Target specificity and developmental functions of the *let-7* microRNA

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Summary
let-7 is a highly conserved microRNA(miRNA) with important functions in a wide variety of biological processes. In the nematode worm *Caenorhabditis elegans (C. elegans) let-7* plays a crucial role in developmental timing, regulating temporal cell fates in the stem cell-like seam cell compartment. Study of *let-7* in this context served as a useful model system for both elucidation of general principles of miRNA function and definition of universal concepts regarding developmental time and stem-cell biology.
In my thesis work, I was interested in the study of molecular aspects of *let-7*-mediated target regulation and its developmental functions. I examined these aspects in three separate, but complementary projects.
First, by performing a genome-wide RNAi screen for suppressors of *let-7* lethality, I identified about 200 novel *let-7* genetic interaction partners. Characterization of these genes revealed a tight connection between *let-7* activity and the cell-cycle. Unfortunately, the screed did not yield any obvious and promising candidate *let-7* target or upstream regulator for further study.
My second project consisted of establishing a novel, quantitative *in vivo* miRNA target reporter system and study of *let-7*-mediated target regulation in different tissues of the worm. This new technique allowed the direct and quantitative visualization of miRNA activity on different targets over time, in various tissues, at a quantitative level. The main finding in these experiments was the demonstration of target specificity among different members of the *let-7* family. Even in the same cell and at the same time, individual targets were differentially affected by the loss of a specific family member, showing that they have intrinsically different target specificity. I examined the sequence requirements of *let-7* specificity towards *lin-41* at the target site level and found that base-pairing at the 3'end of the miRNA contributes to effective and specific repression. However this is probably not sufficient, as I could not transform *lin-41* repression completely to be dependent on another *let-7* family member by mutating the target site. These findings have clearly further implications for our general understanding of miRNA specificity.
Finally, I characterized developmental defects underlying the lethal vulva bursting phenotype of *let-7* mutant worms. I showed that *let-7* expression in the vulva is required for bursting suppression. Contrary to previous assumptions, *let-7* is not involved in the specification of vulva precursor cells at the L3 stage, but probably has a role in later stages of vulva morphogenesis. In this context, *let-60,* the worm *Ras* homologue, is not targeted by *let-7* and loss of the *let-60* 3'UTR and thus miRNA regulation has no functional consequences. By contrast, my experiments show robust regulation of *lin-41* in all vulva cells and suggest that *lin-41* is the key *let-7* target in the vulva. Surprisingly, the *let-7/lin-41* pathway does not influence *lin-29* expression and *lin-29* is, unlike in the hypodermis, not an effector of *let-7/lin-41.* I discovered and characterized a novel function for the heterochronic genes *let-7* and *lin-41* in the vulva and showed that the effectors of the heterochronic pathway can be rewired in different tissues.
In sum, I used different approaches to expand our understanding of the molecular and developmental functions of the *let-7* miRNA in *C. elegans* and my results might have further implications for the target specificity of miRNAs.

Introduction

Target regulation by microRNAs

microRNA modes-of-action: molecular switches or fine-tuners?

MicroRNAs (miRNAs) are short, 20-22 nucleotide (nt) long, RNAs encoded in the genome of animals and plants. Already when *lin-4,* the first miRNA, was discovered in *Caenorhabditis elegans (C. elegans),* the basic principles of miRNA action were evident from genetic and molecular analysis of *lin-4's* interaction with its target gene *lin-14* (Lee et al., 1993; Wightman et al., 1993): antisense complementarity to sites in the target 3'UTR leading to inhibition of gene expression at the post-transcriptional level. Elucidation of the molecular details was greatly accelerated by two sets of key discoveries: first, the identification of other similar short RNAs in *C. elegans (Reinhart et al., 2000; Lau et al., 2001; Lee and Ambros, 2001)* and in a wide variety of other organisms (Lagos-Quintana et al., 2001; Reinhart et al., 2002) fuelled the interest for these tiny RNAs now termed miRNAs. Second, the recognition of the link between the RNA-interference and miRNA pathways provided a new avenue for the biochemical dissection of miRNA function (Grishok et al., 2001).

The numerous studies since then carried out in different model systems firmly established the identity of the core components and basic functions of the miRNA pathway. After its transcription from intergenic or intronic loci, the primary miRNA precursor (pri-miRNA) is cleaved in the nucleus by the Microprocessor complex consisting of Drosha and Dgcr8 proteins (Gregory et al., 2004), the pre-miRNA stem-loop generated is subsequently exported into the cytoplasm, processed further by the Dicer complex (Hutvágner et al., 2001). Finally one of the two strands from the Dicer product is loaded on an Argonaute protein and guides the miRNA-associated silencing complex (miRISC) to target RNAs. Recruitment of the miRISC can lead to degradation and translational inhibition of the target mRNA. Although this framework successfully describes the fate of most miRNAs, three major questions, absolutely required to understand miRNA functions, are still not answered unambiguously and are hotly debated in the field: the exact relationship between mRNA degradation and translational repression induced by miRNAs, the extent of target regulation and the architecture of miRNA-based regulatory networks including specificity of target repression.

The question on the major molecular mechanism responsible for the inhibition of miRNA targets, mRNA degradation vs. translational inhibition, was heavily disputed, but recently the two models were somewhat reconciled with the identification of molecular and kinetic links between these two processes. Historically, inhibition of mRNA translation was first inferred by the observation that miRNA induced changes in target protein levels greatly exceeded decline in target mRNA levels (Wightman et al., 1993). The finding of significant mRNA decay caused by miRNAs (Bagga et al., 2005) made any simple interpretation of experiments analyzing changes in protein and mRNA levels impossible, since decline in mRNA levels will be inevitably followed by a decrease in protein

abundance. As both effects coexist also if assessed on a global scale, e.g. by examining mRNA and protein levels upon experimental manipulation of miRNA and protein levels (Baek et al., 2008; Selbach et al., 2008), the relative importance of translational inhibition vs. mRNA degradation often remained a matter of interpretation.

At the biochemical level, both processes are supported by a wealth of data. It has been clear from the beginning that mRNA degradation occurs, unlike in the RNAi pathway, in a cleavage independent way. This is well explained by the imperfect complementarity between miRNAs and miRNA target sites (Elbashir et al., 2001) as well as by the lost endonucleolytic activity of e.g. human argonautes AGO 1/2/3 (Liu et al., 2004). mRNA degradation is indeed promoted by miRNAs and usually correlates with deadenylation (Giraldez et al., 2006; Wu et al., 2006) and decapping (Behm-Ansmant et al., 2006). miRNAs thus use the general mRNA decay machinery to regulate their target genes. This is explained mechanistically by the direct binding of Argonaute proteins to GW182 effectors (Behm-Ansmant et al., 2006) and thereby recruitment of two deadenylase complexes, the PAN2-PAN3 and CCR4-NOT-CAF1 complexes (Braun et al., 2011), to target mRNAs.

Translational inhibition was directly demonstrated by shift of miRNA targets to monosomal fractions in sucrose gradients (Pillai et al., 2005; Ding and Grosshans, 2009), supporting translational initiation as the step likely inhibited and disfavoring translation elongation initially implicated (Olsen and Ambros, 1999). Although AGO binding to the 5' methylguanosine cap structure of mRNAs (Djuranovic et al., 2010) suggested a competition with eIF4E as the mechanism underlying translational inhibition, recent evidence indicates that sequestration of the eIF4A2 translational initiation factor by the CCR-NOT complex is the main event mediating this miRNA activity (Meijer et al., 2013). This finding also provides an explanation for the fact that recruitment of the CCR-NOT deadenylase complex to mRNAs is able to mediate translational inhibition (Chekulaeva et al., 2011). A longstanding question about the two possible effects of miRNAs on their targets is whether translational inhibition and mRNA degradation are coupled or independent. Although in some special cases the reversibility of translational inhibition implies preservation of the mRNA (Bhattacharyya et al., 2006), this might involve active prevention of mRNA degradation in this case by the RNA binding protein HuR, as translational block is generally linked to mRNA degradation (Schwartz and Parker, 1999). Although at steady state, translational inhibition as assessed by ribosome profiling contributes only to a smaller extent compared to mRNA degradation to the observed decrease of protein output (Guo et al., 2010), careful kinetic analysis of miRNA induced changes provide evidence that translational inhibition occurs first and is followed by mRNA degradation (Bazzini et al., 2012; Djuranovic et al., 2012). Specifically, steep increase of *mir-430* in zebrafish embryos allowed the dissection of the cellular fate of *mir-430* targets in time (Bazzini et al., 2012). Concurrent analysis of miRNA and mRNA levels, length of the polyA tail and translational rates revealed that translational inhibition occurs first, which is independent of polyA tail shortening. This is followed by mRNA deadenylation and degradation. The same observation has been made in *Drosophila* S2 (Djuranovic et al., 2012) cells and HeLa cells (Bethune et al., 2012) examining inducible reporter constructs. Collectively, these experiments suggest that

translational repression is tightly coupled to mRNA degradation and might be even a prerequisite (Meijer et al., 2013).
These recent results unify many hypotheses in one model (Fig 1). However, several open questions remain. It is not clear whether these rules apply in all organisms and to all miRNA-target pairs. Intriguingly, the deadenylation rate of various targets of a given miRNA family was different in *C. elegans* embryonic extracts *in vitro* (Wu et al., 2010) suggesting that the kinetics of miRNA activity are truly context dependent.

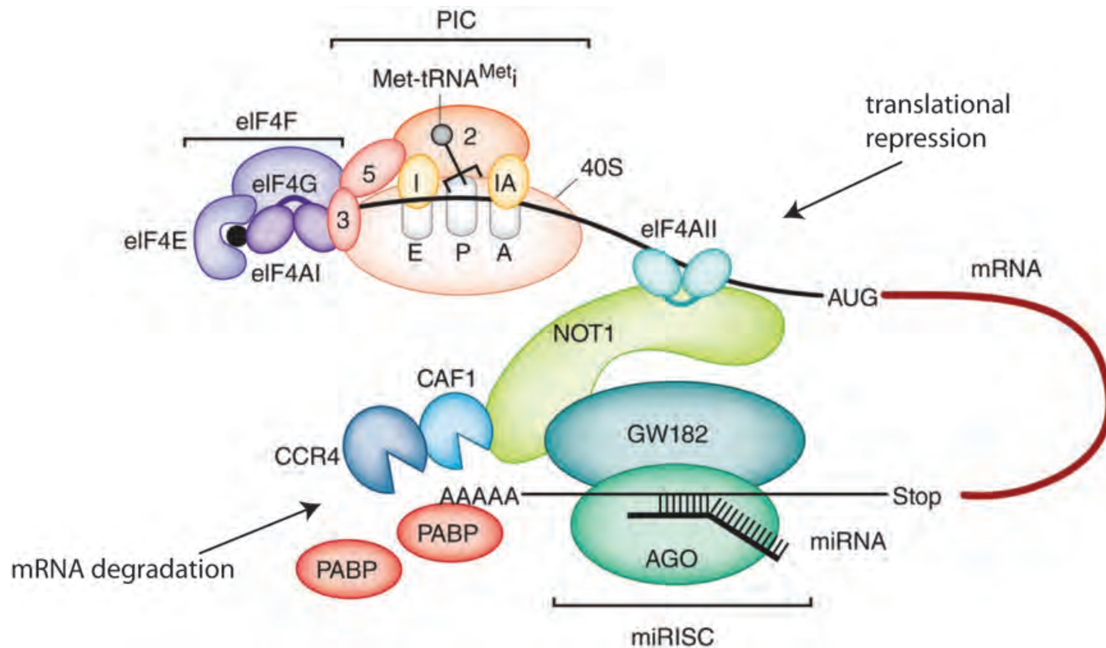

Fig. 1. Current model of miRISC mechanisms of action and interaction partners. See main text for details. Modified with permission from doi:10.1038/nsmb.2582

The other major question concerning the biological role of miRNAs is the extent of target regulation. Whereas early experiments in *C. elegans* led to the conclusion that miRNAs act as switches (Wightman et al., 1993; Reinhart et al., 2000) causing substantial decrease in target protein levels, cell-culture experiments typically showed only modest changes induced by miRNA activity (Baek et al., 2008; Selbach et al., 2008). The biggest difference between these experiments is probably the model system and thus the context used. Developmental model systems involve a physiological transition between two defined cellular states. In this situation miRNAs can either trigger this programmed change or modulate the transition. In any case, miRNA regulation is embedded in a regulatory network involving other concurrent processes. E.g. there is evidence that miRNAs serve to clear maternal transcripts in zebrafish embryos (Giraldez et al., 2006) and zygotic miRNAs deadenylate maternal mRNAs in *C. elegans* embryos (Wu et al., 2010). In these situations, the concurrent shutdown of maternal transcription supports miRNA-mediated repression and results in large changes in miRNA target expression. Similarly,

miRNA targets are often involved in feed-back and feed-forward loops (Rybak et al., 2008; Iliopoulos et al., 2009; Rybak et al., 2009) that can create bistable switches (Alon, 2007) that can be triggered by smaller changes in miRNA target levels. Conversely, measurement of steady state target levels in cell lines reflect in addition to miRNA activity also regulatory mechanisms intended to buffer changes is gene expression, e.g. autoregulation of proteins. This would underestimate the extent of miRNA-mediated gene repression. Nevertheless, there is strong evidence for different levels of miRNA repression even using similar experimental set-ups, e.g. strong repression of the *let-7* target *Hmga2* in cell-lines (Mayr et al., 2007), indicating quantitative differences in miRNA target repression per se. As these differences have major implications for potential biological functions of miRNAs, several studies examined the quantitative aspects of miRNA-mediated gene regulation. Single cell analysis of miRNA target reporters showed that the same miRNA can both confer substantial repression to or fine-tune target gene expression (Mukherji et al., 2011). This was dependent on the relative miRNA and mRNA levels and had a relatively sharp threshold between these two states. Below the threshold, when mRNA levels were low, target expression was inhibited up to 40 fold. Near the threshold, miRNAs regulated their targets in the range of ~2 fold and finally at very high transcript levels, mRNAs escaped miRNA regulation. Importantly, the threshold level was not rigid, but was a function of miRNA levels and number of miRNA binding sites in the target 3'UTR. Furthermore, the level of repression substantially differed between individual cells, pointing towards a serious weakness of population-based studies. A similar conclusion was drawn after analysis of a transfected pool of miRNA target reporters, only the most abundant miRNAs in the cell were able to efficiently repress their targets (Mullokandov et al., 2012). Although this model has yet to be validated *in vivo* and with endogenous targets, it provides a useful framework to think about the dynamic nature and quantitative aspects of miRNA-mediated target regulation. As precise measurement of cumulative miRNA target levels is rarely possible under physiological conditions, determination of the two other factors, miRNA levels and identification of miRNA target sites are the main prerequisites for the estimation of miRNA-mediated target repression.

As the biological function of miRNAs is regulation of target genes, identification of these targets is one of the major goals of miRNA research. A fundamental question in this regard is whether miRNAs regulate a few key targets or influence hundreds of mRNAs concomitantly and how target identity and repression is related to the functional output of miRNA activity. The answer to this question is complicated by the fact that miRNAs not only have several potential targets, but a typical mRNA has predicted binding sites for several different miRNAs (Friedman et al., 2009). Cooperativity and redundancy between miRNAs on the same target mRNA has a huge influence on the experimental analysis of miRNA-mediated target repression. Possible outcomes of miRNA activity range from regulation of two key targets such as in the case of *lin-4* (Wightman et al., 1993; Moss et al., 1997) during *C. elegans* development to low-level repression of hundreds of mRNAs (Baek et al., 2008) upon *mir-124* transfection in cell-culture. The phenotypic consequences of miRNA activity also vary to similar extent, whereas loss of an individual miRNA such as *let-7* can be

lethal in *C. elegans* (Reinhart et al., 2000), the absence of other miRNAs can be well tolerated (Alvarez-Saavedra and Horvitz, 2010). These results imply that miRNA-target relationships represent a continuum from irrelevant, "passenger", interactions to essential regulation vital for an organism. This might explain the discrepancy between the results of studies designed to reveal the functionality of miRNA target regulation and large-scale measurement of miRNA-induced changes in target levels. Identification of a miRNA's role in a specific biological process suggests a switch-like or at least significant regulation of mRNAs and in this scenario usually only a few key targets change. Functional analysis of the targets show accordingly that knock-down of a few or even a single miRNA target can rescue the phenotype of miRNA loss and overexpression of single targets can at least partially recapitulate the effect of decreased miRNA expression. The concept of key targets is corroborated by the finding that loss of an individual miRNA-target interaction can have fatal consequences. A single-nucleotide polymorphism in the *K-Ras* 3'UTR disrupting binding to *let-7* increases lung and ovarian cancer risk (Chin et al., 2008; Ratner et al., 2010) and provides prognostic information in colorectal cancer (Smits et al., 2011). Truncation of the *let-7* target *Hmga2* 3'UTR leads to its overexpression and is involved in oncogenic transformation in various malignancies (Mayr et al., 2007; Peng et al., 2008; Ikeda et al., 2011). Importantly, although *Hmga2* might be the key *let-7* target in some cells, *Hmga2* might not even be regulated in others or its role can change even in the very same cells in different contexts (Copley et al., 2013). The relevance of a specific miRNA-mRNA interaction must be thus functionally determined case-by-case in a defined biological context.
The regulation of target batteries, hundreds of genes, might represent non-functional interactions or might serve some cryptic functions, e.g. buffering against some perturbations, that become evident only under specific circumstances. Such a function has been demonstrated e.g. for *mir-7* in *Drosophila,* as abnormal determination of some olfactory cells was observed in *mir-7* mutant, but not *wt,* animals exclusively upon temperature fluctuations (Li et al., 2009). In *C. elegans,* phenotypes for individual miRNAs mutants can be observed in different sensitized genetic backgrounds (Brenner et al., 2010), indicating that miRNA function can confer robustness to biological processes.

Prediction, identification and validation of microRNA targets

To understand the function of a miRNA, it is essential to know its targets. Information about potential miRNA targets can be obtained either by prediction of candidates using computational algorithms based mostly on general features of a typical miRNA binding site or by experimental identification of the most likely regulated genes. In either case, regulation of candidate miRNA targets has to be validated and, as miRNA-mediated target regulation is highly context dependent, its functional importance determined.
Computational algorithms such as TargetScan (Lewis et al., 2003) or Pictar (Krek et al., 2005) are popular tools for the generation of candidate target lists for a given miRNA. They use some general features of miRNA-target interactions that have been extracted from experimental approaches examining these interactions. The relative importance of the various criteria as well as filters,

such as evolutionary conservation, differs between the specific algorithms, but their overall performance is similar, representing a trade-off between specificity and sensitivity. This is mainly explained by the fact, that although each of the general features used in this algorithms, such as seed match, is characteristic for miRNA binding sites, a big part of the experimentally validated miRNA target sites represents in some way an exemption from the rule. Here, I review the most important rules governing the miRNA-target interaction.

