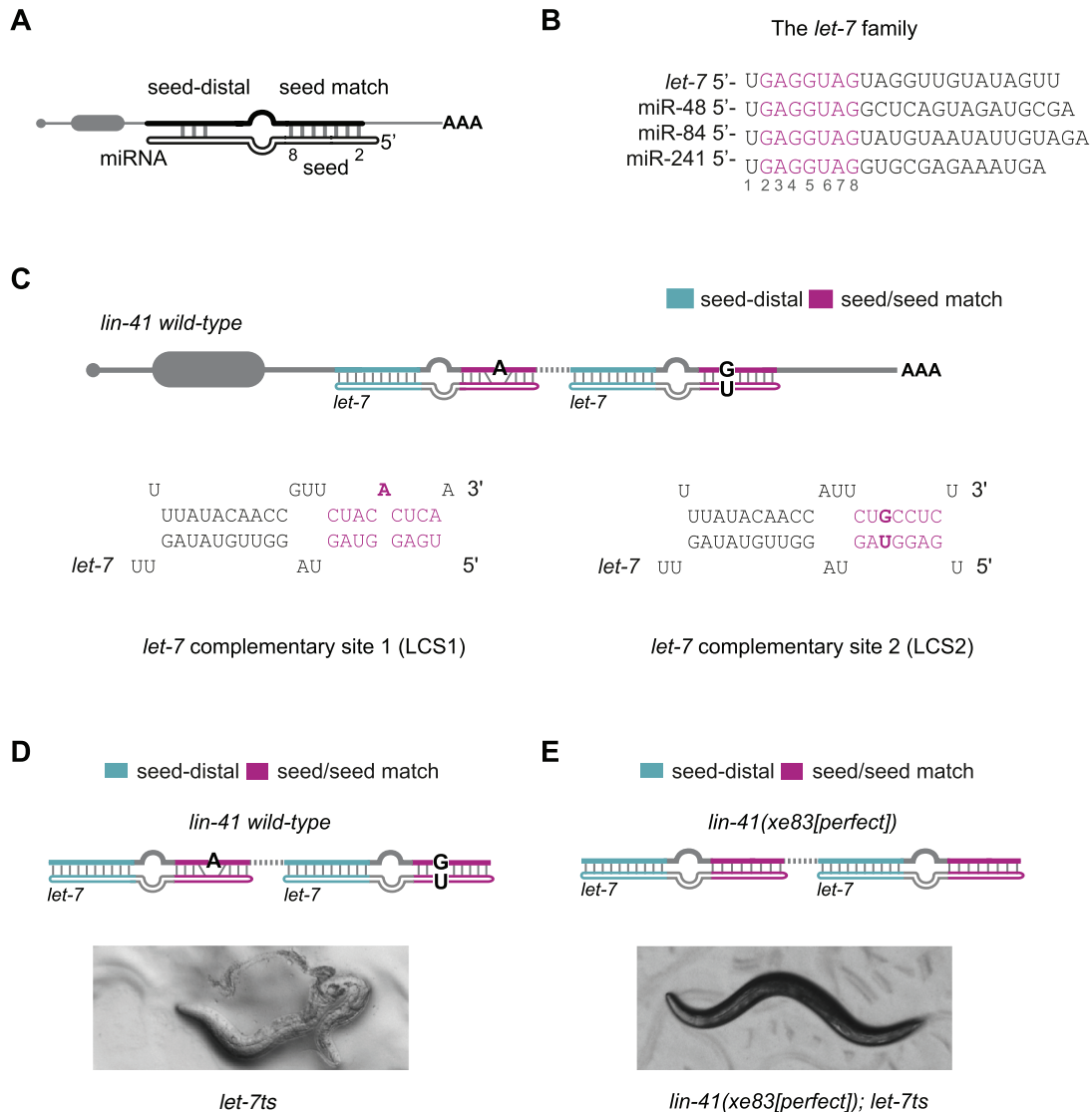
MicroRNAs (miRNAs) are small RNAs of about 22 nucleotides that silence target messenger RNAs by binding to partially complementary sequences in their 3' untranslated regions (3'UTRs). miRNAs are loaded onto an Argonaute

(Ago) protein to form the core of the miRNA-induced silencing complex (miRISC), which induces decay or translational repression of the targets (1). Conceptually, miRNAs can be separated into two parts: the 'seed', comprising nucleotides two through eight, and the 'seed-distal' 3' end (Figure 1A). The seed sequence has emerged as the main determinant for target identification (2). Usually, functional miRNA targets contain 'seed matches', heptamers that base pair with perfect Watson-Crick complementarity to the miRNA seed. These were found to be necessary and sufficient for silencing in studies using ectopic miRNA expression (3–5). Structural and biochemical analyses of miRISC have provided an explanation for these results: the seed of a miRNA bound by Ago exists in a pre-arranged conformation, thus reducing the entropic cost of binding and favoring duplex formation with a target (6–8).

miRNAs frequently occur in families that share the seed sequence but differ in the seed-distal part. Given the reliance of target silencing on seed matches, it is assumed that miRNA family members can function redundantly, and most computational approaches that predict miRNA targets make predictions for miRNA families rather than for individual miRNAs (2). Consequently, it was hypothesized that in order to attain specificity among family members, miRNAs require imperfect seed matches. In this scenario, an imperfect seed match impairs binding and activity of most family members, but extensive seed-distal base pairing would enable a specific family member to compensate for the unfavorable seed binding (3).

However, high-throughput biochemical capture of Ago-bound miRNA/target duplexes revealed numerous instances of interactions that frequently extended beyond the seed, to involve the seed-distal parts of the miRNA (9–12). In cell culture and *in vivo* assays, some of the targets that could base pair through their seed-distal parts were silenced preferentially by specific family members (9,12). Because such specificity also occurred for target sites with perfect seed matches, these findings argued that seed match im-

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**Figure 1.** *let-7* becomes dispensable for viability when the *lin-41* 3'UTR contains perfect seed match sites. (A) Schematic drawing of a miRNA/target duplex with seed (nucleotides 2–8)/seed match and limited seed-distal pairing indicated. Top mRNA, bottom miRNA. (B) The *let-7* family with the seed sequence (nucleotides 2–8) highlighted in magenta. (C) The two *let-7* complementary sites (LCS1 and LCS2) in the *lin-41* 3'UTR of *C. elegans*. Each site contains an imperfect seed match (a bulged A and a G:U wobble, respectively, in bold) to the *let-7* family and an extensive seed-distal pairing to *let-7* only. The sites are separated by 27 nt of intervening sequence (dashed line). (D, E) Representative images of animals carrying the *let-7ts* mutation and (D) wild-type *lin-41* or (E) the *lin-41(xe83[perfect])* allele with perfect seed match to the *let-7* family and unchanged seed-distal region. Animals were grown at 25°C. *let-7ts*: *let-7(n2853)* X, temperature sensitive lesion. miRNA site legend: magenta = seed/seed match; cyan = *let-7* seed-distal binding.

perfections might not be a requirement for miRNA family member specificity.

By contrast, specificity of miRNA silencing through seed mismatches would explain why members of the *let-7* family of *Caenorhabditis elegans* have partially non-redundant functions. Indeed, among four members with overlapping expression patterns (13), *let-7*, miR-48, miR-84 and miR-241 (Figure 1B), only *let-7* is essential for viability (14,15). *let-7* ensures proper development of *C. elegans* by repressing one crucial target, *lin-41* (16,17), whose 3'UTR contains two functional *let-7* binding sites (*let-7* complementary sites, LCSs) (18). Both LCSs contain imperfect seed-matches, which yield a bulged-out nucleotide and a G:U wobble base-pair respectively (Figure 1C). More-

over, both LCSs exhibit extensive complementarity to the seed-distal sequence of *let-7* but to none of its sisters. Here, we test if this miRNA site architecture ensures specific silencing by *let-7* and explore miRNA site architectures as a mechanism for the selectivity of different family members towards distinct targets.

We show that extensive seed-distal pairing favors miRNA silencing by an individual miRNA family member even when the seed match is perfect, but that an imperfect seed match greatly enhances this family member specificity. Thus, we find that perturbing *let-7*-specific regulation of *lin-41*, by introducing a perfect seed match, impairs normal *C. elegans* development through allowing the *let-7* family sisters miR-48, miR-241 and miR-84 to prematurely silence

*lin-41*. Moreover, specificity of targets with perfect or nearly perfect seed matches can be overcome through elevated levels of a miRNA that is incapable of seed-distal pairing. Hence, although sequence-instructed, specificity is not fully hard-wired and can be altered by changes in miRNA expression levels.

Our observations are consistent with a model where *let-7* family miRNAs act as rheostats (19), such that the interplay of target site architecture and miRNA abundance determine the extent of target silencing. This flexible targeting mechanism expands the regulatory potential of miRNA families and indicates that miRNA activity may differ on *bona fide* targets at a given miRNA concentration. Conversely, alterations in miRNA concentrations may then change the miRNA target repertoire, expanding the range of possible biological outcomes, and revealing a need for target validation under physiological conditions to understand miRNA function *in vivo*.

## MATERIALS AND METHODS

### Worm handling and strains

Worms were grown using standard methods at 25°C. The transgenic *unc-54 + miRNA sites* reporter strains were obtained by single-copy integration into the *tTi5605* locus on chromosome II (20). Injected plasmids were cloned using the MultiSite Gateway Technology (Thermo Fisher Scientific) and the destination vector pCFJ150 (21) or Gibson assembly (22). All strains are listed in Supplementary Table S1.

### *unc-54 + miRNA sites* reporters

All *unc-54 + miRNA sites* reporters were constructed using the MultiSite Gateway Technology (Thermo Fisher Scientific) and the destination vector pCFJ150 (21) or Gibson assembly (22). First, the pGB0 vector was obtained via site-directed mutagenesis (23) of the pDONR P2R-P3\_p37 vector to insert the AscI restriction site. Then, the pGB01 plasmid was obtained via LR reaction (Gateway LR Clonase II Enzyme mix, Thermo Fisher Scientific; 11791020) of the three entry vectors pdpy-30 x pGFP::H2B x pGB0 and the pCFJ150 backbone.

All the plasmids listed in Supplementary Table S2 were obtained via Gibson assembly of the digested pGB01 plasmid and gBlocks® Gene Fragments (Integrated DNA Technologies) listed below. All plasmids were verified by sequencing. Transgenic worms were obtained by single-copy integration into the *tTi5605* locus on chromosome II, following the published protocol for injection with low DNA concentration (20). We optimized our previous mCherry reference transgene (16) by replacing the artificial 3'UTR with an endogenous *unc-54* 3' UTR, to achieve more physiologic and brighter expression. The resulting *Pdpy-30::mCherry::H2B::unc-54* transgene was integrated on chromosome I to yield strain HW1454.

### Genome editing

Mutations in the endogenous *lin-41* 3'UTR sequence were obtained by CRISPR-Cas9 to generate the *lin-*

*41(xe83[perfect])*, *lin-41(xe76[ap427-W-C])*, and *lin-41(xe99[48-ized])* alleles. Wild-type worms were injected as described in (24) with a mix containing 50 ng/μl pIK155, 100 ng/μl of each pGB48 and *plin-41*sgRNA, 20 ng/μl repair oligo (see Supplementary Table S4), *dpy-10* co-crispr mix containing 100 ng/ml pIK208 (Addgene plasmid #65630) and 20 ng/ml AF-ZF-827 oligo PAGE purified (IDT). Single F1 roller progeny of injected wild-type worms were picked to individual plates and the F2 progeny screened for the mutated allele using PCR assays and sequencing (Supplementary Table S3). The alleles were outcrossed three times to the wild-type strain.

*let-7 over-expression*. A *let-7(++)* strain (HW 1909 [*xeSi287*, V]) was obtained by injection of the plasmid pGB26, obtained via Gibson assembly of the PCR amplified minimal rescue fragment from (15) and the pIK37 plasmid. Transgenic worms were obtained by single-copy integration into the *oxTi365* locus on chromosome V (universal MosSCI strain #EG8082 (25)).

### Reporter quantification

For confocal assays, worms were grown at 25°C. *Let-7ts* worms were maintained at 15°C and adults were transferred to 25°C for 48 h before imaging. Z-stacks of 0.313 μm μm thickness were acquired in green, red and transmitted light channels at 40× magnification on a Zeiss LSM700 confocal microscope coupled to Zeiss Zen 2010 software equipped with a multi-position tile scan macro. The z-stacks were stitched together and compiled into a single image using scripts in Matlab and Fiji (26).

For data analysis, late L4 worms were selected based on visual inspection of gonad length and vulva morphology (27). Ten to fourteen vulva cells were selected in the 'cell counter' macro in Fiji. Images around these seed points were de-noised using a Richardson-Lucy algorithm and segmented using an Otsu global threshold. Remaining holes were filled using a morphological filter. Signal intensity in the green channel was divided by the red signal intensity for each cell; relative signal intensities were then averaged for each worm. 10–12 vulva cells in 5–10 worms per genotype were quantified, mean signal intensity and SD were calculated and graphed using GraphPad Prism software.

### Confocal analysis of LIN-29 precocious accumulation

Synchronized arrested L1 larvae of animals carrying endogenously tagged LIN-29, *lin-29(xe61[lin-29::gfp::3xflag])* (28), in wild-type or *lin-41(xe83[perfect])* background, were plated on food and incubated at 25°C on 2% NGM agar plates with *Escherichia coli* OP50 bacteria and imaged at the L3 stage (20–22 h after plating). Images were acquired in green and transmitted light channels (with Differential Interference Contrast, DIC) with 40×/1.3 oil immersion objective on a Zeiss LSM700 confocal microscope coupled to Zeiss Zen 2010 software. Further image processing was performed with Fiji (26).