Unlike siRNAs, miRNAs bind to their targets by imperfect base pairing (Lai, 2002), presumably to avoid catalytic activity of slicing-proficient Argonaute proteins. Shortening of the miRNA-mRNA interface has profound consequences for miRNA target site prediction, as it greatly increases the number of potential binding sites that would occur randomly in a typical eukaryotic genome and creates a high background in prediction lists. Moreover, it was clear from the earliest miRNA-target pairs that even different types of mismatches such as smaller or larger bulges and G:U wobbles in the miRNA target site pairing are tolerated (Reinhart et al., 2000), further complicating target prediction. Nevertheless, it became clear that base pairing between miRNAs and their targets is not random and the 5' end of the miRNA, the seed region, carries particular importance. This has been first noted simply by inspection of miRNA targets known at that time (Lai, 2002) and has been validated on a larger scale later showing significant overrepresentation of the seed motif in the 3'UTR of miRNA-responsible genes (Lim et al., 2005; Baek et al., 2008; van Dongen et al., 2008). The seed portion of a miRNA tends to be evolutionary more conserved than its 3' end (Lim et al., 2003; Stark et al., 2003), underscoring the importance of seed base pairing. Match to the seed turned out to be a key feature in computational target prediction (Lewis et al., 2003). The importance of the seed can be explained at the biochemical level by examining the structure of Argonaute proteins, core proteins of the miRISC (Ma et al., 2005; Elkayam et al., 2012; Schirle and MacRae, 2012). The seed is tightly bound in a cleft of the Argonaute protein, displaying the Watson-Crick surface of nucleotides 2-6 for potential base pairing with its target. In addition, nucleotide 7 might also have a crucial role in target binding (Schirle and MacRae, 2012). Importantly, the structure does not speak against a role for the 3' portion of the miRNA in target binding, as these nucleotides also accommodate a specific configuration and are free for target binding (Elkayam et al., 2012). Functionally, a single, exclusively seed-binding miRNA target site is sufficient to confer substantial regulation to a 3'UTR (Brennecke et al., 2005; Farh et al., 2005). The detailed requirements for seed-complementarity have been systematically examined in HeLa cells (Doench and Sharp, 2004) and *in vivo* in *Drosophila* (Brennecke et al., 2005). Both studies revealed base pairing through nucleotides 2-8 in the miRNA as the key determinant of miRNA activity. In HeLa cells luciferase reporter transfections (Doench and Sharp, 2004), binding through the 3' region contributed only to a minor extent to repression. However, the effect of mismatches were examined partly in the context of a 3'UTR having in addition two optimal target sites and as the authors noted that increasing miRNA concentrations could potentially regulate suboptimal targets, the results might not be representative of the physiological contexts with lower miRNA levels and suboptimal target site architecture. Indeed, the importance of non-seed binding (nucleotides at the 3'

end of the miRNA) was evident in the *Drosophila* experiment when only partial seed binding was provided. In this case, compensatory binding through the non-seed portion became necessary for target repression (Brennecke et al., 2005). Although thermodynamic principles also seem to play a role in target recognition, the thermodynamically favorable G:U wobbles significantly decreased or even disrupted target regulation, even though in *C. elegans* the *lin-4* and *let-7* targets *lin-14* and *lin-41* contain G:U bulges in their binding sites (Ha et al., 1996; Reinhart et al., 2000). It remains thus unclear, how the experimental system influences the requirements for seed binding and how miRNA/mRNA concentrations, strength of the targets site and extent of miRNA-mediated target regulation are interrelated.
Despite the success of the seed rule in predicting miRNA-target interactions, up to one-third of such interactions cannot be explained by seed binding (Chi et al., 2012; Helwak et al., 2013). The remaining "non-canonical" binding has been sorted in different categories. Compensatory target sites have a limited binding in the seed region which is compensated by extensive base pairing at the 3' end of the miRNA (Brennecke et al., 2005; Grimson et al., 2007). Bulges opposite to the 5/6th nucleotide of the miRNA are frequently found in functional miRNA target sites if they allow formation of a transient, intermediate base pairing and thus "nucleating" further compensatory interactions on the 3' end of the miRNA (Chi et al., 2012). Centered target sites involve continuous base pairing over typically 11 nucleotides starting from nucleotide 4 or 5 and mediate miRNA-type repression of target mRNAs without siRNA-like cleavage (Shin et al., 2010). As different methods might preferentially detect certain types of miRNA-mRNA interactions, it is currently not known how frequent non-canonical targets are. Interestingly, nonrandom distribution of the different target site classes was observed for about two thirds of miRNAs in one study (Helwak et al., 2013), specific miRNAs thus prefer a particular type of target binding site. Whether this correlates with any sequence feature of the miRNA or has any consequence on the outcome of the miRNA/mRNA interaction is not known. At the mechanistic level, the different types of interactions can be explained by some thermodynamic features and the free energy change (ΔG) upon miRNA binding correlates with miRNA-mediated target repression (Rehmsmeier et al., 2004; Lekprasert et al., 2011). It is not surprising however that it has not been possible so far to integrate all variables of the target site architecture in one unifying model.

Several other factors beside the target site sequence itself influence the miRNA-target interaction. Although miRNA target sites are most commonly located in the 3'UTR of target genes, miRNA-loaded miRISC frequently binds to sites in the coding sequence and rarely to the 5'UTR of target genes (Hafner et al., 2010; Helwak et al., 2013). This binding results only in modest, but significant target repression (Fang and Rajewsky, 2011), probably because the miRISC is displaced by the scanning and translating ribosome (Gu et al., 2009). Within the 3' UTR, target sites at both ends, but excluding the first 15 nucleotides after the stop codon are the most effective (Grimson et al., 2007). The local context could also promote or repress miRNA activity. One factor is the accessibility of the target site, as determined thermodynamically (Kertesz et al., 2007), target sites in an

AU-rich environment confer indeed greater extent of repression than target sites embedded in GC-rich stretches (Grimson et al., 2007).
It has been estimated that a typical mRNA has target sites for ~ 4 miRNAs. Binding of multiple miRNAs and thus recruitment of several miRISC complexes has an additive or even synergistic effect (Doench and Sharp, 2004; Grimson et al., 2007). Cooperation, more than additive effect, was observed when the two miRNA target sites were positioned between 8-40 nucleotides apart (Grimson et al., 2007; Saetrom et al., 2007). Importantly, this principle holds true both for identical and different miRNAs, creating complicated regulatory circuits converging on a single 3'UTR. In fact, such crosstalk is not limited to miRNAs, RNA-binding proteins in the vicinity of the miRNA target site can similarly influence miRNA activity. The effect of RNA-binding proteins can be either negative or positive. Pumilio proteins for instance are able to facilitate miRNA binding to targets site close to a Pumilio response element, as shown for different miRNAs in different organisms (Nolde et al., 2007; Kedde et al., 2010; Miles et al., 2012). On the other hand miRNA-mediated repression can be prevented by the RNA-binding proteins HuR (Bhattacharyya et al., 2006; Kundu et al., 2012) and Dnd1 (Kedde et al., 2007), possibly by competing with miRISC binding.
As computational miRNA target prediction still has a high false-positive and false-negative rate, it is essential to obtain experimental evidence on miRNA targets. On a larger scale, this can be achieved by analyzing gene expression upon manipulation of miRNA activity or by various biochemical methods.
With the exception of some rather exotic examples (Vasudevan et al., 2007), miRNAs negatively regulate their target genes. The expression levels of miRNAs and their targets should be thus anticorrelated. Detection of such a relationship can be exploited to identify miRNA targets. Changes in miRNA levels could be followed in physiological contexts such as development (Farh et al., 2005) or upon experimental manipulation of miRNA levels. miRNA expression can be increased by transfection of synthetic dsRNA mimicking miRNA duplexes (Selbach et al., 2008) or by inducible expression of miRNA expression constructs (Shih et al., 2011). Due to its easiness, this approach has been probably the most popular, overexpression of miRNAs bears the risk of saturating the miRNA machinery (Khan et al., 2009) and by showing regulation of suboptimal, irrelevant targets at supraphysiological miRNA concentrations (Doench and Sharp, 2004). The activity of individual miRNAs can be blocked by transfection of miRNA inhibitors (Hafner et al., 2010), expression of sponges (Ebert et al., 2007) or by using miRNA mutants (Baek et al., 2008). Alternatively, transient knock-down or genetic elimination of miRNA pathway components such as the miRNA processing enzymes Dgcr8 (Wang et al., 2007) or Dicer (Giraldez et al., 2006) can inhibit miRNA activity globally, although as these genes typically have some miRNA unrelated functions (Wagschal et al., 2012), possible unwanted effects have to be considered. Corresponding changes in gene expression upon the above experimental strategies can be followed at different levels. Whereas quantitative PCR, microarray and high throughput sequencing is intended to measure miRNA-induced mRNA degradation (Lim et al., 2005), changes in protein levels can be quantified by stable isotope labeling of amino acids (SILAC) followed by mass spectrometry (Baek et al., 2008; Selbach et al., 2008) or quantitative targeted proteomics (Jovanovic et al., 2010) approaches. Recently,

ribosome profiling was introduced into the toolbox of miRNA target identification (Guo et al., 2010), this method monitors the translational status of mRNAs and can identify translationally repressed targets similarly to polysome profiling used in the past (Nakamoto et al., 2005; Hendrickson et al., 2009). Common to all these approaches is that they cannot directly discriminate between direct and indirect miRNA targets and do not provide information about the functional significance of target regulation, the list of candidate miRNA targets has to be filtered using more or less biased criteria, such as seed-match.
miRNA-target interactions can be directly detected using biochemical methods.
During the last ten years, a number of different strategies have been proposed, including the use of miRNAs as primers for reverse transcription of the bound mRNA (Andachi, 2008) or pull-down of labeled miRNAs and sequencing of the bound targets (Orom and Lund, 2007), most of them are not widely used with the notable exception of approaches relying on immunoprecipitation (IP) of miRISC components, such as Argonaute or TNRC6. IP of these proteins followed by analysis of bound mRNA fragments can indeed identify known and novel miRNA target genes (Karginov et al., 2007). As in the basic ribonucleoprotein-immunoprecipitation/microrarray analysis protocol (RIP-Chip) the reassociation of miRISC with RNA from different cellular compartments or even different cells during the purification process is a major concern, several improved modifications of the original method have been developed recently. In the HITS-CLIP method, the RNA is covalently crosslinked to proteins in very close vicinity before immunoprecipitation. Sequencing of the RNA-fragments obtained in this way was performed in mouse brain (Chi et al., 2009) and also in *C. elegans* (Zisoulis et al., 2010) to identify potential miRNA targets. A further improvement to HITC-CLIP, termed PAR-CLIP, is the use of the photoactivatable nucleoside 4-thiouridine during culturing for the sample which greatly enhances crosslinking efficiency and is converted to an cytosine-like nucleotide by UV light, as this allows more precise mapping of the protein-RNA interaction and discriminates between crosslinked and non-crosslinked, background RNA (Hafner et al., 2010). A different strategy, CLASH, is the introduction of an intermolecular RNA-RNA ligation step after UV-crosslinking and Argonaute IP (Helwak et al., 2013) followed by sequencing and bioinformatic search for RNA-RNA hybrids. Although the methodologies differ considerably and differences in experimental details have distinct biases (Kishore et al., 2011), these high-throughput experiments significantly expanded our catalogue of potential miRNA binding sites and suggest some unexpected functions for miRNAs, such as regulation of non-coding RNAs (Zisoulis et al., 2012; Helwak et al., 2013). A caveat related to these experiments is the use of epitope-tagged and overexpressed proteins, potentially altering levels and composition of the miRISC. Probably the major weakness of CLIP-seq approaches however is the at most semi-quantitative nature of the results. It is not clear how the obtained read numbers correlate with strength of the miRNA-mRNA interaction or with the extent of target regulation. As usual, important biological questions involve analysis of different biological states, it will be interesting to see how CLIP-seq experiments perform in monitoring dynamic changes in miRNA regulation. For such experiments, the complexity and cost of the methods might currently still be prohibitive.

The current gold-standard to prove a miRNA-target interaction is demonstration that regulation can be recapitulated in reporter assays. This involves fusion of the candidate regulatory sequence to a reporter gene and, using appropriate controls, assessment the effects of this sequence on reporter gene expression. Such an assay can be performed not only in cell-culture, but also *in vivo,* eg in *C. elegans* (Wightman et al., 1993), zebrafish (Giraldez et al., 2006) or *Drosophila* (Brennecke et al., 2005). But also for this type of experiment, the devil is in the detail, only the careful choice of the reporter gene, detection method, the regulatory sequence, controls and model system leads to meaningful results. Traditionally, both enzymatic and non-enzymatic, e.g. fluorescent, reporter genes have been used in miRNA target reporter assays. In both cases, the linear relationship between reporter quantity and signal strength must be ensured. If examining dynamic processes, the half-life of the reporter gene has to match the time frame of the expected changes. Whereas e.g. luciferase fulfills these requirements well (Ignowski and Schaffer, 2004), the linearity of the *in vivo* galactosidase assay is not known and results are often presented as percentage of a population with activity above an arbitrary threshold (Reinhart et al., 2000). The commonly used GFP fluorophore has a half-life of up to 26 hrs (Corish and Tyler-Smith, 1999) precluding dynamic analysis of miRNA activity. As regulation of a miRNA target site is context dependent, ideally the entire 3'UTR should be included in the reporter system. Seemingly a trivial point, this requires substantial attention in the case of genes with poorly annotated 3'UTRs and considering frequent alternative polyadenylation of mRNA (Mayr and Bartel, 2009). Controls should rule out unspecific effects during experimental manipulation, such as transfection or genome modification. This can be achieved e.g. by using a non-regulated reporter with similar characteristic as an internal normalizer and scrambled control miRNAs. Even with a normalizer, transient transfection in cell culture or introduction of an extrachromosomal array in *C. elegans* means very different levels of the transfected reporter and/or miRNA across individual cells, resulting in a range of repression levels (Mukherji et al., 2011). For this reason, comparison between different reporters in generally problematic and necessitates reporter systems with stable and defined expression. A typical reporter experiment involves either manipulation of miRNA levels and/or the reporter sequence, commonly mutation of the seed or deletion of the entire target site. In either case, the levels of both reporter and miRNA should be in a physiological range as abnormally high miRNA-target ratios can lead to false positive, the opposite to false negative results (Doench and Sharp, 2004; Mukherji et al., 2011). Similarly, miRNA overexpression yield misleading results, inhibition of an endogenous miRNA is probably more meaningful. The results presented should be not only statistically significant, but also biologically relevant. Finally, unless performed in biological context of interest, reporter assays represent a heterologous system. As miRNA-mediated target regulation is highly cell-type and context dependent, the results cannot be interpolated directly to other systems.

The ultimate part in target validation is demonstration of biological significance. This was clear a priori for the first miRNA-target pair *lin-4* and *lin-14* (Lee et al., 1993; Wightman et al., 1993). In this case, *lin-4* and *lin-14* loss-of-function phenotypes were exactly the opposite and a *lin-14* mutation interfering with

regulation by *lin-4* phenocopied loss of *lin-4*. Such a clear evidence could be rarely obtained for any miRNA-target. More correlative evidence, which is still not always common practice, is provided by the opposite phenotypic effects of miRNA inhibition/ target overexpression and target knock-down/miRNA overexpression, respectively. New developments in gene modifying technologies such as gene conversion using TALE effector nucleases (TALENs) or the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) system promise the possibility to experimentally manipulate miRNA binding sites in their endogenous context allowing the targeted creation of target site disruptions similar to the highly informative natural examples (Wightman et al., 1993; Mayr et al., 2007). Such experiments would bridge the gap between experiments assessing quantitative effects and those investigating functional consequences of miRNA-mediated target regulation.

Modifiers of microRNA target regulation

Given the importance of miRNAs in most biological processes, it is not surprising that their levels and activity are tightly regulated (Fig. 2).

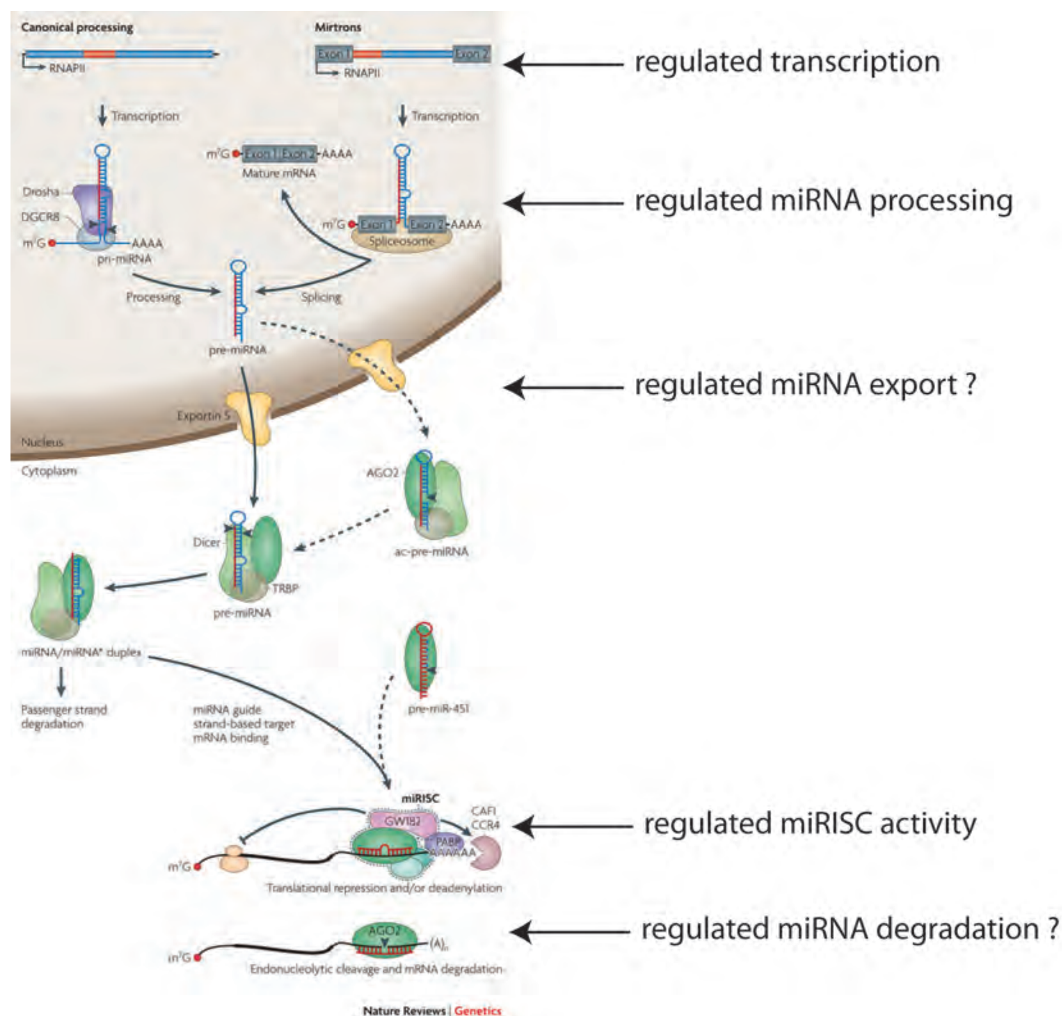

Fig. 2. miRNA activity is regulated at various levels.
Adapted with permission from doi:10.1038/nrg2843.

Regulation occurs at every step on the miRNA's way from its transcription to degradation. The multistep miRNA biogenesis is a common target of regulation (reviewed in (Krol et al., 2010b)). Examples include both miRNA-specific regulatory mechanisms and modulation of global miRNA biogenesis. In addition to homeostatic (auto-)regulation of the miRNA pathway (Han et al., 2009; Martinez and Gregory, 2013), expression levels of miRNA biogenesis machinery components can be regulated by various mechanisms (Qi et al., 2008; Rybak et al., 2009). The necessity for tight regulation of global miRNA biogenesis is exemplified by the fact that decreased miRNA processing promotes malignant transformation (Kumar et al., 2007) and Dicer acts as haploinsufficient tumor suppressor (Kumar et al., 2009). Expression of individual miRNAs or a group of miRNAs can be modulated by RNA-binding proteins recognizing specifc sequences in the pri- or pre-miRNA and either promoting (Davis et al., 2008; Trabucchi et al., 2009) or inhibiting (Yamagata et al., 2009) the miRNA biogenesis machinery.

Biogenesis of the *let-7* family is under extensive control. In *C. elegans,* where *let-7* miRNAs are transcribed from their own promoters, several factors affecting *let-7* transcription have been described. Proper timing of *let-7* transcription is dependent on the presence of a temporal regulatory element in its promoter and is influenced by the activity of other heterochronic genes (Johnson et al., 2003; Roush and Slack, 2009). Other elements in the *let-7* promoter are responsible for *let-7* transcription in the hypodermis and intestine (Kai et al., 2013). Lin-28 proteins inhibit *let-7* biogenesis both at the Drosha and the Dicer processing steps, either by sequestering *pri-let-7* to the nucleolus (Piskounova et al., 2011) or by recruiting the terminal uridyl-transferase Tut4 to *pre-let-7* leading to pre-*let-7* uridylation (Heo et al., 2009) and consequent degradation by the exonuclease Dis3l2 (Chang et al., 2013). Inhibition of *let-7* biogenesis by *lin-28* is conserved in other organisms e.g. in *C. elegans* (Lehrbach et al., 2009; Van Wynsberghe et al., 2011; Vadla et al., 2012) and is important not only during development, but has a profound role also e.g. in human energy metabolism (Zhu et al., 2011) and tumor formation (Viswanathan et al., 2009). Interestingly, Tut4 plays a dual role in regulating *let-7,* in the absence of Lin-28, it mono-uridylates in concert with other Tutases, certain *pre-let-7* RNAs and thereby promotes *let-7* biogenesis at the Dicer step (Heo et al., 2012). Upon growth factor signaling, *let-7* expression is inhibited through MAP kinase- dependendent phosphorylation of the Dicer co-factor TRBP (Paroo et al., 2009).