## RESULTS

### Perfect seed matches in the *lin-41* 3' UTR make *let-7* miRNA dispensable for animal viability

Specific regulation of *lin-41* by *let-7* and not by its sisters was previously speculated (3) to derive from the imperfect seed-matches in the two *let-7* miRNA Complementary Sites (LCS1 and LCS2) in the *lin-41* 3'UTR (Figure 1C (18,29)). When bound by *let-7* family miRNAs, the seed match sequences of LCS1 and LCS2 generate an A-bulge and a G:U wobble pair. Both sites contain seed-distal complementarity to *let-7*, but not to its sisters. However, Broughton and colleagues recently identified a target site in the 3'UTR of *dot-1.1* that appeared specific to the *let-7* family member miR-48 in the absence of seed match imperfections (9). Given this unexpected finding, we tested the possibility that seed mismatches in LCS1 and LCS2 were similarly dispensable for specific recognition by *let-7*. To this end, we generated a *lin-41* allele, *lin-41(xe83[perfect])*, which differs from the wild-type allele in two nucleotides: We eliminated the A bulge in LCS1 and converted the G:U wobble pair of LCS2 into a standard Watson-Crick base pair.

Strikingly, these two nucleotide changes rescued the larval lethality caused by loss of *let-7*, both in the *let-7(mn112)* null mutant strain and the *let-7(n2853)* temperature-sensitive strain (henceforth *let-7ts*), which recapitulates the *let-7* null phenotype at the restrictive temperature, 25°C ((15), Figure 1D and E). Thus,  $\geq 98\%$  ( $N = 3$ , each with  $n \geq 200$  animals) of *lin-41(xe83[perfect]); let-7ts* double mutant animals survived into adulthood, as did 100% ( $N = 2$ ,  $n \geq 98$  animals) of *lin-41(xe83[perfect]); let-7(mn112)* double mutant animals, of which 6% subsequently died as adults. These findings suggest that seed mismatches are required to restrict silencing of *lin-41* to *let-7*, because other *let-7* family members confer silencing in their absence.

### A perfect seed match allows redundant activity of the *let-7* sisters

To confirm that the perfect seed matches of the *lin-41(xe83[perfect])* allele allow redundant binding of the *let-7* family, we monitored the activity of the four miRNAs through a GFP reporter modified from (16) (Materials and Methods). In our assay, each animal contains a red mCherry reporter, which is used as reference during image analysis, and a GFP reporter, which is the miRNA activity sensor (Figure 2A). Both reporters are driven by the ubiquitous and constitutively active *dpy-30* promoter and contain the *unc-54* 3'UTR, generally thought to be devoid of regulatory elements. Finally, each reporter is integrated by Mos1-mediated single copy integration into a distinct genomic location (20).

To monitor *let-7* activity, we generated the reporter '*unc-54 + let-7 sites*' in which only a stretch of 111 nucleotides of the *lin-41* 3'UTR, comprising LCS1 and LCS2, was transplanted into the *unc-54* 3'UTR (Figure 2A). Silencing of this minimal target reporter by *let-7* was comparable to that of a reporter containing the full-length *lin-41* 3'UTR (Figure 2B, C and Supplementary Figure S1A), confirming functionality. We focused our analysis on the vulva because *lin-*

41 repression by *let-7* in this organ is required and likely sufficient to prevent vulval rupturing (16).

As expected, the '*unc-54 + let-7 sites*' reporter was expressed in young L1 or L2 animals (Supplementary Figure S1B), when the *let-7* family levels are low (30). Moreover, it was robustly silenced in older, L4-stage larvae, when *let-7* family levels are high (Figure 2B and C). Finally, it was de-silenced in *let-7ts* animals, but not in animals lacking the three *let-7* sisters (*[mir-48/mir-241(ndf51)V, mir-84(n4037)X]*, henceforth *mir-48/241/84(-)*) (Figure 2C). Therefore, the stretch of 111 nucleotides suffices for efficient and specific *let-7*-dependent silencing.

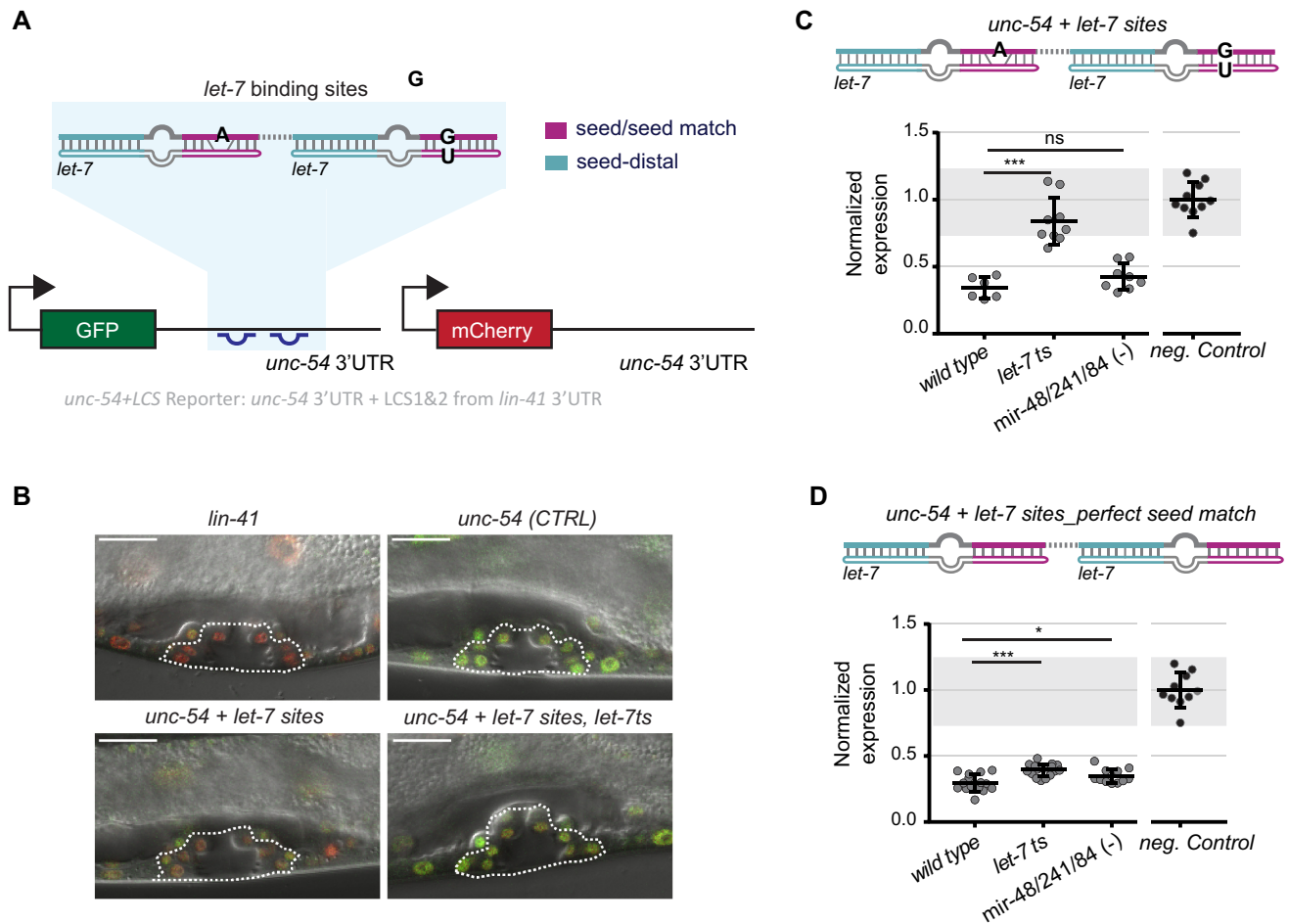
Next, we generated an '*unc-54 + let-7 sites\_perfect seed match*' reporter, modified to contain LCSs with perfect seed matches, as in the endogenous *lin-41(xe83[perfect])* mutation (Figure 1E). Like the '*unc-54 + let-7 sites*' reporter, the new reporter was expressed in young L1 or L2 animals (Supplementary Figure S1B), but robustly silenced in L4-stage larvae (Figure 2D). However, unlike the '*unc-54 + let-7 sites*' reporter, the new reporter was only marginally de-repressed in L4-stage larvae lacking *let-7* (*let-7ts*) or the three *let-7* sisters (*mir-48/241/84(-)*) (Figure 2D).

### A seed-distal match establishes specificity to one miRNA in the presence of an imperfect seed match

Taken together, the genetic interaction and the reporter assay data presented thus far validate the hypothesis that the seed mismatches in the *let-7* complementarity sites of *lin-41* are necessary for specific regulation of *lin-41* by *let-7*, to the apparent exclusion of the other family members. However, this conclusion appears at odds with the results of biochemical miRNA-mRNA duplex identification, which indicate preferential target binding by individual family members even in the presence of perfect seed matches (9,12). Thus, to challenge our finding, we sought to reprogram the LCSs to another *let-7* family member, miR-48, and test the effect of seed match imperfections. We chose miR-48 because its expression levels and spatial expression patterns appear very similar to those of *let-7* (13,14,31).

Because structural data suggest that base pairing between nucleotides 13–16 of the miRNA and a target may be favored (8), we started out by generating a reporter with seed-distal base pairing to only these nucleotides. However, this reporter failed to be silenced even in wild-type conditions, i.e. with both *let-7* and miR-48 present (Figure 3A and Supplementary Figure S2A). Hence, it appears that more extensive seed distal complementarity is required for functionality of targets with a sub-optimal seed match. Indeed, an '*unc-54 + miR-48 sites*' reporter that emulated the LCS architecture by carrying a central bulge in the seed sequence and an extensive seed distal match to miR-48 (Supplementary Figure S2B), was silenced in L4 stage animals. Moreover, and in agreement with our predictions, the '*unc-54 + miR-48 sites*' reporter was repressed at the L4 stage in both the presence and absence of *let-7* miRNA, but became de-repressed when miR-48 was absent (Figure 3B).

Consistent with our results for the *let-7* reporters, the specificity of the '*unc-54 + miR-48 sites*' reporter was largely lost when we modified it to contain perfect seed matches: the resulting '*unc-54 + miR-48 sites\_perfect seed match*' reporter



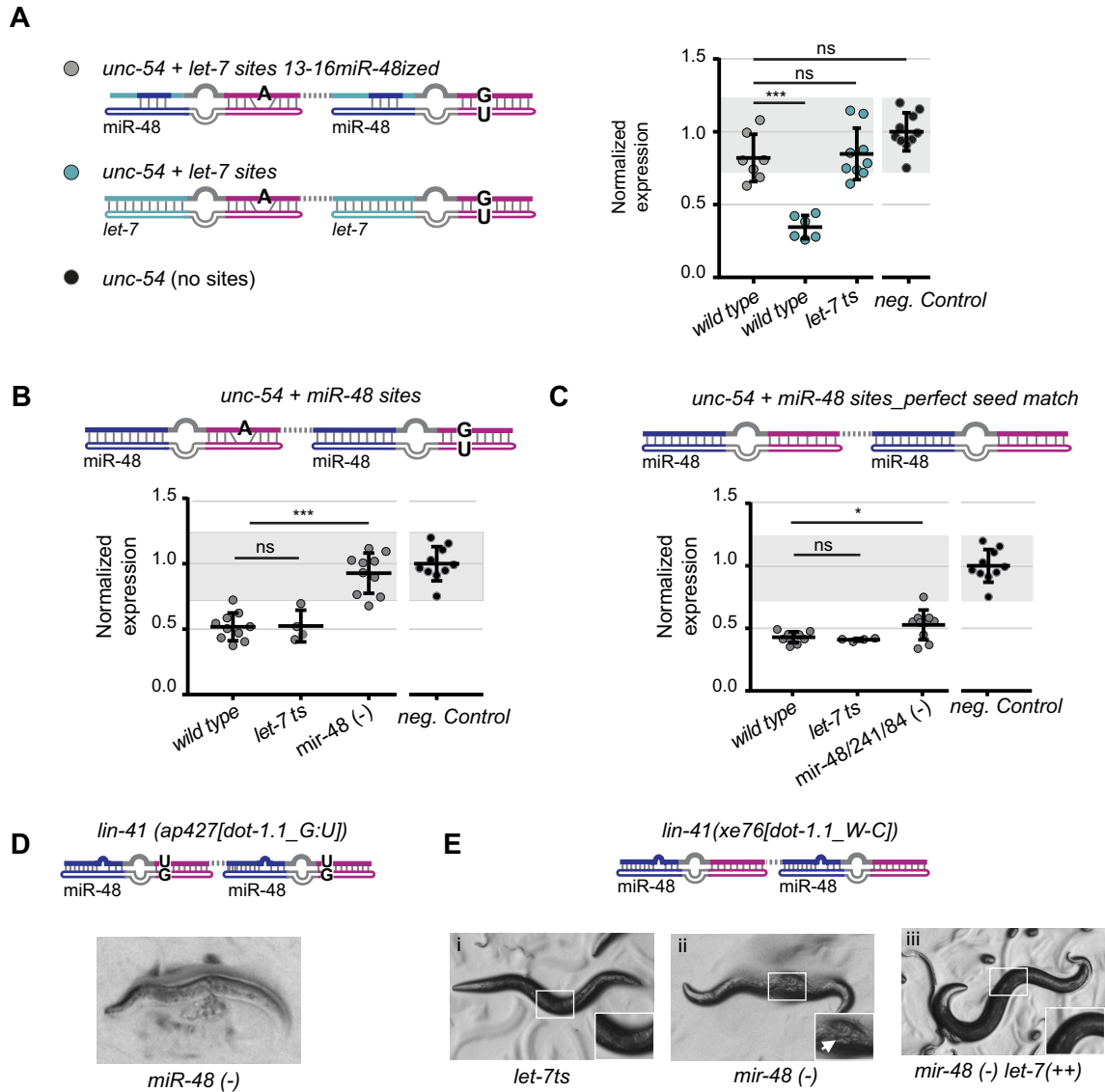
**Figure 2.** Redundant activity of the *let-7* family in the presence of a perfect seed match. (A) Schematic of the reporters used to monitor miRNA activity *in vivo*. The depicted GFP transgene *unc-54 + let-7 sites* reporter contains 111 nucleotides of the *lin-41* 3'UTR (shaded in blue), which harbor the two *let-7* binding sites and the 27 nt-long intervening sequence, grafted into the heterologous, unregulated *unc-54* 3'UTR. Worms also contain a red mCherry reporter for normalization. Transcription of the single-copy integrated reporters from the ubiquitously active *dpy-30* promoter is constitutive. miRNA site legend: magenta = seed/seed match; cyan = *let-7* seed-distal binding. (B) Representative confocal images of the vulvae of animals carrying the red mCherry reporter (for normalization) and GFP reporters with the indicated 3'UTRs. These are '*lin-41* 3'UTR full-length', '*unc-54*' (CTRL, unregulated) and '*unc-54 + let-7 sites*' in wild-type and '*unc-54 + let-7 sites*' in the *let-7ts* background. Images are merged GFP, mCherry and DIC channels. Red color indicates a greater, and green color a lesser degree of reporter repression. Dashed lines outline the vulvae of the animals, which confirm appropriate late Larval stage 4 (L4). Scale bars 15  $\mu$ m. (C, D) Quantification of (C) '*unc-54 + let-7 sites*' reporter, (D) '*unc-54 + let-7 sites\_perfect seed match*' reporter. Each dot represents the average of the GFP signal intensity, obtained by confocal imaging, divided by the mCherry intensity for a single animal per condition. 10–12 vulva cells were quantified per worm. Mean values are normalized to the average value of the GFP/mCherry ratio of the negative control *unc-54* 3'UTR reporter, which is not silenced. Horizontal line and error bars indicate mean values per condition  $\pm$  SD. \* $P < 0.05$  and \*\*\* $P < 0.001$ , two-tailed unpaired t-test. For reference, data obtained for the *unc-54*, Neg. Control reporter are replotted in panel D; gray shading is bounded by the min-max values of this control.