Once loaded into an Argonaute protein, miRNAs are thought to be remarkably stable. As the majority of the miRNA is buried in Argonaute (Elkayam et al., 2012) or engaged in target binding, sequence-specific regulation of miRISC loaded with specific miRNAs is difficult to imagine. One possibility of miRNA-specific regulation of miRISC on certain mRNAs is crosstalk to other RNA-binding proteins with a nearby binding site, as discussed in the previous section. Nevertheless, the TRIM-NHL protein TRIM32 binds to Ago1 in mouse neural progenitor cells and potentiates activity of only a subset of miRNAs, including *let-7* (Schwamborn et al., 2009). The mechanism of this activity is not known. Similarly ill defined is the role of *nhl-2* in the modulation miRNA activity. Although genetic evidence supports a positive role of *nhl-2* in promoting the

function of certain miRNAs (Hammell et al., 2009; Karp and Ambros, 2012), the molecular details of *nhl-2* function have not been elucidated. Activity of the miRISC can be modulated by post-translational modifications. Ago2 is subject to phosphorylation by the AKT3 kinase and this phosphorylation event shifts the balance between non-enzymatic target repression and slicing of miRISC towards cleavage, although this plays probably only an insignificant role *in vivo*, (Horman et al., 2013). This phosphorylation event slightly alters Ago2 localization and binding to GW proteins (Horman et al., 2013). Even if these results are far from being conclusive, given the likely relevance of subcellular compartmentalization of miRNA activity, e.g. in P-bodies (Liu et al., 2005) or at the ER (Stalder et al., 2013), as well as the potentially dynamic composition of the miRISC, post-translational modifications of miRISC components induced by different signaling pathway is a exciting area for further research.

The life of a miRNA ends with its unloading from Argonaute and its degradation. It has been recently shown that these are active processes. In *C. elegans* the 5'-3' exonucleases *Xrn-1* and *2* are involved in miRNA degradation (Chatterjee and Grosshans, 2009), the identity of the "miRNAse" in other organisms is not clear. Intriguingly, miRNAs have different half-lives in different biological contexts, and individual miRNAs differ in their decay rate. In retina cells e.g., some miRNAs show fast turn-over, but this is prevented by blocking electric activity (Krol et al., 2010a). As exonucleases like Xrn-1/2 are typically processive and not sequence-specific, miRNA release from Argonaute might be the rate-limiting step of miRNA degradation. In line with this, Ago2-bound siRNA was resistant to micrococcal nuclease *in vitro* and stable for a long period of time (De et al., 2013). *In vivo,* each miRNA molecule can direct the repression of more than one molecule of mRNA (Baccarini et al., 2011), miRNA unloading is thus not 1:1 coupled to target binding. Nevertheless, evidence for the involvement of target binding in miRNA release is accumulating. In one study, decay of a *tet*-regulated transgenic miRNA was accelerated by expression of a target mRNA (Baccarini et al., 2011). This coincided with untemplated addition of uridines to the 3' end of the miRNA (Baccarini et al., 2011). Such uridylation if reminiscent of results in *Drosophila* S2 cells, where miRNAs are tailed by addition of untemplated nucleotide and trimmed by the exonuclease *Nibbler* (Ameres et al., 2010; Han et al., 2011; Liu et al., 2011). Although uridylation-induced miRNA unloading and degradation is an attractive hypothesis, trimming of longer miRNAs is probably a step required for proper miRISC assembly in *Drosophila* (Han et al., 2011). To complicate matters further, target availability was found to stabilize miRNA levels in *C. elegans.* So far, the effect of target availability potentially modulating miRNA levels on other targets *in trans* has not been examined. At this point, it is not known how target levels shape miRNA stability under physiological conditions and which proteins are required for this regulation.

Finally, as miRNA activity is heavily influenced by the miRNA:target ratio (Doench and Sharp, 2004; Mukherji et al., 2011), changes in expression levels of a given target has a kinetic effect on regulation of other targets. According to this so-called competing endogenous RNA (ceRNA) hypothesis, all RNAs containing a particular miRNA target site are competing with each other for miRNA binding and could in principle regulate each other *in trans* by sequestering or diluting away miRNAs (Salmena et al., 2011). Importantly, this principle could apply to all kinds of RNA, including not only coding mRNAs, but also pseudogene mRNAs,

long non-coding RNAs or the recently discovered circular RNAs. As precise quantification of all potential miRNA targets in the cell is difficult and competition has indeed a strong quantitative aspect, experimental validation of this hypothesis in general is challenging. Functional proof of specific examples (Poliseno et al., 2010; Hansen et al., 2013; Memczak et al., 2013) warrants further testing the general importance of this model.

The let-7 microRNA and the *C. elegans* heterochronic pathway

Temporal regulation of larval development

The nematode worm *C. elegans* develops from an embryo to an adult by progression through four consecutive larval stages each separated by a molt. Each larval stage, is characterized by the stereotypic execution of an appropriate developmental program involving cell divisions, cell differentiation and production of an appropriate cuticle. In *wild-type (wt)* animals, these events are in their order invariant, although their absolute timing depends on the total length of larval development, which is largely influenced by the environmental temperature. Heterochrony describes alterations in timing of developmental events. Certain events can thus occur too early or too late relative to other events and relative to number of molts completed, in sum at the inappropriate stage. Precocious mutations lead to execution of certain events too early, thus skipping a certain event at that stage. Retarded phenotypes mean execution of the program too late or in other words the previous event is reiterated and displaces the next consecutive event to a later stage (Fig. 3). These phenotypes show that during *C. elegans* larval development each cell has a temporal identity. Although misspecification of temporal identity generally leads to apparently normal execution of the cell's characteristic developmental program at the novel, inappropriate, time point, this has dramatic, sometimes fatal consequences for the animal. Detection of such phenotypes allowed the identification of genes involved in specification of temporal identity, the core components of the heterochronic pathway: *lin-4, lin-14, lin-28* and *lin-29* (Chalfie et al., 1981; Ambros and Horvitz, 1984). As the number of genes with a heterochronic phenotypes increased, the proposed architecture of the heterochronic pathway became more and more elaborate. Based on genetic data and expression pattern observed by different more or less accurate methods, heterochronic genes have been e.g. assigned a role as "early timer" and "late timer", a master regulator vs. effector, or categorized into different hierarchies of regulation. These simple models are clearly unable to describe the genetic and functional data available to date. The remarkable robustness and yet adaptability of temporal regulation in larval development could be only achieved by a system featuring redundant and parallel pathways as well as feed-back and feed-forward loops, essentially prohibiting the proposal of simple models. A peculiar characteristic of the network is the extensive involvement of miRNAs in heterochronic regulation. To date heterochronic miRNAs include *lin-4* and the *let-7* family. As heterochronic phenotypes of miRNA pathway mutants are similar of those of *lin-4* or *let-7* (Grishok et al., 2001) and as *lin-4* and *let-7* are among the miRNAs with the highest expression, other miRNAs might play only a minor role in the heterochronic pathway.

In the hypodermis, the developmental processes under temporal control are on one hand cell division events, on the other differentiation events such as production of a specific cuticle or cell-cell fusions. The hypodermis is further divided into lateral and ventral compartments. The term lateral hypodermis is often used interchangeably with the seam-cell compartment, although it includes anatomically the much larger *hyp7* syncytium. At the beginning of larval development the *hyp-7* contains only 23 nuclei, to sustain animal growth, 116 cells with their nuclei will fuse until adulthood. 98 of these cells originate from seam cell divisions, which can be thus regarded as a stem-cell compartment. After hatching, 9 seam cells are present from three different lineages: H1-2, V1-6 and T. The T lineage largely differs between hermaphrodites and males, as it is responsible for formation of the sensory rays required for mating in males. In hermaphrodites, T lineage descendants exit the cell-cycle and differentiate already at the L3. H and V lineage cells divide at each larval stage in a stem-cell like manner: the anterior daughter cell fuses to the *hyp7* syncytium whereas the posterior daughter cell remains in the midline, elongates in its shape and continues dividing. The V cells, but not the H cell undergo a symmetric proliferative cell division at the early L2 stage, both daughter cells adopt the seam cell fate and do not fuse to the *hyp7.* After their last division in the L4 stage, all seam cells exit the cell cycle and finally fuse to each other after the L4/adult molt. This coincides with the production of specialized cuticular structures called alae.
Heterochronic mutants have been defined by observation of these events. In the early days, when researchers were still familiar with lineaging, this was performed by complete lineage analysis, at least in the lateral hypodermis. Importantly, the precise nature of e.g. a reiterated event was inferred from the observation of a specific division pattern occurring in a certain lineage, typically in the H or T lineage. However, this also means to some degree an interpolation of an observation in one lineage to another. This might be of importance, as in some cases, as shown for the H lineage in *lin-14* mutants, the progenitor cells are contrary to the prediction not exact copies of their parents (Chalfie et al., 1981) and for some alleles, there is a discrepancy between fates reiterated among different lineages (Ambros and Horvitz, 1984). It is in particular impossible to directly distinguish between asymmetric cell divisions of L3/L4 stages in V1,2,3,4,6 seam cell descendants. Later on, lineage analysis was often substituted by the use of surrogate endpoints, such as timing of alae synthesis, quantification of seam cell numbers and fusion as assessed by fluorescent reporters or expression of adult-specific collagen markers. An interesting observation is the variability of phenotypes in different alleles of the same gene, e.g. various *lin-14* alleles differ not only in the penetrance of a given phenotype, but also in the specific division reiterated or skipped (Ambros and Horvitz, 1987). This suggests graded effects and distinct thresholds for gene-activity. Another unanswered question in the heterochronic pathway is whether or not genes can be separated into regulators of timing and effectors. The first model, supported by the correct execution of otherwise normal developmental programs at the wrong time in heterochronic mutants, predicts that heterochronic regulation is superimposed onto a layer of master genes coordinating the appropriate developmental programs themselves. Candidate master effectors in this model are *lin-14, hbl-1* and the zinc finger transcription factor *lin-29.* So far, only *lin-29's* role as a master

regulator is supported by direct evidence showing that it can both upregulate the cell-cycle inhibitor *cki-1* (Harris and Horvitz, 2011*),* the fusogen *aff-1* (Friedlander-Shani and Podbilewicz, 2011) and the adult-specific collagen *col-19* while repressing the larval-specific *col-17* collagen (Liu et al., 1995; Rougvie and Ambros, 1995). In line with this, genetic screens for *lin-29* suppresors were unable to identify any gene acting between *lin-29* and the genes responsible for hypodermal phenotypes such as the collagens (Liu et al., 1995). On the other hand, the nuclear hormone receptor *nhr-25* , a downstream target of *lin-29,* has a conserved *let-7* binding site in its 3'UTR and *let-7* directly regulates cell-cycle genes in other organisms (Johnson et al., 2007), suggesting a direct regulation of developmental genes in parallel to *lin-29.* In line with this, *mir-84* overexpression could partially rescue a *lin-29* putative null allele (Hayes et al., 2006), although the resulting phenotype has not been characterized in detail and alternative explanations remain possible. Another enigma in the heterochronic pathway is the iterative nature of certain developmental events. Whereas the larval-to-adult transition occurs only once in the life of a worm and thus involves defined transition from one state to another. The situation during the larval stages is different, as certain seam cells undergo multiple times the same program of asymmetric cell-division and molting. This implies some kind of periodicity on gene expression, the genes required for cell-division and molting must accumulate and decrease in sync with these periodical events. In addition, stage specific factors must be present in order to execute the stage specific division patterns observed in certain lineages and some cue must link completion of the appropriate number of divisions to termination of larval development. Furthermore, the regulatory system must somehow adapt to environmental conditions, e.g. changes in temperature and consequent prolongation of development. This behavior is difficult to explain with a stepwise role for distinct regulators at each transition and with a strictly linear model of the heterochronic pathway.

Based on experiments and caveats described above, we can group genes with similar phenotypes together: Genes with a retarded loss-of-function phenotype include: *lin-4, let-7 family, lin-46 and lin-29.* The opposite group causing precocious defects consists of *lin-14, lin-28, lin-41, lin-42* and *hbl-1.*

The regulatory relationships between heterochronic miRNAs and their targets are described in later sections.

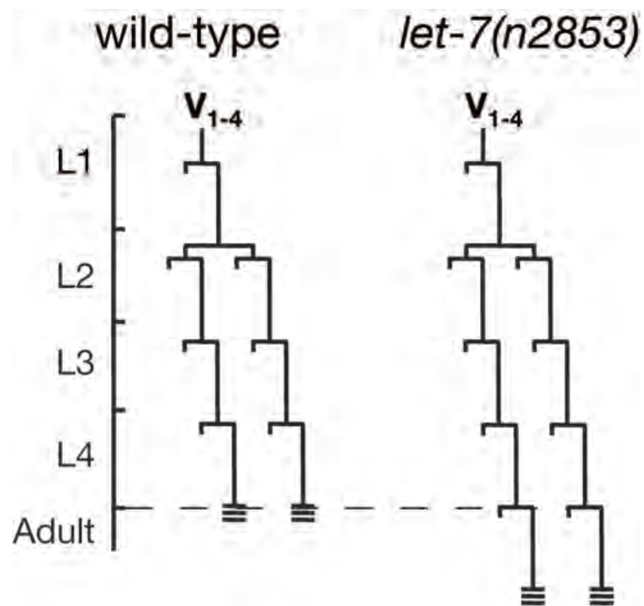

Fig. 3. Retarded hypodermal phenotype of *let-7(n2853)* worms
Loss of *let-7* leads to reiteration of the L4 division program in the V_{1-4} seam cell lineages.

The *C. elegans* vulva is developmentally closely related to the hypodermis as it originates from the ventral hypodermal P cell lineage. Vulva development involves several distinct stages. First, some of the P cells gain the competence to become vulva precursor cells. These cells are specified further by signals originating in the anchor cell to adopt the 1°, 2° or 3° fates (Fig. 4), undergo multiple rounds of cell divisions to finally form 22 vulva cells. Ongoing cell-cell signaling further subdivides the cells into ultimately seven subtypes and crosstalk to the uterine precursor cells ensures proper induction of distinct uterine cell-types. Starting from the early L4 stage, vulva cells invaginate and execute a complex morphogenesis program consisting of cell-movements and cell-cell fusions enabling formation of seven ring-like structures, the vulva toroids. Finally, establishment of connections to neighboring organs, seam, *hyp7,* uterus, vulval muscles and neurons, is necessary for the execution of the primary function of the vulva, namely egg-laying. So far, the early steps of vulva development have been shown to be under heterochronic control. *lin-14* or *lin-28* loss-of-function mutants execute the first two vulva precursor cell (VPC) divisions already at L2 stage, one stage earlier than *wild-type* (Euling and Ambros, 1996). Specifically, loss of *lin-14* or *lin-28* results in precocious entry into the S-phase of the cell-cycle (Euling and Ambros, 1996). Although the VPC progeny is superficially normally specified, apparently by the same signals as in *wt,* consistent with the idea of heterochrony, precocious VPCs show some abnormalities such as altered polarity of cell-divisions resulting in egg-laying defects (Euling and Ambros, 1996). As in these experiments *lin-14* or *lin-28* is inactivated in all tissues where expressed, it is not clear whether precocious VPCs gained the competence to precocious specification or heterochronic mutations advanced and synchronized the inductive signal from the anchor cell. In contrast to *lin-28, lin-14* has in addition to inhibiting cell-cycle progression also a more direct role in VPC signaling. Although *lin-4* loss-of-function or *lin-14* gain-of-function mutations have, similar to the hypodermis, delayed VPC

divisions (Chalfie et al., 1981; Ambros and Horvitz, 1987), this is not a typical retarded phenotype. Cells born after the delayed division are competent in their ability to respond to VPC 1° inductive signal, just like *wt* cells (Li and Greenwald, 2010). The normal cell fate is therefore simply delayed, but no characteristic event is reiterated. In contrast, the *vulvaless lin-14* phenotype is explained by the observation that *lin-4* blocks *lin-12* activity, the main lateral signal specifying the 2° cell fate (Li and Greenwald, 2010). The *lin-4/lin-14* genes thus provide a temporal gate for *lin-12* action. An interesting finding of the above study is that contrary to the hypodermis, *lin-28* downregulation does not contribute significantly to *lin-4* activity. Furthermore, *lin-14* acts as a vulva not as a classical heterochronic gene, but rather as a regulator of a specific signaling event. A similar function has been assigned to *mir-84* in restricting 1° VPC inductive signal to P6p cells by repressing *let-60* (Johnson et al., 2005), but this idea is supported only by indirect evidence using *mir-84* overexpression suppressing *let-60* multivulva phenotype.
Although *lin-4* and *let-7 family* miRNAs as well as other heterochronic genes are widely expressed, their role in other tissues is not well understood. There is evidence that they are involved in some aspects of neuronal development (Olsson-Carter and Slack, 2010; Thompson-Peer et al., 2012; Zou et al., 2012; Zou et al., 2013) and this might involve some kind of temporal identity in a broader sense, but they are not truly heterochronic functions. An interesting question is the function the heterochronic pathway in the *hyp7* synctium and in the intestine. Cells in both tissues undergo endoreplication starting from the L2 stage, but otherwise they do not have any readily apparent temporally regulated function. In the intestine, *lin-4* might be involved in the timing of the first endoreplicaion cycle (Ouellet and Roy, 2007). Recently, *let-7* has been implicated in innate immunity through activity in the intestine (Liu et al., 2013), adding an other unexpected and probably heterochrony-unrelated function to *let-7's* repertoire.

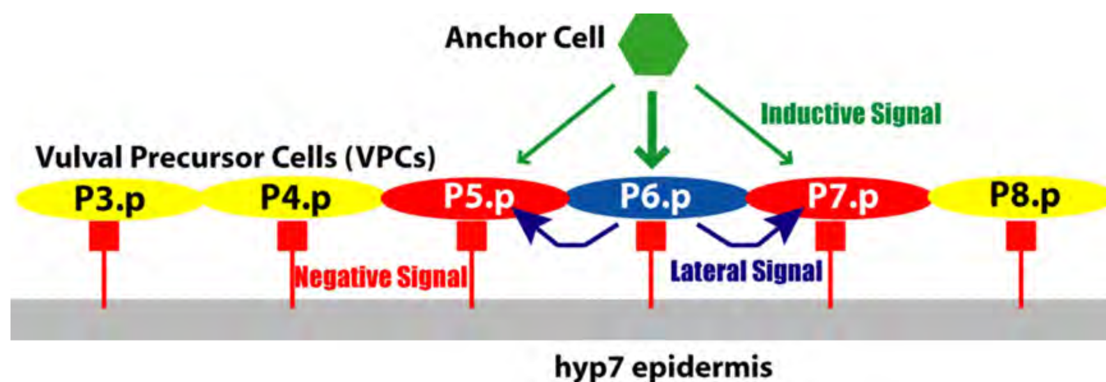

Fig. 4. Vulva precursor cell specification during the L3 stage
The inductive signal consists of LIN-3/EGF secreted by the anchor cell, which is transduced by LIN-23/EGFR and LET-60/RAS in P6.p. The lateral signal is mediated by *lin-12 /Notch.* Figure adapted from (Sternberg, 2005).

The let-7 microRNA: a special member of the family

let-7 was the second miRNA discovered in genetic screen for *lin-14/egl-35* suppressors (Reinhart et al., 2000). Identification of *let-7* RNA in a wide-variety

of species was a milestone in miRNA research (Pasquinelli et al., 2000). *let-7* is indeed conserved in its sequence at every nucleotide from worms to humans. Additionally, developmental regulation of *let-7* expression suggested also a conservation of function (Pasquinelli et al., 2000), especially since some of its targets were conserved. On a larger scale, this might be the case, *let-7* generally promotes exit from the cell-cycle, induces differentiation and counteracts tumor formation. Similar to its role in the *C. elegans* hypodermis, *let-7* defines temporal identity of neuroblasts located in the mushroom body of *Drosophila* brain (Kucherenko et al., 2012). Interestingly, *let-7* is regulated by ecdysone signaling and not *lin-28* and targets the Abrupt transcription factor, which is only observed in this model system, showing the context dependency of *let-7* regulators and targets. On the other hand, *let-7* functions cannot be generalized. The concept of heterochrony is not readily adaptable to other organisms and *let-7's* role in development has not been systematically studied in any model system apart from *C. elegans*. There is also increasing evidence for important *let-7* functions after development, i.e. during adult life, as shown by its involvement in glucose metabolism (Zhu et al., 2011). The search for *let-7* phenotypes is largely complicated by the possible redundancy between *let-7* family members. The *let-7* family includes a variable number of members in different organisms. miRNAs are grouped together based on a common seed-sequence. In most organisms miRNAs of a given family are designated by a small letter, e.g. *let-7 a-i* in humans. For historical reasons *C. elegans let-7* miRNAs have their own numbering, *e.g mir-48.* In *C. elegans let-7* is essential, presence of the other six family members cannot compensate for its absence. So far, characterization of let-7 knockout mice has not been reported, although some *let-7* deletion strains are available and apparently viable (mirKO-database; Park et al., 2012). It will be very informative to see whether crosses of these strains reveal redundancy between family members and whether loss of all *let-7* activity is compatible with life. A hint on the importance of *let-7* function comes from experiments manipulating levels of Lin-28 proteins. *Lin-28a* and *b* both block the biogenesis of the entire *let-7* family (Viswanathan et al., 2008). Although *Lin-28* has *let-7* unrelated functions, e.g. direct regulation of mRNAs (Xu et al., 2009; Cho et al., 2012; Hafner et al., 2013), when contribution of such processes to observed phenotypes is ensured, *Lin-28* overexpression and knock-down are good approximations for *let-7* knock-down or overexpression, respectively. *Lin-28* knock-out is predicted to cause a precocious expression of the *let-7* family. *Lin-28a* and *Lin-28b* knock-out mice are both viable, although smaller than their *wt* littermates (Zhu et al., 2010). *let-7* and *let-7* target levels have not been analyzed in this mice, it is therefore not clear whether *let-7* is regulated in early development primarily transcriptionally or precocious *let-7* expression is surprisingly tolerated. Constitutive overexpression of *let-7a/d/f* is also viable with some metabolic abnormalities, although in this experiment transgenic *let-7* contained the *wt* loop sequence in *pre-let-7* allowing regulation by *Lin-28* proteins (Frost and Olson, 2011) and can thus also not conclusively rule out a function for *let-7* during mouse development. Overexpression phenotypes of *Lin-28a* or *b* are probably dependent on expression levels. Moderate *Lin-28a* overexpression from a leaky inducible transgene is viable, but induction of *Lin28a* from the transgene resulted in gut abnormalities leading to death of the animals (Zhu et al., 2010). Developmental phenotypes of the inducible *Lin28b*

transgenic mice have not been described (Zhu et al., 2011). Modulation of *Lin-28* and as a consequence *let-7* levels in the above studies had a clear influence on glucose metabolism affected the timing of some developmental events, such as puberty. As a variant in the *let-7* target gene *Hmga2* is associated with height in population-based studies (Weedon et al., 2007) and the presence of a single-nucleotide polymorphism in the *LIN28B* gene correlates with earlier onset of puberty (Ong et al., 2009), *let-7* has been proposed to play a role in developmental timing of growth and puberty, analogous to their role in heterochronic regulation in *C. elegans.*
Redundancy and specificity among *let-7* family has two aspects, expression patterns and target specificity. There is ample evidence indicating that in mice and humans different *let-7* family members are expressed in different tissues and are differentially induced or inhibited by external signals (Cairo et al., 2010; Qian et al., 2011).
In mammalian cells, redundant target specificity among *let-7* family members is generally assumed, although this has not been investigated systematically and there is at least one report describing a differential effect of the overexpression of either *let-7b* or *let-7i* (Cimadamore et al., 2013).
In *C. elegans,* the *let-7* family comprises seven members: *let-7, mir-48, mir-84, mir-24, mir-793, mir-794, and mir-795* (Fig. 5). *let-7* clearly stands out from this group as it is essential. *let-7's* role in seam cell development is relatively well characterized, *let-7* loss-of function mutants reiterate a larval type seam cell division after the L4 molt and the seam cells fail to fuse together and do not secrete alae (Reinhart et al., 2000). *let-7* overexpression has the opposite phenotype, *let-7* is thus a *bona fide* heterochronic gene regulating the larval-to-adult transition. The three *let-7* "sisters" *mir-48/84/241* are also expressed or at least transcribed in the hypodermis as shown by promoter::GFP fusions (Esquela-Kerscher et al., 2005) and are also involved in the heterochronic control of hypodermis development. Among the three sisters, *mir-48* has a more prominent role in the hypodermis. *lin-58,* a regulatory mutation in the *mir-48* gene was identified in a screen for suppressors of the retarded *col-19::GFP* expression of *lin-4* mutants (Abrahante et al., 1998). Interestingly, *lin-58* suppressed retarded *col-19* expression and alae formation, but failed to suppress the supernumerary molting and vulvaless *lin-4* phenotypes. *lin-58* alone showed a precocious phenotype resulting in seam cell fusion and alae formation at the L3/L4 molt (Abrahante et al., 1998). As expected, combination of the precocious regulatory mutation with *mir-48* overexpression from multicopy arrays enhanced the observed phenotype (Li et al., 2005), but this finding did not reveal physiological *mir-48* target(s). More informative are the phenotypes of *mir-48* deletion mutants (Abbott et al., 2005). *mir-48* deletion leads to a supernumerary adult molt after seemingly normal larval-to adult transition. The penetrance of this phenotype was enhanced by the additional deletion of *mir-84.* Interestingly, in this double mutant, the seam cells displayed adult characteristics at the L4 molt, as they stopped dividing and secreted alae, whereas the *hyp7* cells failed to express the adult-specific collagen *col-19.* Intriguingly, deletion of the third sister, *mir-241,* caused a very different phenotype, as these *mir-48/241* double mutants or *mir-48/84/241* triple mutants have increased seam cell numbers at the L3 stage, due to reiteration of the symmetric L2 division in five V-lineage cells. Genetic experiments indicate, that this phenotype is not caused by *lin-28*

repression. Furthermore, *mir-48/84/241* likely interact with the *lin-46* heterochronic gene to downregulate the *hbl-1* transcription factor. The role of *lin-46* or *hbl-1* in the adult phenotype of *mir-48/84* has not been determined. *mir-84* cooperates not only with *mir-48,* but also with *let-7* itself in regulating of molting (Hayes et al., 2006), as deletion of *mir-84* enhanced the supernumerary molting phenotype and reduced the penetrance of adult specific *col-19* expression in a weak *let-7* mutant background. This study has to be interpreted with caution. The use of the relatively poorly characterized *let-7(mg279)* and in some experiments the temperature-sensitive *let-7(n2853)* allele at intermediate temperature does not allow to strictly define a genetic epistasis between *let-7* and *mir-84.* Furthermore, redundancy at the target level has not been demonstrated, lineage analysis has not been done and the identity of the cuticle, whether larval as in *let-7* mutants or adult as in *mir-48/84* double mutants, has not been determined. Interestingly, the reported enhancement of *let-7* phenotypes can be mostly assigned to the *hyp7* compartment rather then to seam cells.
In sum, the function of *let-7* family members, especially their site of action and their interaction with each other and with other heterochronic genes is not completely understood. To answer the open questions, careful and quantitative examination of miRNA expression patterns and target regulation is needed.

let-7: T**GAGGTA**GTAGGTTGTATAGTT
mir-48: T**GAGGTA**GGCTCAGTAGATGCGA
mir-84: T**GAGGTA**GTATGTAATATTGTA
mir-241: T**GAGGTA**GGTGCGAGAAATGA

Fig. 4. Alignment of *let-7* and its sisters.
Bold nucleotides indicate the common seed. Nucleotide sequences are shown at the DNA level from 5' to 3' .

microRNA-target relationships in the heterochronic pathway

The heterochronic pathway relies on the activity of *lin-4* and *let-7* family miRNAs. Information about the targets of these miRNAs has been gained mainly by genetic epistasis experiments and target reporter assays as the application of other methods commonly used in miRNA target identification such as quantification of RNA and protein levels is limited by the issue of analyzing a mixture of different tissues. Analysis of miRNA-target relationships is complicated by the fact that heterochronic miRNAs might have overlapping targets and by possible, but not proven, redundancy between members of a given miRNA family.
Expression of the *lin-4* miRNA starts from the early L1 stage and remains high until adulthood (Esquela-Kerscher et al., 2005), consistent with a role in preventing execution of earlier, L1, cell fates. The main *lin-4* targets in this context are *lin-14* and *lin-28. lin-14* encodes a nuclear protein that is highly expressed in embryos and in L1 worms, but is completely absent in older worms from the L2 stage on (Ruvkun and Giusto, 1989) and this downregulation is

dependent on *lin-4* activity (Arasu et al., 1991). *lin-14* has seven putative *lin-4* binding sites in its 3'UTR and these binding sites are necessary and sufficient to recapitulate *lin-14* regulation in a *lacZ* reporter (Ha et al., 1996). Interestingly, *lin-28* positively regulates *lin-14* expression and this is independent of *lin-4* and *lin-4* binding sites in the *lin14* 3'UTR (Arasu et al., 1991). The molecular mechanism underlying this observation is not known. Genetically, loss of the *lin-4* binding sites in the *lin-14* 3'UTR is phenotypically very similar to that of loss of *lin-4 (Ambros and Horvitz, 1987),* indicating that downregulation of *lin-14* is the main function of *lin-14,* at least to define the L1/L2 transition. Unfortunately, several issues remain. Targeting of *lin-28* is also well supported by experimental evidence. Expression of a functional *lin-28::Gfp::lin-28* transgene is post-transcriptionally downregulated in a *lin-4* and *lin-4* binding site dependent manner *(Moss et al., 1997).* Deletion of the *lin-4* binding site from the *lin-28* transgene not only resulted in continuous (mis-)expression of *lin-28,* but also caused retarded phenotypes. Most surprisingly, the phenotype was different from that of *lin-14(gain-of-function)* or *lin-4(loss-of-function).* Instead of reiterating the L1-like symmetric division, *lin-28* deregulated animals continuously reiterated the L2 proliferative division (Moss et al., 1997). By examining *lin-4* phenotypes in a complex *lin-14* background that is insensitive to *lin-4* regulation and has yet *wt* levels, it has been also shown genetically, that *lin-28* regulation by *lin-4* is relevant. This indicates a hierarchy between *lin-14* and *lin-28* deregulation: upon loss of *lin-4,* both genes are deregulated, but high *lin-14* levels lead to continuous reiteration of L1 fates, precluding appearance of the characteristic L2 proliferative division. Yet, it cannot be concluded that the *lin-14* and *lin-28* have explicitly separate roles in regulating the L1/2 and L2/3 transitions respectively. *lin-14* and *lin-28* positively crossregulate each other *(Arasu et al., 1991; Moss et al., 1997),* deregulation of one thus is probably linked to the deregulation of the other. Furthermore, lineage analysis of a handful *lin-14 alleles* showed that *lin-14* has two genetically separable activities, loss of activity *a* leads to precocious execution of L2 cell fates, whereas loss of activity *b* results in precocious L3/4 fates (Ambros and Horvitz, 1987). A simple model would predict that this is expression level dependent, activity *a* corresponding to high *lin-14* levels and activity *b* corresponding to intermediate levels. Alternatively, activity *b* could correspond to positive regulation of *lin-28.* Unfortunately, the molecular identity of the alleles used in the above study are not known in all cases, *lin-14* levels have not been examined and no epistasis analysis with *lin-28* has been carried out (Ambros and Horvitz, 1987). This leaves us with the conclusion, that *lin-4* is the key regulator of both L1/L2 and the L2/L3 progression by downregulating *lin-14* and *lin-28. lin-14* is probably more important for the L1/L2 transition, although it is clearly involved also in the next transition, possibly by promoting *lin-28* expression. Taken together, appearance of *lin-4* influences developmental transitions at two consecutive stages. This is by the mutual regulatory relationship between *lin-14* and *lin-28* creating a temporal gradient in their expression pattern as well as by the action of other heterochronic genes at the L3 stage, namely *lin-66,* the *let-7* miRNAs *mir-48/84/241* (Morita and Han, 2006) and *daf-12* (Antebi et al., 1998). What are the targets of *lin-14* and *lin-28* in early larval development? This question has gained surprisingly little attention. *lin-14* encodes a nuclear protein (Ruvkun and Giusto, 1989), whereas *lin-28* is cytoplasmic (Moss et al., 1997). Neither the molecular

function nor the targets of these proteins is known. The very strong genetic interaction between *lin-28* and the cytoplasmic scaffolding protein *lin-46* suggests a common function and/or common targets of these genes. Additionally, *lin-28* regulates biogenesis of *let-7 (Lehrbach et al., 2009; Vadla et al., 2012),* but this represents probably more a coupling of *lin-28* activity in L2 to promotion of the subsequent stage by *let-7*, similar to the relationship between *lin-14* and *lin-28.*

Following *lin-4,* the *let-7* sisters *mir-48/84/241* are the miRNAs with a major role in the heterochronic pathway. These miRNAs are expressed earlier than *let-7* and have been proposed to act as a "middle timer" by repressing *lin-28* and *hbl-1.* Indeed, deletion of all *let-7* sisters leads to reiteration of the L2 proliferative seam cell division (Abbott et al., 2005). It is not entirely clear whether *lin-28* downregulation contributes to this phenotype. Although the *lin-28* precocious phenotype is epistatic to the *mir-48/84/241* retarded phenotype, the *mir-48/84/241* could enhance the *lin-46* retarded phenotype also in the absence of *lin-28.* This has been interpreted to indicate *lin-28* independent activity of the *let-7* sisters (Abbott et al., 2005). The mutual suppression of *lin-28* and *lin-46* (Pepper et al., 2004), makes this relationship complicated, as the molecular mechanism underlying the genetic interaction between *lin-28* and *lin-46* is not clearly understood. In the above study, no change in the expression of a *lin-28::Gfp::lin28* reporter or endogenous *lin-28* levels was observed. As a contrast, by examining expression of a hypodermal *col-10:lacZ:lin-28* reporter, at least an interaction between the *lin-4* and *let-7* binding sites in the 3'UTR was found (Morita and Han, 2006). Molecularly, *hbl-1* is clearly a better target of the *let-7* sisters. Both an *hbl-1*::*Gfp*::*hbl-1* reporter and *col-10::lacZ::hbl1* reporter were downregulated from the L3 stage (Abrahante et al., 2003; Lin et al., 2003; Abbott et al., 2005) and this was partially dependent on the presence of *mir-48/84/241 (Abbott et al., 2005).* However, several questions remain. First, *hbl-1* seems to be strongly regulated also at the transcriptional level and is probably already absent in the seam cells by the L3 stage (Lin et al., 2003). Regulation by the *let-7* sisters at that stage therefore should not affect its function in the seam cell. Second, *hbl-1* possesses not only *let-7* binding sites, but also *lin-4* binding sites in its 3'UTR and *lin-4* is indeed involved in *hbl-1* regulation in the ventral nerve cord (Lin et al., 2003). The derepression of the *col-10::Gfp:*reporter was observed accordingly only in about a third of animals examined at the L3 stage (Abbott et al., 2005). Third, *hbl-1* seems to play a more important role in later stages, at the larval-to-adult transition, since loss of *hbl-1* induces precocious execution of the adult developmental program, seam cell fusion and alae formation (Abrahante et al., 2003; Lin et al., 2003). Moreover, after precocious fusion, seam cells divide again, suggesting that *hbl-1* is involved more in the regulation of differentiation rather than inducing seam cell proliferation. Additionally, it is not clear whether the genetic interaction between *let-7* miRNAs is reflected in redundancy in the regulation of common targets. Furthermore and most importantly, it is a major question why the *let-7 siters* cannot substitute for *let-7* activity in the *let-7* mutant, although knock-down of *hbl-1* by RNAi rescues *let-7* phenotypes (Lin et al., 2003).

Compared to *lin-4* and the *let-7* sisters, the targeting space of *let-7 proper* have been extensively explored by genetic, molecular and biochemical methods.

Collectively, theses experiments support a primary role for *lin-41* as a *let-7* target and proposed more than a dozen putative targets of unknown significance.
lin-41 is probably the key target of *let-7* mediating *let-7* effects in the hypodermis. *lin-41* has been discovered in a genetic screen for *let-7(n2853)* suppressors (Slack et al., 2000). Indeed, loss of *lin-41* completely suppressed the vulva bursting phenotype and partially restored proper timing of the larval-to-adult transition in *let-7(n2853)* animals (Slack et al., 2000). Loss of *lin-41* has a hypodermal phenotype opposite to *let-7,* namely precocious seam-cell fusion and alae formation. *Lin-41* protein levels, as assessed by a Gfp reporter, decrease during the L4 stage, the time when *let-7* is active. This downregulation was recapitulated by a hypodermal *col-10::lacZ* reporter assay and was dependent on *let-7* (Slack et al., 2000). The sequence requirements for *lin-41* 3'UTR regulation were extensively analyzed with this reporter system (Vella et al., 2004a; Vella et al., 2004b). Although the *lin-41* 3'UTR contains five predicted *let-7* binding sites, two sites termed LCS (*let-7* complementarity site) 1 and 2 were both necessary and sufficient for repression of the reporter in the L4 stage. Interestingly, this pair of target sites has some special features that contribute to efficient repression of *lin-41:* LCS1 and 2 are separated by a 27nt spacer, which is required both in specific sequence and length for regulation. LCS1 and 2 differ in the architecture of the seed base-pairing. *let-7* binding to LCS1 results in formation of a one nucleotide bulge in the seed region, whereas *let-7* binds to LCS2 by forming a G:U wobble at position six of *let-7*. Altering these features decreased or abolished regulation of the *lin-41* 3'UTR reporter. The two sites cooperate in a remarkable way, as either two LCS1 or two LCS2 separated by the linker sequence are not able to mediate repression, but reversing the order of the two had no negative effect. Furthermore, base pairing at the 5' end of the target site, in addition to interaction through the seed, is also necessary for 3'UTR regulation. The principles underlying these special requirements are not known.
Genetic evidence indicates that *lin-41* is the key, but not the only *let-7* target. E.g. suppression of the *let-7* retarded alae phenotype by the *lin-41* null allele is not complete (Slack et al., 2000). A candidate *let-7* target would be *hbl-1* as it has an important role in promoting the larval-to-adult transition, suppresses *let-7* phenotypes and has several predicted *let-7* target sites in its 3'UTR (Abrahante et al., 2003; Lin et al., 2003). Interestingly, this regulation could not be confirmed by a reporter assay in the hypodermis (Lin et al., 2003). This result indicates that genetic suppression of *let-7* loss is a good indication, but not a proof of being a *let-7* target. *let-7* targets have been predicted computationally on a larger scale and validated by suppression of *let-7* lethality (Grosshans et al., 2005). Showing again the high false-positive rate of target prediction, only 9 of 73 candidates suppressed *let-7* bursting. Of these candidates, the 3'UTR of *daf-12, let-60*, *pha-4, lss-4* and *die-1* conferred regulation to a reporter, although the extent of repression differed considerably (Grosshans et al., 2005). Similar to strategies in cell lines, novel *let-7* targets have been proposed based on mRNA and protein quantification or cloning of *let-7* complementary mRNAs. Cloning of mRNAs using a labeled miRNA primer as a primary screening method identified 40 putative *let-7* targets, five suppressing *let-7* bursting to some extent and one, an uncharacterized gene, causing vulva bursting upon overexpression (Andachi, 2008). Analysis of predicted *let-7* targets by targeted mass spectrometry in *wt vs.*

let-7(n2853) worms revealed regulation of 29 proteins, ten showing a genetic interaction with *let*-7 (Jovanovic et al., 2010). In another study, more than two thousand genes were differentially expressed at the mRNA level in wt *vs. let-7(n2853)* (Hunter et al., 2013). Although a handful of short-listed candidates suppressed *let-7* bursting or seam cell overproliferation, only three of them passed the criteria for *bona fide let-7* targets set by the authors and even these are somewhat special, as e.g. two have *let-7* target sites in their CDS (Hunter et al., 2013).