continued to be silenced extensively in both *let7ts* and *mir-48/241/84(-)* animals (Figure 3C). However, silencing appeared marginally impaired in the absence of the *let-7* sisters (Figure 3C), mirroring an analogous result for the '*unc-54 + let-7sites\_perfect seed match*' reporter in *let-7ts* animals (Figure 2D). We conclude that the imperfect seed match and the extensive 3' pairing are both important determinants for the robust target specificity of the *lin-41* sites.

#### A G:U wobble base-pair in a peripheral seed match location promotes miRNA specificity

The duplexes formed between *let-7* and *lin-41* contain a bulge between nucleotides 4–5 in LCS1 and a G:U wobble base-pair at position 6 in LCS2 (Figure 1C). We won-

dered if such centrally located 'imperfections' were required for specificity. We turned to the miRNA binding site in the *dot-1.1* 3'UTR, which had been shown to be specific to miR-48 (9). Broughton *et al.* found that substitution of the *let-7* complementary sites in the endogenous *lin-41* 3'UTR by two copies of the *dot-1.1* site rendered animals insensitive to loss of *let-7* (9), but made them depend on the presence of miR-48. This finding was attributed to the fact that the site features an extensive seed-distal match to miR-48 (Figure 3D and Supplementary Figure S2C). However, we noticed that the *let-7* family/*dot-1.1* predicted duplexes exhibited not only perfect Watson–Crick pairing from nucleotides 2–7, but also a G:U wobble pair at position 8 (Supplementary Figure S2C). Although hexameric seed match



**Figure 3.** Imperfect seed matches and extensive 3' pairing confer target specificity. (A–C) Reporter quantification as in Figure 2, from which the negative control data (black dots) are also replotted for reference; gray shading is bounded by the min-max values of the negative control. (A) The '*unc-54 + let-7 sites 13–16miR-48ized*' reporter contains *let-7* complementary sites modified to pair miR-48 at position 13–16 but not other seed-distal nucleotides (gray dots). Results from the unmodified '*unc-54 + let-7 sites*' reporter in wild-type and *let-7ts* mutant background are from Figure 2C and included for reference (cyan dots). (B) The '*unc-54 + miR-48 sites*' reporter combines extensive seed-distal complementarity to miR-48 with seed match imperfections whereas (C) the '*unc-54 + miR-48 sites\_perfect seed match*' reporter contains extensive seed-distal complementarity to miR-48 and perfect seed matches. Horizontal line and error bars indicate mean values per condition  $\pm$  SD. \* $P < 0.05$  and \*\*\* $P < 0.001$ , two-tailed unpaired *t*-test. (D) Animals carrying the *lin-11 (ap427[dot-1.1\_G:U])* allele die in the absence of miR-48. (E) Survival of strain *lin-11(xe76[dot-1.1\_W-C])* upon manipulation of *let-7* and miR-48 activity. In this strain, a U at position 8 in the two target sites of the *lin-11 (ap427[dot-1.1\_G:U])* allele has been converted to a C, to permit Watson-Crick instead of G:U wobble base-pairing with the *let-7* family seed sequence (Supplementary Figure S2C and D). This allele was crossed into a (i) *let-7ts*, (ii) *mir-48(-)* or (iii) *mir-48(-) let-7(++)* background, where *let-7(++)* denotes *let-7* overexpression from a single copy integrated transgene. Insets magnify the central part of the animal body to reveal egg retention (arrow), i.e. and egg-laying defective (Egl) phenotype. *let-7ts*: *let-7(n2853)* X, temperature-sensitive lesion, grown at the restrictive temperature 25°C; *mir-48(-)*: *mir-48(n4097)* V; *mir-48/241/84(-)*: *mir-48/mir-241(ndf51)* V, *mir-84(n4037)* X.

sites, with complementarity to nucleotides 2–7, are considered canonical and functional (2), genome-wide studies also suggested that they are less functional than heptameric sites that match nucleotides 2–8 (6,32,33). Since G:U wobble base pairs elsewhere in seed-seed match duplexes appear detrimental to silencing (3,4,34–36), we wondered if this 'peripheral G:U' in seed match position 8 might affect silencing and specificity.

To test this hypothesis, we modified the endogenous target sites in *lin-11* to those of *dot-1.1*, but with the G:U wobbles at positions 8 converted to Watson-Crick G:C pairs, yielding allele *lin-11(xe76[dot-1.1\_W-C])* (Supplementary Figure S2D). We then compared the reliance of this and the *lin-11 (ap427[dot-1.1\_G:U])* strain, which carried the unmodified G:U-wobble-containing *dot-1.1* sites, on *let-7* and miR-48 for survival. Whereas both strains



were insensitive to loss of *let-7* (Figure 3E(i) and (9)), *lin-41(ap427[dot-1.1.G:U])* but not *lin-41(xe76[dot-1.1.W-C])* required miR-48 for survival into adulthood (Figure 3D and E(ii)). We conclude that the G:U wobble at position eight repels binding by all *let-7* family members such that only miR-48 can exert repression by compensating through extensive complementarity of its 3' seed-distal sequence. Collectively, our data thus reveal that bulges or wobbles in different positions of a seed match can serve to avoid redundancy of the *let-7* family and confer strong target specificity.

### miRNA abundance affects silencing *in vivo*

Although our experiments provided strong evidence that seed mismatches are required for robust specificity among *let-7* family members, we consistently observed evidence of residual specificity even for targets that contained a perfect seed match. In target reporters containing perfect seed matches, we observed modest but reproducible de-silencing specifically when the family member with seed-distal match was lost (Figures 2D and 3C), and phenotype (Figure 3E(ii)). In fact, although *lin-41(xe76[dot-1.1.W-C]); mir-48(-)* animals survived into adulthood, they exhibited an egg-laying (Egl) defect (Figure 3E(ii)), 93%,  $n = 132$ , i.e. a partial vulval dysfunction that is consistent with incomplete repression of *lin-41* (16).