The above results reflect on one hand the experimental difficulties to identify miRNA targets in *C. elegans.* On the other, it also shows that the heterochronic pathway is truly non-linear, complicating the validation of predicted target. Finally, an intriguing possibility is that in *C. elegans* and specifically in the heterochronic pathway, target regulation differs from the situation in other experimental systems such as cell-culture. Heterochronic miRNAs might have only a few, or even a single, key targets and these targets might be those that were identified in genetic screens. The elucidation of their wiring and understanding of the principles governing heterochronic patterning requires analysis of miRNA target regulation in space and time in quantitative manner as well as more functional studies intended to examine the effect altered miRNA-mediated gene regulation at the individual target level.

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REVIEW

LIN-41/TRIM71: emancipation of a miRNA target

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***lin-41* (lineage variant 41)/*TRIM71* (tripartite motif 71) is well known for being a conserved target of the *let-7* (lethal 7) microRNA (miRNA), a regulatory relationship found in animals evolutionarily as distant as *Caenorhabditis elegans* and humans. It has thus been studied extensively as a model for miRNA-mediated gene silencing. In contrast, the developmental and molecular functions of LIN41 have historically received less attention. However, LIN41 proteins are now emerging as important regulators of cell proliferation and differentiation in stem and progenitor cells. Moreover, LIN41's functions appear to involve two distinct molecular activities; namely, protein ubiquitylation and post-transcriptional silencing of mRNAs. Thus, LIN41 is ready for a scientific life of its own.**

Caenorhabditis elegans lin-41 (lineage variant 41) was originally discovered more than a decade ago as a target of the highly conserved *let-7* (lethal 7) microRNA (miRNA) (Reinhart et al. 2000; Slack et al. 2000). This was also the time when miRNAs were beginning to emerge as a large and important class of regulators of gene expression in plants and animals (Lagos-Quintana et al. 2001; Lau et al. 2001; Lee and Ambros 2001; Reinhart et al. 2002). However, very few miRNA targets had been validated, and among the known targets, *lin-41* stood out in that orthologous proteins could be identified in other organisms, including mice and humans (Slack et al. 2000). Yet more strikingly, even the regulation by *let-7* was conserved for these orthologs (Kloosterman et al. 2004; Schulman et al. 2005; Lin et al. 2007). It is thus hardly surprising that *lin-41* quickly became an intensely studied model miRNA target. Somewhat paradoxically, then, a focus on the mechanism of *lin-41* regulation by *let-7* also meant that our understanding of the molecular and developmental functions of LIN-41 itself has lagged behind. Recent studies have begun to change this and identified LIN-41 and the orthologous tripartite motif 71 (TRIM71) proteins as regulators of stem and progenitor cell proliferation and differentiation that can silence mRNA and drive protein ubiquitylation (Rybak et al. 2009; Chang et al. 2012; J Chen et al. 2012; Loedige et al. 2012). Here we discuss these exciting novel insights into the molecular and developmental biology of *lin-41*. For simplicity, we avoid, in the following, species-specific nomenclatures and refer to the gene and mRNA as *LIN41* and the protein product as LIN41, respectively.

[*Keywords*: RING domain; development; differentiation; stem cell; self-renewal; ubiquitin]
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Prelude: *LIN41* as a target of the *let-7* miRNA

LIN41 is a member of the TRIM-NHL family of proteins (for review, see Wulczyn et al. 2011). The family name derives from the tripartite motif of RING (really interesting new gene) finger, B-box(es), and coiled-coil domain (accordingly also named RBCC) and, typically, six NHL repeats at the C terminus (Fig. 1). (Here, NHL stands for NCL-1, HT2A2, and LIN-41, after the proteins in which this motif was first discovered [Slack and Ruvkun 1998].) Initially, *LIN41* was identified as a suppressor of phenotypes caused by a *let-7* loss-of-function (*lf*) mutation as well as a regulator of temporal cell fates in *C. elegans* larvae (Slack et al. 2000), as discussed in more detail in a later section. The genetic data supported *let-7* functioning as a negative regulator of *LIN41*, and parallel work identified *let-7* as a short noncoding RNA (Reinhart et al. 2000), now known as a miRNA, which had potential to bind to partially complementary sites in the *LIN41* 3′ untranslated region (UTR). Subsequent studies could confirm direct regulation of *LIN41* by *let-7* and identified sequence elements that generate functional *let-7* target sites (Vella et al. 2004a, b; Long et al. 2007) and revealed *LIN41* mRNA degradation (Bagga et al. 2005) and translational repression (Ding and Großans 2009) as modes of *let-7* activity. Collectively, these findings were highly influential in building a general framework for our understanding of miRNA function, helped in part by the fact that *let-7* sequence and function appeared highly conserved in animals. Specifically, *let-7* orthologs, known as *let-7a* in some organisms, are 100% conserved in sequence and present in most animals (Pasquinelli et al. 2000; Lagos-Quintana et al. 2002). Moreover, *let-7* was shown to repress fly (O'Farrell et al. 2008), zebrafish (Kloosterman et al. 2004), mouse (Kanamoto et al. 2006; Rybak et al. 2009), chicken (Kanamoto et al. 2006), and

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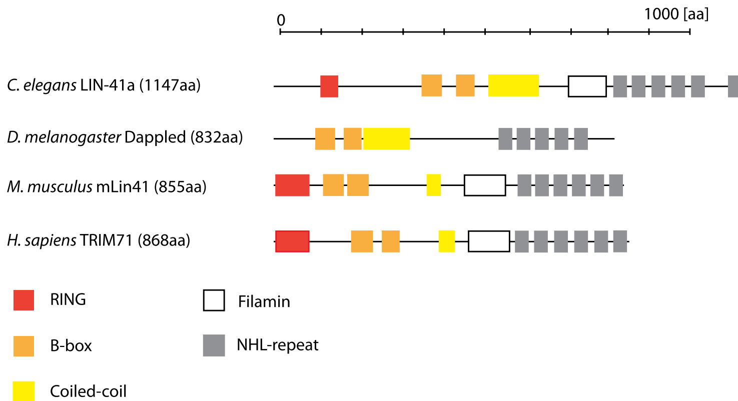

Figure 1. Domain architecture of LIN41 proteins from different species. The indicated domains were identified using SMART (http://smart.embl-heidelberg.de) and CDART (http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi) algorithms. Domain sizes are approximately to scale. Note that two isoforms have been reported for *C. elegans*, LIN41a and LIN41B, which only differ by three amino acids (Slack et al. 2000).

human (Lin et al. 2007) *LIN41* orthologs in 3′ UTR reporter assays. Finally, ectopic expression of *let-7* decreased endogenous LIN41 protein levels in a mouse embryonic carcinoma cell line (Rybak et al. 2009).

Surprisingly then, whereas extensive research on *let-7* over the past decade has established it as a regulator of a wide range of processes in development (Mondol and Pasquinelli 2012), metabolism (Zhu et al. 2011), and human disease (Büssing et al. 2008), a contribution of *LIN41* repression to these *let-7* functions has not been investigated. This is despite the fact that the evolutionary conservation of *LIN41* regulation by *let-7* makes a strong case for *LIN41* being a key *let-7* effector, even when taking into account that most experiments involved the use of reporters and/or *let-7* overexpression rather than regulation of endogenous *LIN41* under physiological conditions. By extension, the evident importance of *LIN41* regulation suggests that *LIN41*'s yet elusive molecular functions might have significant impact on cellular homeostasis, and this idea is strongly supported by recently published data discussed in the following sections.

An emerging theme: LIN41 controls cell proliferation and differentiation in vertebrate and invertebrate development

C. elegans seam cells divide in an asymmetric, stem cell-like fashion in a characteristic pattern during each larval stage (Sulston and Horvitz 1977). At the larval-to-adult (L/A) transition, the seam cells exit the cell cycle, fuse, and differentiate to secrete specialized collagenous structures called alae. The timing of these events is governed by the heterochronic pathway (Ambros and Horvitz 1984). Reduced levels of LIN41 lead to precocious execution of the L/A transition, as evidenced, for instance, by alae secretion and cell cycle exit in larvae. In contrast, *LIN41* overexpression—or *let-7lf*—results in a retarded L/A transition, which promotes continued division of even adult seam cells, while preventing them from fusing and secreting alae (Reinhart et al. 2000; Slack et al. 2000). Epistasis analysis placed *LIN41* downstream from the *let-7* miRNA and upstream of the *lin-29* zinc finger transcription factor (Slack et al. 2000). Indeed, it has been suggested that *let-7* acts primarily through *LIN41* because RNAi-mediated depletion of *LIN41*, but not another putative target, *hbl-1*, almost completely suppressed aberrant seam cell divisions in *let-7(0)* mutant adult animals (Vadla et al. 2012). However, we note that *lin-41lf; let-7(0)* double-mutant animals generate alae, like wild-type animals, only at the L4-to-adult molt (Slack et al. 2000). In contrast, precocious alae formation at the L3 molt, a hallmark of *lin-41lf* single-mutant animals, is rare (Slack et al. 2000). These observations are inconsistent with the notion of a simple, linear pathway. It is therefore possible that distinct *let-7* targets are key to regulating proliferation and differentiation, respectively, although this remains to be determined.

Although the hypodermis is the tissue where *LIN41* function has been studied in most detail, *LIN41* appears to play broader roles in *C. elegans* development. In addition to the hypodermis, a GFP-LIN41 fusion protein was observed in neurons, muscle cells, and the somatic gonad, in all of which it might accumulate in a *let-7*-insensitive manner (Slack et al. 2000). As *LIN41* mutant worms display gut defects (Del Rio-Albrechtsen et al. 2006) and are sterile due to a failure to produce oocytes (Slack et al. 2000), widespread expression appears functional, although the basis of the respective phenotypes remain to be determined. In *C. elegans* males, *LIN41* further functions in morphogenesis of the tail tip. This process is temporally regulated (Nguyen et al. 1999) and highly sensitive to *LIN41* dosage, as demonstrated by graded RNAi and allelic complementation studies (Del Rio-Albrechtsen et al. 2006). Similar to the hypodermis, reduced *LIN-41* activity results in precocious, increased activity in retarded phenotypes (Del Rio-Albrechtsen et al. 2006). Mutations altering the LIN41 N terminus outside annotated domains cause the male tail tip but no hypodermal phenotypes, suggesting either a different threshold or, alternatively, different functions or interaction partners of LIN41 in these two tissues. Indeed, LIN-29, which we discuss in more detail in a later section, is a major effector of LIN41 functions in the hypodermis but dispensable in the tail (Del Rio-Albrechtsen et al. 2006).

The conservation of *LIN41* sequence and regulation by *let-7* in various animals as well as the fact that *LIN41* expression is temporally and spatially regulated during development in animals where this has been investi-

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gated, strongly imply that LIN41 proteins have important functions in a wide range of animals and developmental contexts. This notion is supported by studies of specific loss-of-function phenotypes in zebrafish (Lin et al. 2007) and mice (Maller Schulman et al. 2008; J Chen et al. 2012). In zebrafish, knockdown of *LIN41* leads to partially penetrant embryonic lethality, with surviving embryos showing developmental abnormalities such as a short trunk, abnormal yolk shape, and an S-shaped tail (Lin et al. 2007). Mice carrying a homozygous *LIN41* "gene trap" mutation, which depletes *LIN41* by providing a strong splice acceptor site that will cause endogenous *LIN41* transcripts to missplice and terminate prematurely, start to die at around embryonic day 8.5 (E8.5), and all embryos are dead by E13.5–E16.5 (Maller Schulman et al. 2008; J Chen et al. 2012). The most striking phenotype of these *LIN41lf* mice is a neural tube closure defect (Maller Schulman et al. 2008; J Chen et al. 2012), although this is apparently not the cause of lethality (Maller Schulman et al. 2008). J Chen et al. (2012) could subsequently pinpoint decreased proliferation rate and increased differentiation of neural progenitor cells, caused at least in part through defects in fibroblast growth factor (FGF) signaling, as the basis of the neural tube closure defect.

It is also in mice where *LIN41* expression has been studied in the most detail, including by analysis of endogenous promoter activity (Maller Schulman et al. 2008; Yu et al. 2010; J Chen et al. 2012), mRNA in situ hybridization (Lancman et al. 2005; Schulman et al. 2005), immunohistochemistry (Rybak et al. 2009; Yu et al. 2010), and Western blot analysis (Rybak et al. 2009; Yu et al. 2010; J Chen et al. 2012). Generally, *LIN41* expression decreases over development (Schulman et al. 2005; Yu et al. 2010; J Chen et al. 2012). In particular, following ubiquitous expression early, *LIN41* transcription becomes gradually restricted to some neural tissues and the limb buds during embryogenesis (Maller Schulman et al. 2008). In neural tissue, LIN41 protein levels, measured by Western blotting, are high in neuroepithelial cells of early embryos (until E11.5) but undetectable as neurogenesis—and thus neural differentiation—proceeds (J Chen et al. 2012).

LIN41 expression not only correlates temporally with proliferative processes, but is also generally high in the proliferative, progenitor compartments of tissues where this has been characterized in more detail. Whole-mount staining of early embryos at E7 showed that LIN41 distribution is similar to that of the pluripotency factor Oct4, with staining in the embryonic ectoderm and, less strongly, the ectoplacental cone (Rybak et al. 2009). LIN41 was also detected in the interfollicular stem cells of both embryonic and adult epidermis as well as in postnatal testis, where it was found in gonocytes, early spermatogonia, and spermatocytes (Rybak et al. 2009). Interestingly, LIN41 mRNA or protein and *let-7* display largely inverse expression patterns in skin and testis, with *let-7* staining, for instance, being visible in the subrabasal cell layer rather than the basal stem cell layer, where LIN41 accumulates (Rybak et al. 2009). Taken together with the demonstration that LIN41 levels in mouse embryonic stem (mES) cells decrease during differentiation in parallel with up-regulation of *let-7* (Chang et al. 2012), this suggests that *let-7* plays a major role in shaping *LIN41* expression during mouse—and, possibly more global, animal—development.

At the same time, it is worth keeping in mind that even for a notorious miRNA target such as *LIN41*, regulation by a miRNA is just one among many regulatory inputs. For instance, whole-mount in situ hybridization against endogenous *LIN41* mRNA in FGF signaling-deficient mice suggests that expression of *LIN41* in limb buds is dependent on FGF signaling, similar to the situation in chickens in which Shh and Fgf signaling induces *LIN41* expression (Lancman et al. 2005). Interestingly, *LIN41* itself appears to promote FGF signaling during mouse embryonic brain development (J Chen et al. 2012), which implies that LIN41 might stimulate its own expression. Furthermore, the *LIN41* promoter and first intron contain three putative E-boxes that can be bound by the *MYC* transcription factor in cell lines (YL Chen et al. 2012). In luciferase reporter experiments, these elements can mediate transactivation of the *LIN41* promoter by ectopic *MYC* expression (YL Chen et al. 2012). Although the physiological significance of this finding is not clear, *MYC* is, similar to *LIN41*, highly expressed and has an important function in ES cells (Kim et al. 2010). Moreover, in addition to transcriptionally activating *LIN41*, *MYC* relieves it from post-transcriptional repression by repressing *let-7* directly (Chang et al. 2008) and through increased transcription of the *let-7* inhibitor *LIN28* (Chang et al. 2009; Dangi-Garimella et al. 2009). This exquisite regulation by at least two different mechanisms might point to a need to ensure sufficiently high *LIN41* expression in ES cells in a robust and faithful manner.

Taken together, *LIN41* expression in mice and worms appears to be a marker of a uni- or multipotent self-renewing state. Given this expression pattern as well as the functions of *LIN41* in mouse neural progenitor (J Chen et al. 2012) and *C. elegans* seam cells (Slack et al. 2000), an emerging principle of *LIN41* activity in developmental processes and tissues seems to be promotion of proliferation and inhibition of differentiation. Consistent with this notion, *LIN41* has been suggested to have a tumor-promoting role in human hepatocellular carcinoma (HCC): It is overexpressed in many tumor samples, and high levels correlate with poor patient survival (YL Chen et al. 2012). Moreover, *LIN41* overexpression in HCC cell lines resulted in increased tumor growth in a subcutaneous xenograft model, which can probably be explained by an increased proliferation rate (YL Chen et al. 2012). In this regard, LIN41 may generally, in diverse organisms and developmental events, function as an important effector of the LIN28–*let-7* "switch" between a self-renewing, proliferative stem cell or progenitor state, and differentiation (Melton et al. 2010). This idea is consistent with available data indicating that LIN28, a small RNA-binding protein, promotes proliferation and represses differentiation at least in part through repressing *let-7* biogenesis (Rybak et al. 2008; Viswanathan et al.

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2008; Vadla et al. 2012) and that *let-7* conversely promotes differentiation and represses proliferation while repressing *LIN41*. How LIN41 in turn controls the two processes of differentiation and proliferation is less clear, and we discuss in the next section potential molecular targets of, and mechanisms of regulation by, LIN41 (Fig. 2).

A multitude of targets: cell cycle mRNAs and a signaling protein as LIN41 effectors

Genetic analysis in *C. elegans* identified *lin-29* as a factor functioning downstream from *lin-41* in the control of differentiation and proliferation of seam cells (Slack et al. 2000). LIN-29 is a transcription factor and appears to provide a direct link to the cell cycle machinery, as it can stimulate transcription of the cell cycle inhibitor *cki-1* (Hong et al. 1998). Moreover, LIN-29 promotes cell differentiation, although, with the exception of collagen genes (Rougvie and Ambros 1995), the relevant targets in this process largely remain to be established. The fact that *lin-29* mRNA accumulates two larval stages before LIN-29 protein becomes detectable (Rougvie and Ambros 1995; Bettinger et al. 1996), that *LIN41lf* mutations cause precocious LIN-29 accumulation in seam cells (Slack et al. 2000), and that a GFP-LIN41 fusion protein localizes to the cytoplasm (Slack et al. 2000) was taken to speculate that LIN41 could be a translational repressor of *lin-29* (Slack et al. 2000).

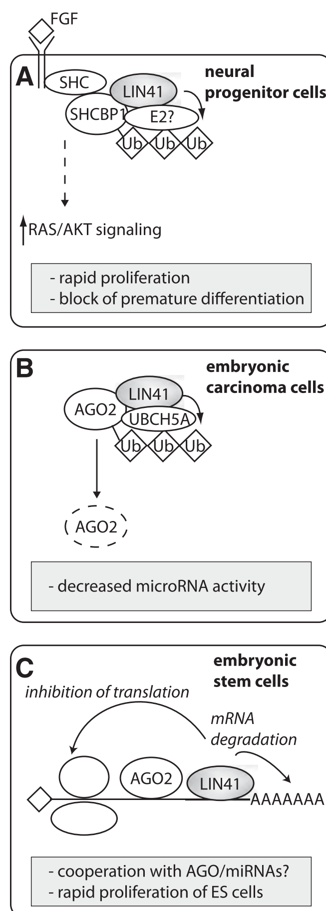

Figure 2. Proposed models of LIN41 function. (*A*) LIN41 ubiquitylates SHCBP1 to augment FGF signaling, increase proliferation rate, and inhibit premature differentiation in neural progenitor cells (J Chen et al. 2012). (*B*) LIN41 ubiquitylates AGO, inducing its degradation by the proteasome, leading to decreased miRNA activity (Rybak et al. 2009). (*C*) LIN41 induces degradation and translational inhibition of target mRNAs such as *CDKN1a*, promoting rapid proliferation of ES cells (Chang et al. 2012; Loedige et al. 2012). See the text for details.

Although experimental data for such an activity of LIN41 on *lin-29*—and, in fact, for *lin-29* as a direct target of LIN41—are still lacking, evidence is now accumulating that LIN41 proteins are indeed post-transcriptional, potentially translational, silencers of mRNAs. Consistent with this notion, modulation of LIN41 levels changes the accumulation of numerous mRNAs and proteins in mouse and human cells, and in several instances, the 3′ UTRs of potential target genes are sufficient to recapitulate these effects for target reporters (Chang et al. 2012; Loedige et al. 2012). Moreover, LIN41 can directly repress translation of a luciferase reporter mRNA when artificially tethered to its 3′ UTR of a luciferase reporter (Chang et al. 2012; Loedige et al. 2012). Silencing of either type of reporter is typically accompanied by some decrease in mRNA levels (Loedige et al. 2012). Finally, for a subset of these targets, it was shown that ectopically expressed LIN41 could coimmunoprecipitate the relevant endogenous mRNAs (Loedige et al. 2012). Thus, at least when expressed in HEK293 or mES cells, LIN41 displays several hallmarks of a translational repressor, although the mechanisms of repression remain to be elucidated.

How is LIN41 recruited to mRNAs? The LIN41 paralog and translational repressor BRAT might provide a model, as it is thought not to bind mRNAs directly, but rather in a complex with the pumilio protein PUF and the unspecific RNA-binding protein Nos (Sonoda and Wharton 2001). However, although both the *hb* mRNA and the *cyclin B* 3′ UTR contain Nos response elements (NREs), which recruit a NOS/PUF complex, BRAT only binds to the complex on the *hb* NRE (Sonoda and Wharton 2001). Thus, mRNA features such as specific sequences or secondary structures seem to contribute to BRAT recruitment. It has not been possible so far to establish the requirements of target repression by LIN41. Although it interacts with several RNA-binding proteins, these interactions are mostly RNA-dependent (Loedige et al. 2012) and thus presumably not responsible for recruitment of LIN41 to RNA. Moreover, human pumilio proteins are not required for LIN41 activity in HEK293 cells, and on the mRNA side, there is no apparent consensus sequence or binding motif that would explain the interaction with LIN41 (Loedige et al. 2012).

Insights into LIN41's mode of action come from structure–function analyses. The coiled-coil and the filamin domain mediate mRNA repression in tethering assays but alone are not sufficient to recapitulate *lin-41* activity in the context of a free mRNA (Loedige et al. 2012). Moreover, whereas a construct lacking the TRIM domain (i.e., RING, B-box, and coiled-coil domains) can coimmunoprecipitate LIN41 target mRNAs, a construct lacking

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the NHL domain cannot (Loedige et al. 2012). Combined with the fact that NHL domains are structurally similar to the more widely studied WD40 propellers, which have been shown to mediate both protein–protein interaction (Xu and Min 2011) and RNA binding (Lau et al. 2009; Castello et al. 2012), it seems possible that the NHL domain could mediate specific, and possibly direct, binding of target mRNAs. A domain swap experiment with Trim32 supports a key role of the NHL repeats in determining target specificity: Trim32 containing the NHL repeats of LIN41, but not wild-type Trim32, represses luciferase reporters containing the 3′ UTRs of LIN41 targets, whereas LIN41 containing Trim32 NHL repeats is inactive (Loedige et al. 2012).

Is mRNA silencing a major activity of LIN41 in regulating cell differentiation and proliferation in development? In support of this notion, the cyclin-dependent kinase inhibitor *Cdkn1a* was identified as a *LIN41* target in mES cells, where *LIN41* promotes G1-to-S-phase transition and thus increases proliferation (Chang et al. 2012). Specifically, Cdkn1a levels were increased when *LIN41* was depleted by siRNAs, and combined knockdown of *LIN41* and *Cdkn1a* rescued the slow proliferation rate as well as the G1-phase accumulation of *LIN41*-deficient cells (Chang et al. 2012). These data are consistent with, although not a direct proof of, *LIN41* regulating cell proliferation in these cells through repression of *Cdkn1a*.

Loedige et al. (2012) sought to obtain a more global picture of the effects of *LIN41* on gene expression and found that ectopic expression of *LIN41* in HEK293 cells caused the expression levels of >800 genes to change significantly, whereas knockdown of *LIN41* in mES cells affected ∼100 genes. The 3′ UTRs of selected dysregulated genes were sufficient to confer post-transcriptional regulation by LIN41 to a reporter gene, with targets thus validated including genes with cell cycle-promoting but also inhibitory roles. For instance, the retinoblastoma-like transcription factors Rbl1 and Rbl2 as well as E2F7 are inhibitors of the cell cycle; their repression might thus explain the increased proliferation rate observed in ES cells with high levels of LIN41. On the other hand, LIN41 also repressed positive regulators of the cell cycle such as cyclin D and cyclin E or Myb. The significance of these findings is currently not clear. It is also currently unknown which target mRNAs, if any, might mediate LIN41's effects on differentiation.

In addition to regulating mRNAs, mouse LIN41 was recently shown to bind to and stabilize Shcbp1 protein in mouse neural progenitor cells. Shcbp1 is a putative mediator of FGF signaling, and *Shcbp1* knockdown impairs FGF signaling in neural progenitor cells (J Chen et al. 2012). FGF signaling, like LIN41 activity, promotes neural precursor proliferation and represses differentiation (Guillemot and Zimmer 2011). Moreover, neuroepithelial cells from *LIN41*-deficient mice have decreased activity of the FGF pathway in vivo and are hyporesponsive to FGF stimulation in vitro (J Chen et al. 2012). Although it remains to be formally demonstrated that LIN41 affects FGF signaling by stabilizing SHCBP1 in neural progenitor or other cells, it therefore seems possible that a major function of LIN41 in this developmental context is to promote FGF signaling to control cell proliferation and differentiation status.

Finally, as we discuss next, LIN41 has been shown to polyubiquitylate Argonaute (Ago), the core component of the miRNA-induced silencing complex (miRISC), which mediates mRNA degradation and translational repression by miRNAs. Since global impairment of miRNA activity has been shown in several examples to promote cell proliferation (Kumar et al. 2007; Hill et al. 2009; Melo et al. 2009, 2010), it is conceivable that ubiquitylation-dependent Ago degradation would mediate (some of) LIN41's effects on cell proliferation and differentiation.

LIN41 and miRNAs: a complex relationship

Several TRIM-NHL family members have been found to regulate gene expression by modulating the miRNA pathway, suggesting a model of how LIN41 could regulate targets such as *lin-29*. For instance, *C. elegans nhl-2* (Hammell et al. 2009), *Drosophila mei-P26* (Neumüller et al. 2008), and mouse *Trim32* (Schwamborn et al. 2009) influence miRNA activity either negatively or positively by interacting with Ago proteins. Intriguingly, a proteomic analysis identified LIN41 in immunoprecipitates of *C. elegans* Dicer, the enzyme that processes precursor miRNAs to mature miRNAs (Duchaine et al. 2006). However, this interaction has not been examined in great detail, and a functional relevance remains to be determined. In contrast, an interaction between LIN41 and Ago has been observed in various human and mouse cell lines. Specifically, several studies showed that LIN41 localizes at least in part to P-bodies, as assessed by immunofluorescence staining of endogenous LIN41 in KH2 ES cells (Chang et al. 2012) and embryonic carcinoma cells (Rybak et al. 2009) or staining tagged and overexpressed LIN41 in HEK293 (Loedige et al. 2012) and HeLa cells (Rybak et al. 2009). LIN41 not only resides in these sites of mRNA storage and degradation where Ago is also found (Kulkarni et al. 2010), but in fact physically interacts with Ago (Rybak et al. 2009; Chang et al. 2012; J Chen et al. 2012; Loedige et al. 2012), albeit in a largely RNA-dependent manner (Loedige et al. 2012).

Functionally, LIN41 was found to promote Ago ubiquitylation in vitro and in mouse embryonic carcinoma cells, where this resulted in proteasome-mediated Ago decay (Rybak et al. 2009). Repressive effects of LIN41 on Ago levels and on the function of several miRNAs have recently also been reported for HCC cell lines, although an involvement of Ago ubiquitylation was not examined in this study (YL Chen et al. 2012). Irrespective of mechanism, the observation of altered Ago levels raised the attractive hypothesis that LIN41, by antagonizing miRNA activity globally, could constitute a double-negative feedback loop with *let-7* to create a bistable switch at the crossroad between the stem cell state and differentiation. The notion also provided an alternative explanation to earlier genetic data, which had shown that *LIN41* depletion in worms could suppress developmental phenotypes seen with reduced activity of core components of

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the miRNA pathway (Grishok et al. 2001; Büssing et al. 2010). Since these phenotypes, vulval bursting and alae defects, are the same that are also found in *let-7lf* animals, it was originally assumed that the *let-7* defect was predominant in the miRNA pathway mutant animals and accordingly suppressed upon *LIN41* knockdown. The new data (Rybak et al. 2009) offered the alternative possibility that also in *C. elegans*, LIN41 could more directly modulate miRNA activity.

However, although ubiquitylation activity of LIN41 on Ago was confirmed in an independent study (Loedige et al. 2012), the functional consequences remain unclear. In particular, no changes in Ago2 levels or stability could be observed upon *LIN41* knockdown in mES cells (Chang et al. 2012) or *LIN41* overexpression in HEK293 cells (Loedige et al. 2012). Similarly, there was no difference in Ago2 levels between a wild-type and a *LIN41*-overexpressing neural progenitor cell line (J Chen et al. 2012). More importantly, neural progenitor cells of wild-type and *LIN41lf* mice had similar Ago2 levels and ubiquitylation patterns in vivo (J Chen et al. 2012).

Although it is unclear why the results in the earlier (Rybak et al. 2009) and later (Chang et al. 2012; J Chen et al. 2012; Loedige et al. 2012) studies differ, collectively, these data indicate that regulation of Ago2 levels and thus global miRNA activity through polyubiquitylation by LIN41 does not seem to be a general phenomenon. As both studies that observed Ago2 decline upon LIN41 overexpression used transformed cell lines (Rybak et al. 2009; YL Chen et al. 2012), different LIN41 cofactors, altered baseline Ago2 stability, or levels in malignant cells might explain the observed differences.

Regardless of the possibility that LIN41 may alter miRNA activity globally, several LIN41 targets are also under miRNA control, and it has been suggested that similar to NHL-2, LIN41 and miRNAs may collaborate in target repression. For instance, in mES cells, *Cdkn1a* is regulated by both miR-302 and LIN41 via its 3′ UTR. Consistent with LIN41 activity depending on miR-302 activity and vice versa, a reporter containing a 59-nucleotide (nt) fragment of the *Cdkn1a* 3′ UTR that contained a validated miR-302-binding site was equally desilenced whether LIN41 and miR-302 were inactivated individually or jointly (Chang et al. 2012). Moreover, whereas *LIN41* overexpression in wild-type ES cells promoted their proliferation, this effect was abrogated in *Dgcr8* knockout cells, which lack most miRNAs (Chang et al. 2012). In contrast, examination of endogenous *Cdkn1a* mRNA showed that joint depletion of *LIN41* and miR-302 additively stabilized its levels, which would argue against their cooperation (Chang et al. 2012). However, Cdkn1a protein levels were not examined in this experiment, whereas the reporter assay determined luciferase activity, which would integrate both mRNA and protein level changes. An intriguing but speculative possibility could be that LIN41 and miR-302 activities on mRNA levels are independent, whereas further repression of protein accumulation might require a cooperative mechanism.

In contrast to the findings on *Cdkn1a* regulation, examination of a larger set of LIN41 targets revealed that many of these remained unchanged upon Ago depletion (Loedige et al. 2012), suggesting that LIN41 can silence them independently of miRNA activity or can at least tolerate compromised miRNA activity. Moreover, ectopic expression of both *miR-302* and *LIN41* in HEK293 cells additively silenced an *E2F7* reporter (Loedige et al. 2012). Although the latter experiment leaves open the possibility that LIN41 and miRNAs cooperate under physiological conditions with presumably lower endogenous expression levels, these results show that LIN41 does not, per se, require miRNAs to achieve mRNA silencing. However, with the emerging view of 3′ UTRs as platforms for regulation through RNA-binding proteins and miRNAs, we predict that more examples of cooperation—and, indeed, antagonism—between LIN41 and miRNAs will emerge, with the rules of interaction depending on the sequence or architecture of individual 3′ UTRs.

An enigmatic RING domain: To ubiquitylate or not to ubiquitylate?

Ubiquitylation is a post-translational protein modification that not only results in proteasome-mediated degradation, but also alters protein function in many different ways (Komander and Rape 2012). It is catalyzed by a multisubunit enzyme complex, which obtains its substrate specificity from its E3 subunit. A large class of these E3 ligase subunits contains a characteristic RING domain, which is also found in the TRIM domain of most LIN41 proteins. Accordingly, and as an alternative to LIN41 functioning as a translational repressor, it was suggested to destabilize target proteins such as LIN-29 through polyubiquitylation (Slack et al. 2000).

However, a few observations seemed to argue against ubiquitylation as a major molecular activity of LIN41 in developmental processes. First, the putative *Drosophila* LIN41 *dappled/wech* lacks a RING domain (O'Farrell et al. 2008). Second, although screens for heterochronic mutants and *let-7lf* suppressors identified many *LIN41lf* and null alleles, no point mutations affecting the RING domain were identified (Slack et al. 2000). In contrast, several point mutations affected the NHL domain. Finally, LIN41 function in hypodermal and male tail development is highly dosage-sensitive (Del Rio-Albrechtsen et al. 2006), which would be unexpected if its major role was catalytic.

Nonetheless, ubiquitylation activity of LIN41 has now been reported in several studies. LIN41 can autoubiquitylate in vitro and in cells (Rybak et al. 2009; Loedige et al. 2012), and it has been speculated that this may be used as an autoregulatory mechanism in vivo (Del Rio-Albrechtsen et al. 2006), although experimental evidence for this is currently lacking. A second validated target is Ago, although, as discussed above, the consequences of Ago ubiquitylation are unclear, with Ago destabilization not observed in most instances.

Recently, J Chen et al. (2012) identified *Shc-binding protein 1* (*Shcbp1*) as a possible mediator of LIN41's functions in regulating mouse neural development, as discussed in an earlier section. Strikingly, Shcbp1 is ubiquity-

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lated in vivo and binds to LIN41, as determined by yeast two-hybrid and coimmunoprecipitation analysis of tagged, ectopically expressed proteins in HEK293 cells. Somewhat counterintuitively, Shcbp1 levels are reduced in embryonic neuroepithelium from *LIN41lf* mice, and conversely, *LIN41* overexpression in HEK293T cells increases the stability, and thus levels of, Shcbp1. It is, however, unclear whether stabilization and ubiquitylation of Shcbp1 are linked. This is because expression in HEK293T cells of a RING domain-less LIN41, which is inactive in promoting autoubiquitylation, continues to promote significant, albeit reduced, ubiquitylation of Shcbp1 and stabilizes Shcbp1 to almost the same degree as does wild-type LIN41. Thus, it will be important to identify the sites on Shcbp1 that are targets of ubiquitylation to test whether Shcbp1 mutated to prevent ubiquitylation is detabilized. Similarly, it will be interesting to determine the ubiquitylation status of endogenous Shcbp1, whose levels decline in sync with those of LIN41 during mouse embryonic brain development.

Taken together, it is now well documented that LIN41 has E3 ligase activity. However, it is still unclear in which contexts and to what extent ubiquitylation contributes to LIN41 function, including effects on LIN41 targets that are still elusive. Furthermore, a major question is whether the ubiquitylating activity of LIN41 represents a function separate from translational repression. Possibly, the RING and other domains of LIN41 jointly induce mRNA degradation and translational repression, as recently observed with another RING domain protein and translational repressor, MEX-3C (Cano et al. 2012).

Outlook and conclusion

Converging results from research in vertebrates and invertebrates have now helped to generate a picture of LIN41 as a key effector of the *let-7* miRNA pathway that promotes cell proliferation and inhibits differentiation to control various developmental processes. The emerging fact that LIN41 is itself a translational repressor further increases the sophistication of the *LIN28*/*let-7*/*LIN41* network architecture: *let-7* directly represses cell cycle genes and thus proliferation (Johnson et al. 2007). To promote differentiation, it further down-regulates transcription factors that are important for progenitor states (Großans et al. 2005; Melton et al. 2010). At the same time, repression of *LIN41* by *let-7* derepresses cell cycle inhibitors, which provides a further break on proliferation. Finally, differentiation-inducing factors are also likely to be released from LIN41-mediated repression, although their identities need to be revealed. This dual function—repression of direct targets and liberation of *LIN41* targets from repression—might be important to achieve robust changes in gene expression and thus cell fates.

Despite the evident importance of LIN41 as an effector of *let-7*, we predict that *LIN41* may also emerge as a critical node in developmental events not controlled by *let-7*. The *lin-28* heterochronic gene has at least three distinct molecular activities in mammalian cells, including some not requiring *let-7* (Polesskaya et al. 2007; Rybak et al. 2008; Viswanathan et al. 2008; Cho et al. 2012), and by analogy, *LIN41* might influence development by several distinct mechanisms. The widespread expression and complex biological output of *LIN41*, together with its intricate domain architecture, also favor such a scenario, as they suggest that the activity of *LIN41* might be modulated at various levels. Nonetheless, how such modulation occurs and how LIN41 promotes translational silencing and mRNA degradation mechanistically remains to be determined. Moreover, although there is now little doubt that LIN41 can support ubiquitylation, the physiological significance of this activity requires further study. We speculate that the E3 ligase activity of LIN41—or (given the activity of overexpressed, RING-less LIN41) of a LIN41-binding partner—has more specific functions in particular processes. Examination of the rescue of *LIN41lf* mouse phenotypes by a ubiquitylation-deficient *LIN41* transgene may offer one way forward.

Finally, the *Drosophila LIN41 dappled/wech* is required for a functional link between integrins and the cytoskeleton—and thus muscle attachment to the body wall—by virtue of its interaction with Talin and the integrin-linked kinase ILK (Loer et al. 2008). Although it remains to be determined whether the canonical, RING finger-containing LIN41 proteins in other animals share this function, it is intriguing that endogenous TRIM71 in adult mice is also detected at sarcomeric Z disks of adult muscles, where it colocalizes with ILK (Loer et al. 2008).

At any rate, the recent exciting progress on the developmental, cellular, and molecular functions of LIN41 has clearly demonstrated that, beyond serving as a model miRNA target, LIN41 is a worthy subject of scientific examination in its own right.

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Results

1. A genome-wide RNAi screen for let-7 suppressors

Specific aims and contribution:

In order to identify novel genetic interaction partners of *let-7* with a potential role in the heterochronic pathway as modulators or targets of *let-7*, I conducted a genome-wide RNAi screen for suppressors of *let-7* bursting. Suppression of *let-7* lethality is a characteristic of essentially all known *let-7* targets in *C. elegans* (Slack et al., 2000; Lin et al., 2003; Grosshans et al., 2005) and the key *let-7* target *lin-41* has been identified in forward genetic screen for *let-7* suppressors (Slack et al., 2000). Similarly, two factors involved in modulating *let-7* biogenesis, *lin-28* and *pup-2,* interact genetically with *let-7* (Lehrbach et al., 2009). Finally, the entirely novel pathway of active miRNA degradation was discovered by virtue of *let-7* suppression upon knock-down of the *xrn-2* "microRNAse" (Chatterjee and Grosshans, 2009). Previous work in the Grosshans lab demonstrated the feasibility of a larger scale RNAi screen and the use of vulva bursting as an appropriate screening read-out (Ding et al., 2008).

I performed the initial screening and candidate validation with Magdalene Rausch, a graduate student colleague in the lab. The further follow-up was carried out in close collaboration, I focused on the analysis of a novel miRNA target reporter and effects of chemical cell-cycle inhibition on *let-7* phenotypes, whereas she quantified *let-7* and target levels. Characterization of the cell-cycle regulators *cdk-1* and *cdc-25.2* was performed by Hrishikesh Bartake.

Publication 2:

A genetic interactome of the let-7 microRNA in C. elegans

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Genomes and Developmental Control

A genetic interactome of the *let-7* microRNA in *C. elegans*

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ABSTRACT

The heterochronic pathway controls temporal patterning during *Caenorhabditis elegans* larval development. The highly conserved *let-7* microRNA (miRNA) plays a key role in this pathway, directing the larval-to-adult (L/A) transition. Hence, knowledge of the genetic interactome of *let-7* has the potential to provide insight into both control of temporal cell fates and mechanisms of regulation and function of miRNAs. Here, we report the results of a genome-wide, RNAi-based screen for suppressors of *let-7* mutant vulval bursting. The 201 genetic interaction partners of *let-7* thus identified include genes that promote target silencing activity of *let-7*, seam cell differentiation, or both. We illustrate the suitability of our approach by uncovering the mitotic cyclin-dependent kinase CDK-1 as a downstream effector of *let-7* that affects both seam cell proliferation and differentiation, and by identifying a core set of candidate modulators of *let-7* activity, which includes all subunits of the condensin II complex. We propose that the genes identified in our screen thus constitute a valuable resource for studies of the heterochronic pathway and miRNAs.