We wondered if this partial specificity could be overridden by increased levels of another miRNA family member. Since we were unable to overexpress *mir-48*, we tested this possibility by overexpressing *let-7*. *Mos1*-mediated single copy integration (25) of a genomic fragment, known to rescue *let-7* lethality (15), to a locus on chromosome V that is ~5 cM apart from *mir-48*, yielded a ~2-fold increase in expression levels (data not shown). Consistent with our hypothesis, *lin-41(xe76[dot-1.1.W-C])* animals that overexpressed *let-7* were no longer Egl in the absence of miR-48 (Figure 3E(iii), compare to E(ii)). We conclude that, *in vivo*, increased miRNA levels can override the specificity imparted by seed-distal pairing.

### Seed match imperfections maintain specificity upon miRNA overexpression

Since the modest preferential silencing imposed by the seed-distal pairing to miR-48 could be overcome by increasing the levels of *let-7* in the presence of a perfect seed match (Figure 3E(ii) and (iii)), we wondered about the effect of *let-7* over-expression on sites with more extensive target specificity. Hence, we examined two reporters specific to miR-48 that harbored imperfect seed matches: the previous '*unc-54 + miR-48 sites*' (Figures 3B and 4A) and the new '*unc-54 + dot-1.1 sites*' reporter, obtained by inserting two copies of the binding sites from the *dot-1.1* 3'UTR (Figure 4B). Consistent with the *in vivo* data ((9) and Figure 3E), silencing of both reporters was dependent on miR-48 but not *let-7* (Figures 3B, 4A, B and Supplementary Figure S2E). However, the response of the two reporters differed when we overexpressed *let-7* in the absence of miR-48. The '*unc-54 + miR-48 sites*' reporter, with central seed mismatches, was insensitive to a doubling of *let-7* expression (Figure 4A). By contrast, silencing of the '*unc-54 + dot-1.1 sites*' reporter, with

peripheral seed mismatches, was restored to almost wild-type level in the same conditions (Figure 4B). This suggests that for miR-48 targets with extensive seed-distal pairing, sensitivity to *let-7* levels depends on seed match quality.

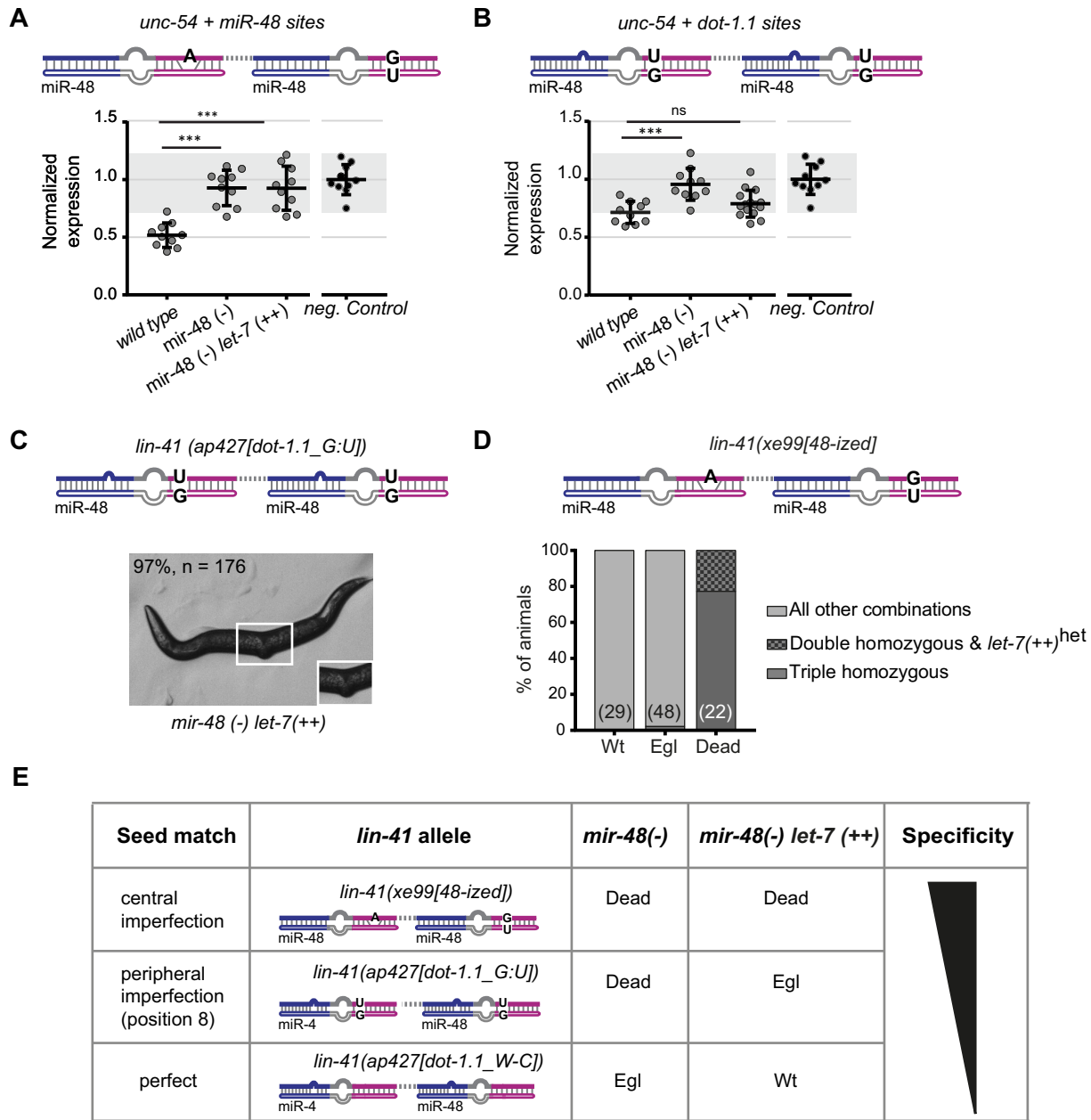
To confirm this result on a functional level, we tested whether *let-7* overexpression could suppress the dependence on miR-48 of animals carrying *lin-41* alleles analogous to those in the miR-48-specific reporters, namely the *lin-41(ap427[dot-1.1.G:U])* allele and the newly generated *lin-41(xe99[48-ized])* allele (Figure 4C and D, respectively). As predicted by the reporter assay, overexpression of *let-7* rendered *lin-41(ap427[dot-1.1.G:U]); mir-48(-)* double mutant animals viable, although Egl (Figure 4C). By contrast, we were unable to obtain viable animals of the *lin-41(xe99[48-ized])I; mir-48(-) let-7(++) V* genotype (Figure 4D). Instead, we readily observed dead animals, which had burst through the vulva. Genotyping revealed that such animals were homozygous for the three alleles of interest, *lin-41(xe99[48-ized])*, *mir-48(-)*, and *let-7(++)* (Figure 4D). [Note that *mir-48(-)* and *let-7(++)* are closely linked loci on chromosome V, explaining why we did not find dead animals that were *lin-41(xe99[48-ized]); mir-48(-)* double mutant but lacked the *let-7* over-expression transgene.] In contrast, randomly selected wild-type animals were never doubly homozygous for *lin-41(xe99[48-ized])* and *mir-48(-)*, irrespective of *let-7* transgene status, and only one Egl animal was found to be *lin-41(xe99[48-ized]); mir-48(-) let-7(++)* mutant. Hence, although an increase in *let-7* levels can overcome the specificity to miR-48 imposed by seed-distal matches in combination with a perfect seed (Figure 3E) or in the presence of peripheral seed mismatches (Figure 4C), it cannot do so with a central seed bulge or wobble (Figure 4D), at least within the physiological ranges of the expression levels that we tested.