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Introduction

Proper organismal development requires faithful temporal and spatial control of gene expression. In the nematode *Caenorhabditis elegans*, the heterochronic pathway controls temporal patterning during larval development by ensuring successive occurrence of specific developmental programs in distinct tissues at the correct time (Ambros and Horvitz, 1984). Heterochronic mutations may thus cause retarded phenotypes, where developmental events characteristic of one larval stage are reiterated during subsequent stages, or precocious phenotypes, where stage-specific programs are skipped in favor of subsequent programs.

A classical example of a developmental process controlled by the heterochronic pathway is the establishment of the adult *C. elegans* hypodermis (skin), which mainly consists of the large multinuclear hyp7 syncytium as well as two sets of lateral hypodermal blast cells called seam cells (Sulston et al., 1983; Podbilewicz and White, 1994). The seam cells are characterized by a stem cell-like, asymmetric division during larval stages that, in most lineages, generates posterior daughters that maintain the proliferative potential and anterior daughters that differentiate and fuse to the hypodermal syncytium (Sulston and Horvitz, 1977). This mechanism allows elongation of the hypodermis proportional to the growth in body size during larval development. Upon transition from larval to adult stage, seam cells cease proliferation and terminally differentiate, i.e., they fuse into a syncytium and express adult-specific collagens to generate an adult cuticular structure known as alae (Singh and Sulston, 1978). These events depend on the *let-7* microRNA, which accumulates strongly during

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the last larval (L4) stage (Reinhart et al., 2000). *let-7* exerts its function by binding to partially complementary sequences in the 3′ untranslated regions (3′ UTRs) of target mRNAs, which silences these through inhibition of their translation or through degradation (Slack et al., 2000, Lin et al., 2003; Abrahante et al., 2003; Großhans et al., 2005; Ding and Großhans, 2009; Bagga et al., 2005). Loss of *let-7* activity leads to failed silencing of its targets and, consequently, continued seam cell proliferation, failed fusion, and sustained expression of larval- instead of adult-specific cuticular collagens (Reinhart et al., 2000). *let-7* mutant animals also display a vulval rupturing phenotype that causes their death (Reinhart et al., 2000), but it is currently unclear if and to what extent this is linked to the retarded heterochronic seam cell phenotypes (Roush and Slack, 2008; Ecsedi et al., 2015).

The sequence of *let-7* is invariant across animal phylogeny (Pasquinelli et al., 2000), and a number of targets are conserved (Slack et al., 2000; Großhans et al., 2005). Indeed, function in inhibition of proliferation and induction of differentiation is a common feature of *let-7* from invertebrates to mammals (Büssing et al., 2008). Thus, *let-7* suppresses self-renewal of embryonic stem cells, promotes neural stem cell differentiation, and acts as a tumor suppressor gene (Takamizawa et al., 2004; Melton et al., 2010; Worringer et al., 2014; Rybak et al., 2008). These functions may involve regulation of a number of direct *let-7* targets, including oncogenes such as *MYC*, *RAS*, and *HMGA2*, but also cell cycle genes such as CDK6 and CDC25A (Johnson et al., 2007; Lee and Dutta, 2007; Sampson et al., 2007; Johnson et al., 2005).

For *C. elegans let-7*, previously identified direct targets include the TRIM-NHL protein LIN-41 (Slack et al., 2000), and the transcription factors DAF-12 (Großhans et al., 2005) and HBL-1 (Lin et al., 2003; Abrahante et al., 2003). In addition, genetic data revealed that hypodermal LIN-41 represses, directly or indirectly, accumulation of the zinc finger transcription factor LIN-29 (Slack et al., 2000), which in turn is needed for expression of the adult-specific collagen *col-19* and the cell cycle inhibitor *cki-1* (Rougvie and Ambros, 1995; Liu et al., 1995; Hong et al., 1998). Hence, *let-7* may promote at least some aspects of the L/A transition by relieving LIN-29 from LIN-41-mediated repression. Whether it additionally exerts direct repression of cell cycle genes is currently unknown.

Here, we conducted a genome-wide study for genetic interactors of *let-7*. The purpose of this study was two-fold. First, we sought to identify downstream effectors to obtain a better understanding of *let-7* function in the heterochronic pathway. Second, we aimed to establish a genome-wide collection of modulators of *let-7* activity to identify candidate components of the miRNA pathway (Ding et al., 2008; Hunter et al., 2013; Großhans et al., 2005; Büssing et al., 2010; Parry et al., 2007). We illustrate the suitability of our approach for these purposes by identifying 201 suppressors of *let-7* mutant vulval bursting, establishing the mitotic cyclin-dependent kinase CDK-1 as a downstream effector of *let-7*, and uncovering a core set of candidate modulators of *let-7* activity that include all subunits of the condensin II complex.

Materials and methods

A genome-wide RNAi screen for suppressors of let-7(n2853) bursting

RNAi by feeding (Timmons et al., 2001) was performed using primarily the RNAi library from the Ahringer group (Kamath et al., 2003) supplemented with unique clones from the Vidal library (Rual et al., 2004). The two libraries together are predicted to target 18′578 loci representing ~94% of *C. elegans* protein coding genes (Kim et al., 2005). L1 stage *let-7(n2853)* worms synchronized by hatching overnight in M9 buffer were grown in 96-well plates at a concentration of 25 worms per well in S-medium liquid culture with RNAi bacteria; double-stranded RNA production was induced by IPTG (4 mM final concentration in the bacterial growth medium). Wells were scored for surviving adult worms after 70 h of incubation at 25 °C using a dissecting microscope. *let-7(n2853)* animals grown on mock RNAi showed a > 90% penetrant bursting phenotype under these conditions. Bacteria from positive wells were streaked directly from the wells, and a single colony was selected for retesting on RNAi plates at 20 °C and 25 °C as described previously (Ding et al., 2008). For clones scoring positive again, the RNAi plasmid was isolated, sequenced and retransformed into *HT115* bacteria. This new library of positive clones was retested on RNAi plates at 20 °C and 25 °C. Bursting suppression was scored as indicated in the legend of Table S1.

col-19::gfp assay

col-19::*gfp*; *let-7(n2853)* worms ($n > 100$) were tested at 20 °C and 25 °C on suppressor RNAi plates as in the bursting suppressor screen. Worms were scored at two time points (48 h and 56 h for 25 °C and 56 h and 72 h, respectively, for 20 °C) for presence of detectable GFP expression in the hypodermis using a Leica MZ16 FA fluorescence dissection microscope. At the magnification used, it was not possible to differentiate between expression in hyp7 or seam cell nuclei. As *let-7(n2853)* worms, at the permissive temperature of 15 °C, undergo a larval-to-adult transition after an L5 molt and eventually express *col-19::gfp*, we scored suppressors based both on the penetrance and timing of *col-19::gfp* expression as indicated in the legend of Table S3. Certain suppressors (results) were examined further on a Zeiss Z-1 microscope and imaged with Zeiss Axiovision software.

let-7 target and cdc-25.2 and cdk-1 3′UTR reporters

The hypodermal-specific *wrt-2* promoter (Aspöck et al., 1999) and indicated 3′UTRs were amplified using the primers listed in the supplementary methods and inserted into an appropriate Gateway donor vector. *Pwrt-2*, *gfp::h2b::PEST* (pBMF2.7) and individual 3′UTR entry vectors were recombined into the MosSCI-compatible pCFJ150 plasmid. All plasmids were verified by sequencing. Transgenes were integrated in single copy at a defined genomic location as described (Frokjaer-Jensen et al., 2008). Integrant lines were outcrossed at least three times.

For examination of *let-7* activity, reporter worms were subjected to RNAi by feeding as for the suppressor screen and hypodermal differentiation assay. Fluorescence intensity was compared to the empty vector control after 32 h incubation at 25 °C using a Leica MZ16 FA fluorescence dissecting microscope. Repression of the reporter was scored independently by two observers for penetrance and degree of repression. Scores for the *lin-41* 3′ UTR and the control *unc-54* 3′UTR reporters were compared to identify positive hits. Selected suppressors (Results) were imaged further on a Zeiss Z-1 microscope with Zeiss Axiovision software using equal exposure times.

To assess regulation of *cdk-1* and *cdc-25.2* 3′ UTR reporter transgenes by *let-7*, synchronized worms were grown for 36 h at 25 °C on plates. Worms were observed on a Zeiss Z-1 microscope with Axiovision software using Nomarski DIC and fluorescence microscopy.

Gene expression profiling

For microarray analysis synchronized L1 larvae were grown at 25 °C, the restrictive temperature of the temperature-sensitive sterile *glp-4(bn2)* allele (Beanan and Strome, 1992), to L4 stage (33 and 34 h for *glp-4(bn2)* and *glp-4(bn2)*; *let-7(mn112)*, respectively, to adjust for a minor growth delay of *let-7* mutant animals) and harvested in TRI Reagent (MRC). RNA was isolated according to the

manufacturer's instructions. Total RNA (300 ng) was converted to cDNA and amplified with 1 cycle of IVT using the Affymetrix GeneChip WT Amplified Double Stranded cDNA Synthesis Kit, fragmented using the Affymetrix GeneChip WT Double-Stranded DNA Terminal Labeling Kit, and Biotin labeled using the GeneChip WT Genechip WT Terminal Labeling Kit. 7.5 μg of labeled double-stranded cDNA was hybridized to *C. elegans* tiling arrays for 16 h. Scanning was performed with Affymetrix GCC Scan Control v. 3.0.0.1214 on a GeneChip Scanner 3000 with an autoloader. All sequencing data generated for this study have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE52910 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE52910).

Raw data CEL files from tiling arrays were processed in R using a bioconductor and the packages tilingArray and preprocessCore. The arrays were RMA background corrected and log 2 transformed on the oligo level using the following command:

expr <- log 2(rma.background.correct(exprs(readCel2eSet(filenames, rotated=TRUE)))). We mapped the oligos from the tiling array (bpmap file from www.affymetrix.com) to the *C. elegans* genome assembly ce6 (www.genome.ucsc.edu) using bowtie allowing no error and unique mapping position. Expression levels for individual transcripts were calculated by intersecting the genomic positions of the oligonucleotides with transcript annotation (WormBase WS190) and averaging the intensity of the respective oligonucleotides.

miRNA target enrichment analysis

In order to test the identified suppressors of *let-7(n2853)* for enrichment of miRNA targets, ALG-1 binding site locations of L4 stage worms (Zisoulis et al., 2010) were downloaded from the *C. elegans* version ce6 (May 2008) UCSC genome annotation database (http://hgdownload.soe.ucsc.edu/goldenPath/ce6/database/).

Gene annotations were previously downloaded from WormBase for the *C. elegans* genome version WS190, corresponding to UCSC version ce6. ALG-1 binding sites were assigned to the nearest annotated transcript using the BedTools intersect utility (Quinlan and Hall, 2010), and 3217 unique gene IDs were extracted from the resulting list. The number of genes expressed during L4 stage was calculated based on published expression data (Hendriks et al., 2014). To this end, samples from a total of 9 time points of continuous development (28–36 h) were first normalized for library size, averaged and log 2 transformed. We used a cutoff of 4 (in log 2 space) to separate expressed from non-expressed genes based on the bimodal expression distribution, yielding 15,179 expressed genes. An enrichment of putative miRNA targets among the different classes of miRNA suppressors (see main text) was tested by comparison against this baseline frequency of 0.212 (3217 of 15,179 genes) miRNA targets per expressed gene using a hypergeometric test.

Results and discussion

A genome-wide RNAi screen identifies 201 suppressors of the let-7 (n2853) lethality phenotype

To study the *let-7* regulatory network on a global level, we sought suppressors of the temperature-sensitive (*ts*) *let-7(n2853)* vulval bursting phenotype in a genome-wide, RNAi-based screen.

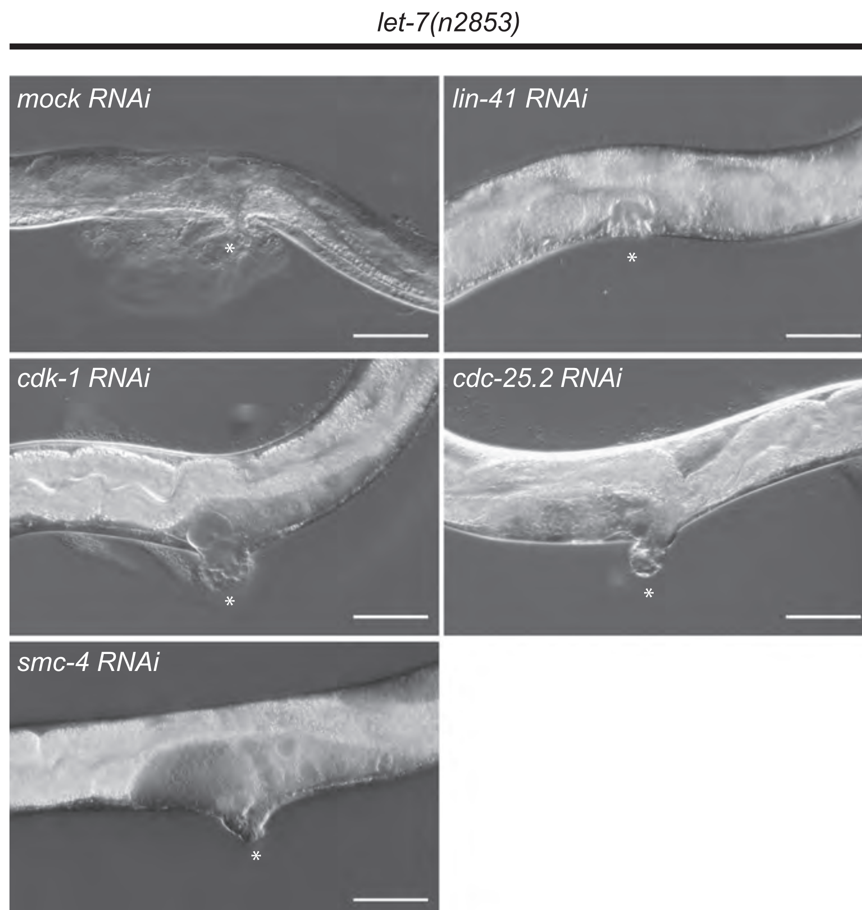

Fig. 1. A genome-wide RNAi screen for suppressors of *let-7(n2853)* bursting. Knock-down of the indicated suppressors by RNAi rescues bursting of *let-7(n2853)* worms. Vulvae are marked with asterisks. Scale bar indicates 50 μm.

let-7(n2853) worms carry a G-to-A point mutation in the seed sequence of the mature miRNA, leading to impaired binding and repression of *let-7* targets as well as reduced expression of the mature miRNA (Reinhart et al., 2000). The resulting vulval bursting phenotype at the L/A transition is highly penetrant at the restrictive temperature of 25 °C. At 15 °C, *let-7(n2853)ts* animals are viable, but seam cells continue to divide and fail to differentiate (Reinhart et al., 2000), whereas at an intermediate temperature, 20 °C, lethality occurs but at reduced penetrance (Großhans et al., 2005).

In a pilot experiment, we had previously used RNAi by feeding against genes on *C. elegans* chromosome I to identify suppressor genes of the *let-7(n2853)* lethality at 20 °C and 25 °C (Ding et al. 2008). To expand the screen from these 2400 genes to a genome-wide scale, we complemented the "Ahringer library" with select RNAi clones from the "Vidal library" to cover $>90\%$ of *C. elegans* genes (Kamath et al., 2003; Rual et al., 2004). Moreover, we streamlined the screening procedure further by performing it in liquid medium, and at only one temperature, 25 °C, followed by rescreening of primary candidates on RNAi plates at both 20 °C and 25 °C. Plasmids from bacteria scoring positive in the second round of screening were isolated, sequenced, and retransformed into bacteria, which were then utilized for a final round of testing for suppression. Through these three rounds of testing, we validated 201 genes as suppressors of *let-7* lethality that were capable of restoring viability of at least 20% of the worms in one or both conditions (Fig. 1 and Table S1). Note that some suppressed animals retained a protruding vulva phenotype, reflecting incomplete suppression or a separate vulval defect due to depletion of the targeted gene (see also below). Our screen also covered the previously screened chromosome I (Ding et al., 2008), permitting us to compare the two datasets. We found that we had rediscovered a high 78% of the candidates identified in the previous study (Table S1), which demonstrates the interactions to be robust and reproducible even under distinct screening conditions.

Modulation of let-7 function by suppressors of vulval bursting

The list of 201 suppressors also contained five out of 61 genes previously identified as enhancers of vulval bursting associated with the weak *let-7(mg279)* hypomorphic allele in a total of 17,900 genes tested by RNAi (Parry et al., 2007). Although few, this constitutes a 7.3-fold enrichment over background (p-Value$=6\times10^{-4}$, hypergeometrical test). Possibly, the activity levels of these specific genes need to be very tightly regulated. Hence, their presumably greater depletion in the RNAi-sensitized strain used in the previous study (Parry et al., 2007) might have resulted in different effects from those seen here. Regardless of this possibility, the finding indicated a need for a better understanding of the suppressor genes. As a first step, we sought to determine whether any of the *let-7(n2853)* suppressor genes were negative regulators of *let-7*-mediated gene silencing. Hence, we developed a GFP-based *let-7* target reporter system to directly analyze *let-7*-activity in hypodermal cells in vivo. We fused the hypodermis-specific *wrt-2* promoter (Aspöck et al., 1999) to a gene encoding a destabilized nuclear GFP (GFP-H2B-PEST) followed by the 3′UTR of *lin-41*, which we chose as the best-characterized target of *let-7* (Vella et al., 2004). In addition to this reporter, which we termed *pREP_lin-41*, we generated control reporters, *pREP_unc54* and *pREP_lin41ΔLCS*, which contained the unregulated *unc-54* 3′UTR and a *lin-41* 3′UTR lacking a 98nt fragment required for *let-7*-mediated regulation (Vella et al., 2004), respectively. All three transgenes were integrated into the same genomic site in single copy through Mos1 transposon-mediated single copy transgene integration (MosSCI) (Frokjaer-Jensen et al., 2008).

The reporter system faithfully recapitulated *let-7*-mediated regulation: all three reporters were highly expressed in the hypodermis of early wild-type larvae. Subsequently, *pREP_lin41*, but not *pREP_unc54* or *pREP_lin41ΔLCS*, showed repression starting during L4 larval stage (Fig. 2A and data not shown). This correlates well with the accumulation of *let-7* during the L4 stage (Reinhart et al., 2000). The differences in expression between the control reporters and *pREP_lin-41* increased further when adult animals were examined. In old adults, even the signal from the control reporters declined substantially, presumably reflecting decreased promoter activity. We confirmed that repression of *pREP_lin41* depended on *let-7* by crossing the reporters into *let-7(n2853)* mutant animals. This resulted in elevated *pREP_lin41* expression levels in L4 and adult stage animals relative to their wild-type counterparts, whereas expression of *pREP_unc54* and *pREP_lin41ΔLCS* remained unaffected (Fig. 2A and data not shown).

Transcriptional profiling data from our lab recently revealed periodic *wrt-2* mRNA accumulation during larval development (Hendriks et al., 2014), and the *pREP_unc54* reporter indeed exhibited increased *wrt-2* promoter activity towards the end of the L4 stage. As the fluctuation of GFP was less than that of the endogenous *wrt-2* mRNA, we could control for this potential source of variability in *pREP_lin41* experiments by the examination of worms carrying the *pREP_unc54* control transgene. Furthermore, a reporter carrying the 3′UTR of the *let-7* target *daf-12* (Großhans et al., 2005) (*pREP_daf12*) was used to test independently for restoration of *let-7* activity. Analyzing the full set of our identified suppressors, we found 73 genes to restore repression of a *let-7* target reporter in the *let-7(n2853)* background while showing no or modest repression of the control 3′UTR upon RNAi ('target reporter positives', Fig. 2B and Table S2).