We conclude that specificity arises through seed-distal pairing of a miRNA, but that it is enhanced in extent and robustness by appropriate seed match architecture (Figure 4E).

### Loss of miRNA specificity impairs robust development

Our results suggest that sites with central seed match imperfections, such as LCS1 and LCS2 in the *lin-41* 3'UTR, are extremely specific to one miRNA, even when a paralogue is highly expressed. We suspected that such robust specificity would be physiologically relevant in the case of *lin-41*. This is because the *let-7* sisters are all expressed prior to *let-7*, in the L2 stage (30). Given their overlapping spatial expression patterns, lack of mechanisms to prevent *let-7* sisters' action on *lin-41* might cause inappropriately early repression of *lin-41*, as speculated previously (2,3). Consistent with this notion, we found that the '*unc-54 + let-7 sites.perfect seed match*' reporter was precociously repressed during the L3 stage, whereas the '*unc-54 + let-7 sites*' reporter was still expressed at the same stage (Figure 5A).

To test whether this precocious repression of *lin-41* had physiological consequences, we examined the accumulation of LIN-29A, a target of LIN-41. In wild-type animals, LIN-41 translationally represses LIN-29A until the L4 stage, when repression is released following *let-7* accumulation and consequent LIN-41 downregulation (28). Premature

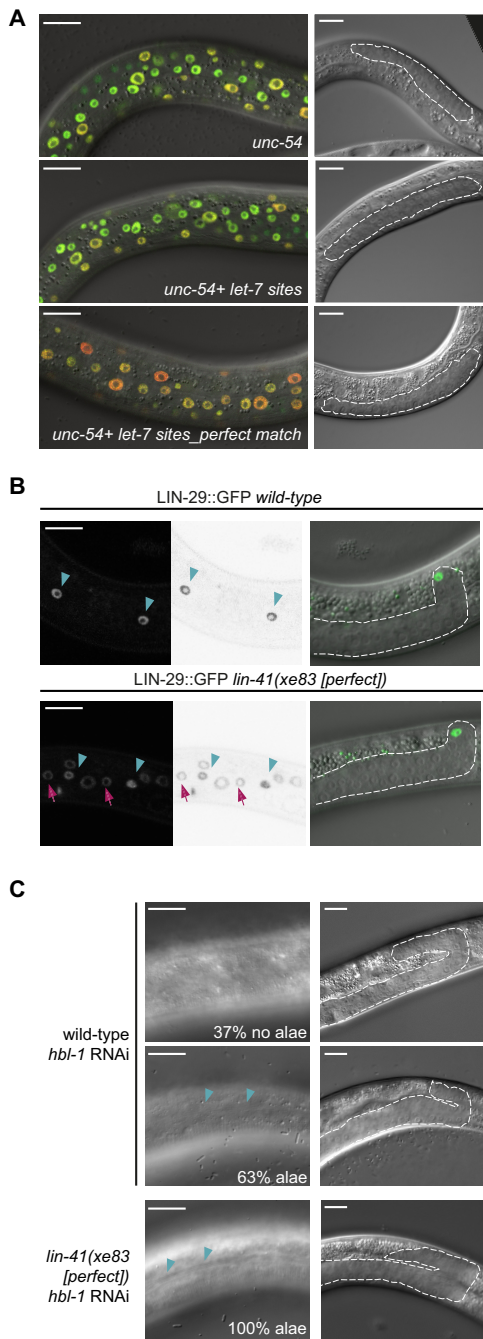


**Figure 4.** Robust miRNA specificity relies on imperfect seed matches. (A, B) Reporter quantification as in Figure 2, from which the negative control data are also replotted for reference. (A) '*unc-54 + miR-48 sites*' reporter and (B) '*unc-54 + dot-1.1 sites*' reporter are assayed in worms of the indicated genotypes. Horizontal line and error bars indicate mean values per condition  $\pm$  SD,  $*P < 0.05$  and  $***P < 0.001$ , two-tailed unpaired *t*-test. (C) Representative image of a viable *lin-41(ap427[dot-1.1\_G:U])*, *mir-48(-) let-7(++)* animal. (D) Progeny ( $n = 99$ ) derived from a cross of *lin-41(xe99[48-ized])* with *mir-48(-) let-7(++)* animals were categorized by phenotype and genotyped to determine the viability of *lin-41(xe99[48-ized])*; *mir-48(-) let-7(++)* 'triple homozygous' mutant animals. (E) Summary of the effect that different site architectures and miRNA abundance have on silencing *lin-41* alleles 'recoded' towards miR-48. *mir-48(-)*: *mir-48(n4097)V*; *unc-54(CTRL)*: wild-type *unc-54* 3' UTR; *let-7(++)*: *let-7* over-expression allele (MosSCI, V).

loss of LIN-41 activity causes inappropriately early activation of LIN-29A and thereby precocious execution of the so-called larval-to-adult transition, which includes fusion of hypodermal seam cells into a syncytium and secretion of an adult cuticular structure termed alae (17). We observed LIN-29A levels through use of a *lin-29(xe61[lin-29::gfp::3xflag])* strain, in which the endogenous *lin-29* locus has been edited to produce GFP-tagged LIN-29A and B isoforms, and in which loss of *lin-41* activity yields a spe-

cific upregulation of only LIN-29A (28). At mid-L3 larval stage, wild-type animals have LIN-29::GFP signal only in their seam cells (Figure 5B). By contrast, animals carrying the *lin-41(xe83[perfect])* allele show additional GFP expression in the major hypodermal syncytium, hyp7, at the same developmental stage (Figure 5B). Therefore, precocious downregulation of *lin-41(xe83[perfect])* is responsible for premature LIN-29 translation and accumulation





**Figure 5.** Developmental robustness requires an imperfect *let-7* seed match in *lin-41*. (A) Representative confocal images of skin cells of animals carrying an *unc-54* 3'UTR reporter (top), an '*unc-54 + let-7 sites*' (center), or an '*unc-54 + let-7 sites\_perfect seed match*' reporter (bottom). At the L3 stage, levels of miR-48 but not *let-7* are already high (39). Scale bars 15  $\mu$ m. (B) Microscopy images of the skin of late L3 worms expressing endogenously tagged LIN-29::GFP (*xe61*) (28) in wild-type and *lin-41(xe83[perfect])* background. Cyan arrowheads point to LIN-29 signal in seam cells, magenta arrows to LIN-29 accumulation in hyp7 cells. Images in the middle are inverted to increase clarity. Worms are staged according to the position of the distal tip cell (green) and gonad length. Scale bars 15  $\mu$ m. (C) Representative images of wild-type ( $n = 27$ ) or *lin-41(xe83[perfect])* ( $n = 36$ ) animals treated with *hbl-1* RNAi. Percentages of animals with the indicated alae status at the L3/L4 transition are indicated. Gonads are outlined to confirm appropriate staging. The strains used, SX346 and HW2144, additionally contain the *mjIs15* and *wIs1* transgenes. Scale bars 15  $\mu$ m.

in the hypodermis, as described for other *lin-41* loss-of-function alleles (17).

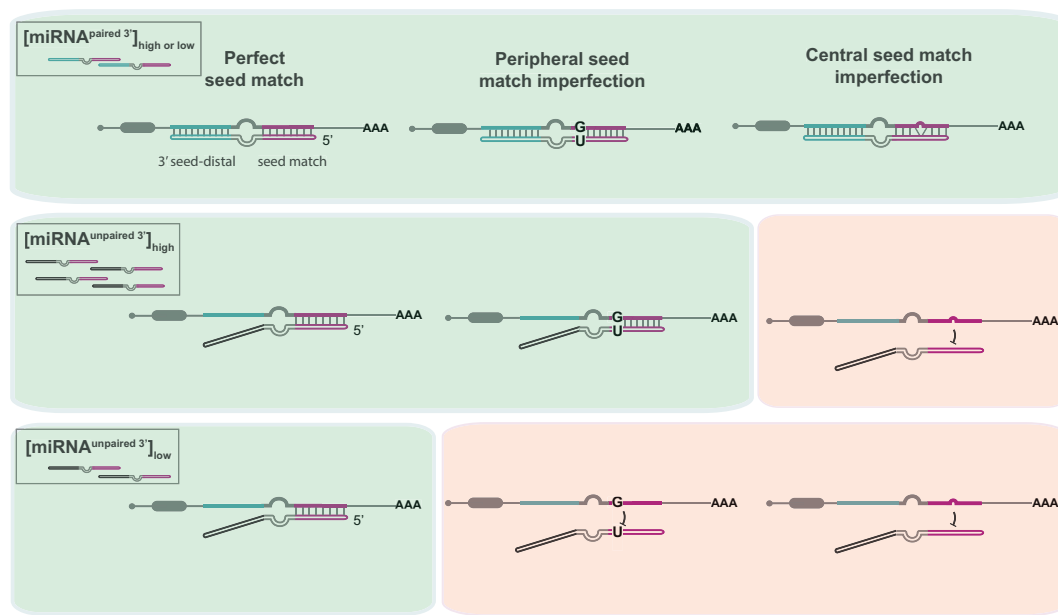
The *lin-41(xe83[perfect])* animals looked superficially wild-type, but the premature upregulation of LIN-29 was sufficient to promote precocious larval-to-adult transition in a sensitized background. Specifically, the transcription factor HBL-1 inhibits larval-to-adult transition, possibly in parallel to LIN-41 (37,38), and its RNAi-mediated depletion causes partially penetrant and partially expressive precocious alae formation (Figure 5C). This phenotype was enhanced when we depleted HBL-1 in *lin-41(xe83[perfect])* mutant animals, resulting in fully penetrant precocious secretion of alae (although weak or patched in some cases) (Figure 5C). We conclude that loss of specificity of repression by *let-7* alone in the *lin-41(xe83[perfect])* background impairs the robustness of temporal patterning through premature LIN-29 accumulation.

## DISCUSSION

It has been an open question to what extent and by which mechanisms miRNA family members can function non-redundantly despite a shared seed sequence. Previously, it was proposed that redundancy was the rule (2). Rare occasions of non-redundant function were hypothesized to require targets with both an imperfect seed match and extensive seed-distal pairing to only one specific family member (3). According to this view, the seed match imperfection impairs silencing by all family members but extensive seed-distal pairing can compensate to facilitate silencing by an individual miRNA. However, this hypothesis has remained untested, and recent observations have challenged it by providing evidence that non-redundant target binding appears wide-spread and that seed-distal pairing may suffice to achieve specificity (9,12).

Our systematic study through gene editing and fluorescent reporter analysis with cell-type resolution resolves the discrepant views on specificity-promoting features for the *let-7* family: We demonstrate that extensive seed-distal pairing to a specific family member suffices to generate a weak but consistent preference for silencing by this family member. However, more robust discrimination requires an imperfect seed match and depends on the quality of such imperfections: a central bulge or G:U wobble base pair, as in the *lin-41* 3'UTR, confers the strongest specificity, while a peripheral G:U wobble base pair, as in the *dot-1.1* 3'UTR, gives an intermediate level. The physiological importance of extensive, seed-mismatch-dependent specificity is evident from the decreased developmental robustness that results when perfect *let-7* seed matches permit promiscuous silencing of *lin-41* by the whole *let-7* family.

Perfect seed matches can still be compatible with selective targeting by individual miRNAs, but the effect depends on miRNA abundance: A moderate increase in *let-7* levels (~2-fold) could overcome the specificity of a binding site that was silenced by miR-48 and had a perfect seed match. However, it only partially did so when the seed match contained a peripheral G:U wobble, and it was insufficient to override sequence-determined specificity when a site contained a central seed match imperfection. This suggests that *in vivo*, miRNA binding site architecture, particularly seed



**Figure 6.** miRNA abundance and architecture of the target site determine mRNA silencing. (top) Extensive complementarity (paired 3') between a miRNA and a target site allows for efficient and specific silencing, independently of the miRNA level and the presence of imperfections in the seed match. (middle) Abundant miRNAs can silence targets carrying a perfect seed match or a nearly-perfect seed match (e.g. a peripheral G:U wobble), even in the absence of complementarity to the sequence outside the seed. A 'central mismatch' repels poorly complementary miRNAs. (bottom) Lowly abundant and poorly complementary miRNAs can silence targets carrying a perfect seed match, but not the ones carrying a seed match imperfection (e.g. peripheral G:U or central bulge). Green shading: functional site; pink shading: nonfunctional site. Magenta: seed/seed match

match quality, and miRNAs abundance act together to determine miRNA activity towards individual targets (Figure 6).

The finding that miRNA activity is determined at the level of individual targets has implications beyond the issue of miRNA family member specificity. It contrasts with a view where a miRNA is globally either 'on' or 'off' in a cell, silencing all of its targets at sufficiently high concentrations and none at low ones. Variable, target site-dependent activity was already entertained in the early days of the miRNA field when miRNAs were likened to rheostats, whose activity is adjusted by two features, namely the extent of target site complementarity to the miRNA and miRNA abundance (19). A lack of explicit experimental testing of such context-dependent function (4) and the rising popularity of the 'seed-match only' model caused this hypothesis to fade from view. We propose that it is time to revisit the idea of miRNAs functioning as rheostats and subject it to further testing.

We note that target validation experiments that rely, as often done, on ectopic miRNA expression appear to make the implicit assumption that miRNAs are uniformly active on their targets. However, if the goal of target validation is to provide insights into pathway biology, physiology and/or pathology, our results and those of others (34) strongly suggest that it must be conducted in a relevant physiological context, avoiding ectopic expression or overexpression of miRNAs. Ideally, validation will also involve functional studies such as those offered by direct manipulation of individual miRNA/target interaction through genome editing. We predict that such efforts will reveal a more nuanced picture of dynamic, context-dependent miRNA target reper-

toires, and thereby improve our understanding of the diversity of biological outcomes that miRNA-mediated gene regulation can achieve *in vivo*.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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