A subset of the suppressors affect let-7-dependent hypodermis differentiation

It was conceivable that some suppressors modulated vulval development and/or morphogenesis in a *let-7*-independent manner, thus preventing bursting indirectly. Consistent with this notion, we frequently observed protruding vulva (Pvl) phenotypes upon suppressor RNAi on wild-type as well as on *let-7(n2853)* animals (Table S1). Therefore, we wished to examine suppression of another *let-7* mutant phenotype, outside the vulva. We utilized a previously established *Pcol-19::gfp* reporter (Abrahante et al., 1998) to examine whether hypodermal cell differentiation was also restored upon depletion of the suppressor genes. Transcription of *col-19*, an adult-specific cuticular collagen gene, requires the zinc-finger transcription factor LIN-29 (Rougvie and Ambros, 1995; Liu et al., 1995) (Fig. 3A), which, however, does not accumulate in *let-7(n2853)* mutant animals (Reinhart et al., 2000). Accordingly, *Pcol-19::gfp* is not expressed in *let-7* mutant animals (Fig. 3B). By contrast, depletion of 102 of the 201 *let-7* suppressor genes resulted in GFP accumulation in adult animals ('col-19 positives', Fig. 3B and Table S3). Hence, depletion of these genes restores at least some aspect of hypodermal cell differentiation, further supporting their function in the heterochronic pathway.

let-7 suppressor genes can be grouped into four functional classes

Taken together, the results of the three different assays that measure restoration of viability, *let-7* target gene repression, and restoration of seam cell differentiation, yield four different groups of suppressor genes (Fig. S2). 'Suppressor-only' genes are positive for restoration of viability, but none of the other assays.

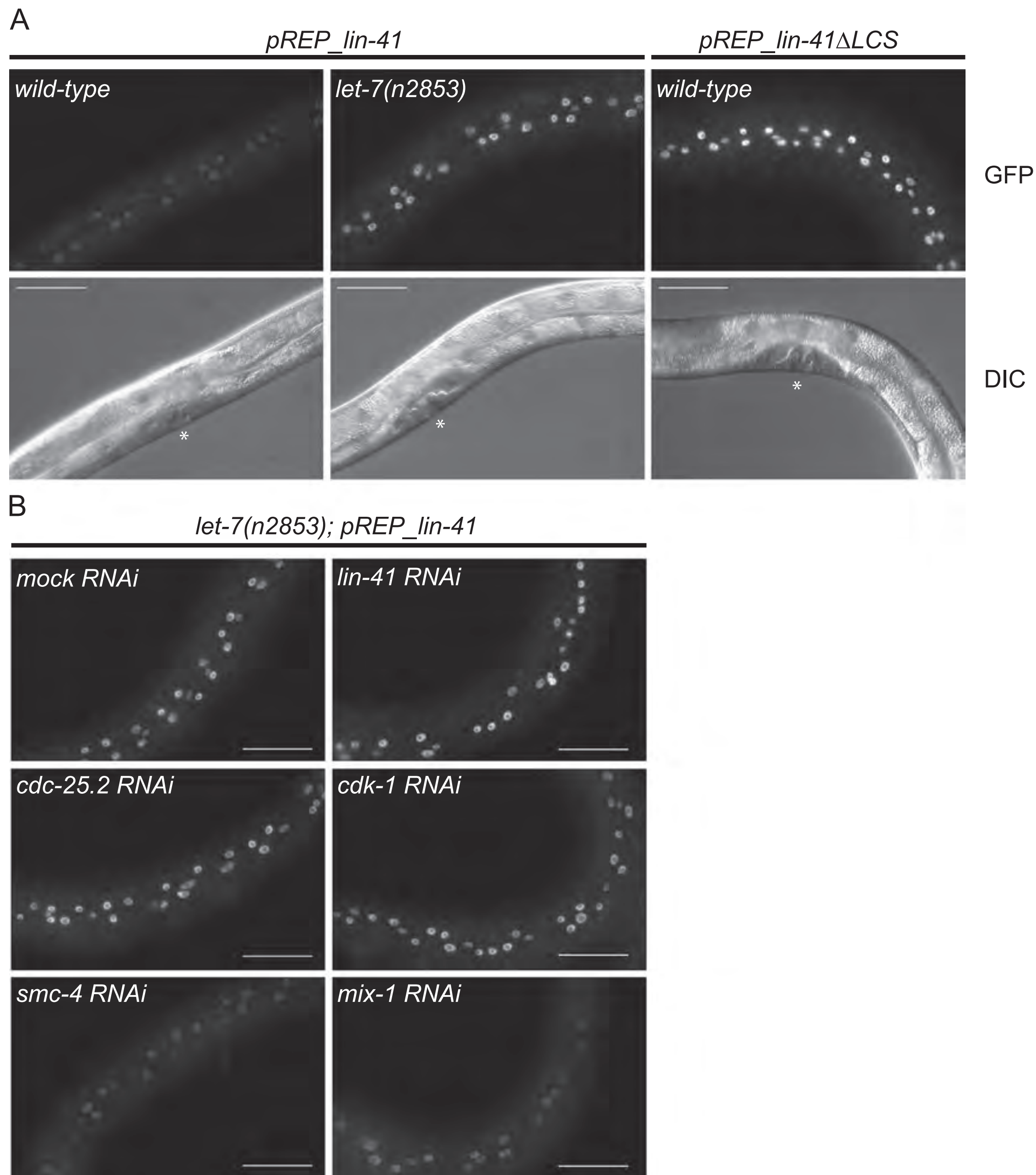

Fig. 2. *let-7* suppressor RNAi restores repression of a *let-7* target reporter. (A) Repression of a *let-7* target reporter (*Pwrt-2::gfp::lin-41 3′UTR*, "*pREP_lin-41*") in late L4 worms depends on *let-7* and is lost upon mutation of the *let-7* complementary sites (*pREP_lin-41ΔLCS*). Vulvae are marked with asterisks. (B) GFP intensity in *pREP_lin-41, let-7 (n2853)* worms subjected to the indicated RNAi; pictures were taken at the young adult stage. RNAi against *smc-4* and *mix-1*, but not against the other genes, causes repression of the reporter. Scale bar indicates 50 μm.

These genes may be enriched for false positive hits, modulate *let-7* functions that are currently unknown, or act in tissues other than the hypodermis.

The three other classes contain genes that are all positive for restoration of viability, and additionally one or both of the other assays. Thus, 'target reporter-only' genes are positive for target reporter repression, but not for *Pcol-19::gfp* expression. In a linear model, where increased *let-7* target repression would proportionally enhance *let-7*-dependent cellular differentiation, these genes may be false positive hits. However, it seems equally possible that modulation of the developmental phenotype, measured by *Pcol-19::gfp* expression, needs restoration of target gene repression beyond a certain threshold, and/or that the sensitivities of the two assays differ. Finally, the genes in this class may only alter activity of some *let-7* target genes, with hypodermis differentiation depending at least in part on some targets whose activity we have not measured here.

Genes in the 'col-19-only' group affect *Pcol-19::gfp* expression without apparent effects on *let-7* target gene silencing. These genes might act downstream of, or in parallel to, *let-7*, potentially as direct *let-7* targets or indirect effectors, and we provide a detailed dissection of one example below.

Finally, a group of 36 genes scored positive in both the target reporter and the *col-19* expression assays (Table 1) and constitute the 'double-positive' class. Although the mechanisms by which these genes function remain to be established, they are strong candidates for modulators of *let-7* activity. Notably, this list includes all five members of the *C. elegans* condensin II complex, namely *smc-4*, *mix-1*, *kle-2*, *capg-2*, and *hcp-6* (Csankovszki et al., 2009) as well as *plk-1*, the *C. elegans* orthologue of Polo-like kinase

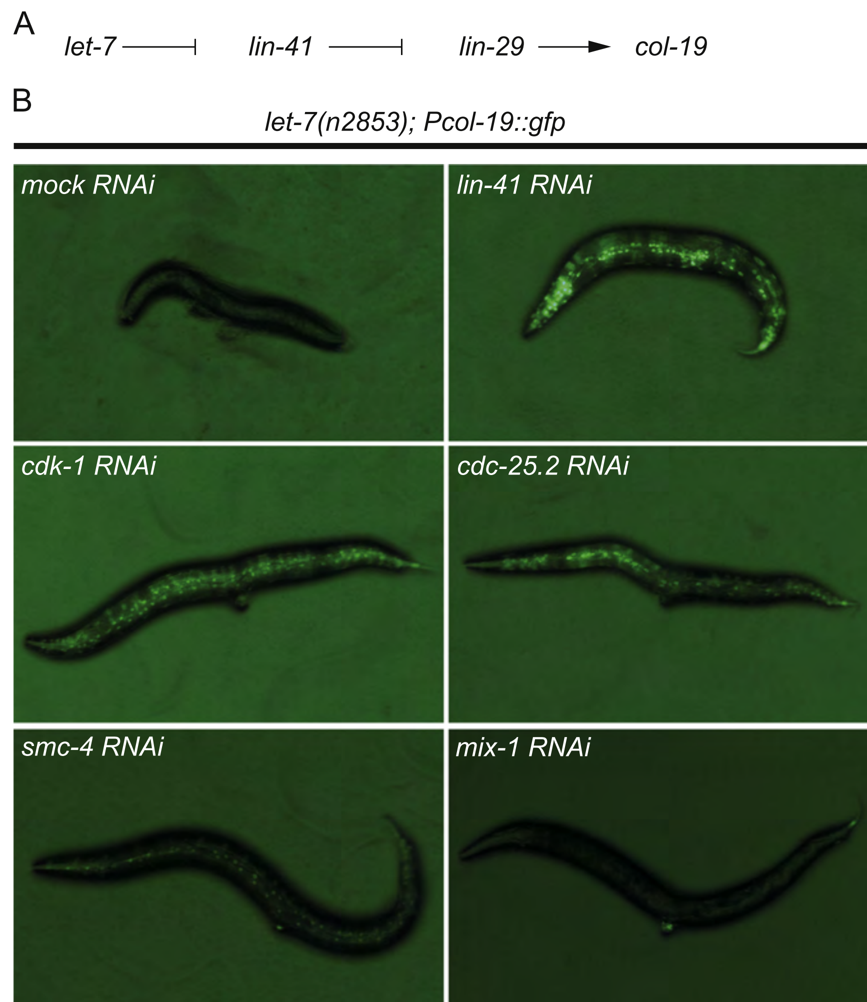

Fig. 3. RNAi of *let-7(n2853)* suppressors restores hypodermis differentiation. (A) Activation of the adult-specific *col-19* promoter is controlled by *let-7* through activation of the transcription factor LIN-29. (B) Expression of *col-19::gfp* in *let-7(n2853)* worms subjected to the indicated RNAi; pictures were taken at the young adult stage (100 × magnification). RNAi against *cdk-1* and *cdc-25.2* but not against *smc-4* or *mix-1* causes upregulation of the reporter.

(Ouyang et al., 1999), a known regulator of condensins in human HeLa cells (Abe et al., 2011). RNAi of the condensin II complex has been shown to result in chromosome condensation and segregation defects both in mitosis and meiosis (Hagstrom et al., 2002; Stear and Roth, 2002), but in addition to its structural functions, the complex was reported to bind to interphase chromatin in *C. elegans* where it acts as a transcriptional repressor (Kranz et al., 2013). Although we have currently no mechanistic explanation for the ability of condensin II to modulate *let-7* activity, the identification of this entire complex further corroborates the robustness of our analysis, and makes condensin II a particularly interesting candidate miRNA pathway factor.

Most novel suppressors are unlikely to be direct let-7 targets

Zisoulis et al. (2010) previously identified candidate miRNA targets through their association with the miRNA Argonaute protein ALG-1. Interestingly, we found that 81 out of 201 suppressors as well as 41 out of the 102 'col-19 positive' suppressors were also bound by ALG-1. This represents a moderate enrichment of 1.9-fold for both classes compared to the 3217 ALG-1 bound mRNAs in a total of 15,179 genes expressed in L4 (total suppressors: p-Value$=5.1\times10^{-10}$, 'col-19 positives': p-Value$=9.9\times10^{-6}$, hypergeometric test; see *Methods*). To determine whether a subset of these genes was indeed regulated by *let-7*, we compared gene expression patterns of wild-type and *let-7(mn112)* null mutant worms at the late L4 stage using *C. elegans* tiling arrays. Because *let-7* activity has not been reported in the germline, we performed these experiments in germline-less *glp-4(bn2)* mutant animals (Beanan and Strome, 1992), to examine gene expression levels specifically in somatic tissues (Fig. 4 and S1). Analysis of the data did reveal robust overexpression of the published *let-7* targets *lin-41* (4.17 fold) and *daf-12* (2.1 fold) in *let-7(mn112)* compared to wild-type worms. By contrast, most of the novel suppressors did not change in *let-7* mutant worms. This finding implies that, consistent with the moderate enrichment of ALG-1 binders, the majority of *let-7* suppressors are not direct *let-7* targets. This notion is also supported by our recent finding that vulval bursting of *let-7* mutant animals is explained by dysregulation of only LIN-41 (Ecsedi et al., 2015). Alternatively, some of these genes may either be *let-7* targets regulated through mechanisms that do not involve substantial mRNA degradation, e.g., translational control, or their downregulation may occur in only a subset of tissues, making detection impossible in whole worm RNA.

let-7 regulates CDK-1 expression in a LIN-29-dependent manner

Since gene expression profiling failed to reveal new *let-7* targets or downstream effectors, we sought to find specific examples of such genes by examining the 'col-19-only' suppressors. Previous work on cultured cells revealed that *let-7* targets include a cyclin-dependent kinase, CDK6, and a CDK-regulating phosphatase, CDC25A (Johnson

Table 1
List of suppressors positive for both target reporter repression and hypodermis differentiation assay ('double-positive' genes). Shown are all genes which upon RNAi rescue both adult hypodermis formation (*Pcol-19::gfp* reporter assay) as well as repression of a *let-7* target reporter (*Pwrt-2::gfp-H2B-PEST::lin-41-3′UTR* or *Pwrt-2::gfp-H2B-PEST::daf-12-3′UTR*) in *let-7(n2853)* animals. *Pcol-19::gfp* reporter assay: weak (+), medium (++) or strong (+++) activation of GFP upon RNAi. *let-7* target reporter: weak (+), medium (++) or strong (+++) repression of GFP upon RNAi.

	Predicted gene	*col-19* activation				Target reporter repression			Function
		25 °C		20 °C					
		49 h	58 h	56 h	72 h	*lin-41*	*daf-12*	*unc-54* (ctrl.)	
Cell cycle/chromosome maintenance and segregation	hcp-6	–	++	–	+++	++	+++	+	Condensin II subunit
	capg-2	–	++	–	+++	++	+	–	Condensin II subunit
	kle-2	+	+++	–	+++	+	–	–	Condensin II subunit
	smc-4	–	++	–	++	++	–	–	Condensin II subunit
	mix-1	–	–	–	++	+++	–	–	Condensin II subunit
	scc-3	+	+++	–	+++	–	+	–	Cohesin subunit
	cyb-3	+	++	–	+++	++	–	–	Cyclin B
	plk-1	+	++	–	+++	+	+	–	Polo-like kinase
	knl-2	–	++	–	++	+++	++	–	Kinetochore associated
	him-1	++	+++	–	+++	+	–	–	Structural maintenance of chromosome family
DNA/replication	lig-1	+	++	–	+++	+	–	–	DNA ligase
	Y47D3A.29	–	+	–	–	–	++	–	DNA polymerase alpha subunit
	pri-1	–	++	–	++	+++	++	–	DNA primase
	ruvb-2	–	+	–	+	++	+	–	Recombination protein homolog
	rpa-1	–	+	–	+	–	++	–	Replication protein A homolog
mRNA biogenesis	rpb-7	–	+	–	+	++	+	–	RNA Pol II subunit
	cpsf-2	–	–	–	+	++	++	–	Cleavage and polyadenylation specificity factor
	symk-1	–	+	–	–	+	+	–	Cleavage and polyadenylation factor
	prp-21	+	+	–	++	–	+	–	Splicing factor related
	uaf-1	–	–	–	++	++	++	–	Splicing factor related
Ribosome biogenesis	C37H5.5	–	+	–	+	–	+	–	Nucleolar complex protein 3 homolog
	C47E12.7	–	+	–	–	–	+	–	Ribosomal RNA processing protein 1 homolog
	K12H4.3	–	+	–	–	–	+	–	Ribosome biogenesis protein BRX1 homolog
Nuclear transport	npp-3	–	+	–	+	–	++	–	Nuclear pore protein
	npp-9	–	+	–	–	++	++	–	Nuclear pore protein
	npp-6	+	++	–	+	+++	+++	+	Nuclear pore protein
	xpo-2	+	+	–	++	+	+	–	Nuclear export receptor
Other	aco-2	+	++	–	++	++	–	–	Aconitase
	pyp-1	+	+	+	++	+	–	–	Pyrophosphatase, nucleosome remodeling?
	ani-1	+	+	–	++	+	–	–	Actin binding protein
	dut-1	–	–	–	++	+	–	–	DeoxyUTPase
	toe-1	–	–	–	+	–	+	–	Target of ERK kinase MPK-1
	nhr-25	+++	+++	+++	+++	+	+	–	Nuclear hormone receptor
	T06E6.1	–	–	–	+	–	+	–	
	F44G4.1	–	–	–	+	+	–	–	
	C16A3.4	–	+	–	–	–	++	–	
Control	hda-1	–	–	–	–	–	–	–	Randomly chosen 'suppressor-only'

et al., 2007). Although the functional relevance of these interactions remained unclear, *let-7* has a conserved function in regulation of cell proliferation (Büssing et al., 2008). We were thus intrigued by the identification of *ncc-1*/*cdk-1* (Mori et al., 1994; Boxem et al., 1999) and its activating phosphatase *cdc-25.2* (Kim et al., 2010) among this class of suppressors of vulval bursting. To place the two genes in the pathway, we tested whether their depletion suppressed also vulval bursting caused by the *let-7(mn112)* null mutation, which we found to be the case. We observed 97% rescue of bursting for *cdk-1* RNAi and 99% rescue for *cdc-25.2* ($n>200$ each). About half of the surviving worms were vulvaless (data not shown). Although suppression of bursting might therefore, in part, be indirect, restoration of *col-19::gfp* expression in the hypodermis supported specificity of the genetic interaction (Fig. 3, Table S3). To examine this further, we analyzed the formation of adult alae in *let-7(mn112)* mutant animals. Strikingly, whereas only 9% ($n=32$) of *let-7(mn112)* animals on mock RNAi displayed any alae, 51% ($n=47$) of animals on *cdk-1(RNAi)* and 41% ($n=27$) of animals on *cdc-25.2(RNAi)* did. Similar to the *lin-41(RNAi)* positive control, knockdown of *cdk-1* and *cdc-25.2* virtually always resulted in partial, rather than complete alae, whereas the occasional animals on mock RNAi typically exhibited weak but complete alae. Hence, *cdk-1* and *cdc-25.2* exhibit hallmarks of a downstream effector of *let-7*.

Based on these results it seemed possible that *cdk-1* and *cdc-25.2* were direct targets of *let-7*. Because *let-7* targets that are regulated in a tissue-specific manner and/or through translational repression might not be evident from whole animal gene expression studies by microarray, we generated *cdk-1* and *cdc-25.2* 3′ UTR reporters to assess their potential for regulation by *let-7*. When we analyzed these reporters, *pREP_cdk1* and *pREP_cdc-25.2*, respectively, we found them both to be repressed in L4 stage animals in both seam cells and the hyp7 syncytium relative to the unregulated *pREP_unc54* control reporter (Fig. 5). For *pREP_cdk1* this repression was more pronounced in hyp7 than the seam, whereas the opposite was true for *pREP_cdc-25.2*. However, whereas the positive control *pREP_lin-41* was efficiently derepressed in the *let-7(n2853)* mutant background, this was not observed for *pREP_cdk-1* and *pREP_cdc25.2* in either tissue. We conclude that although the 3′ UTRs of these two mitotic genes

might confer post-transcriptional repression at the L4 stage, when *let-7* is present, this seems unlikely to be a consequence of *let-7* function.

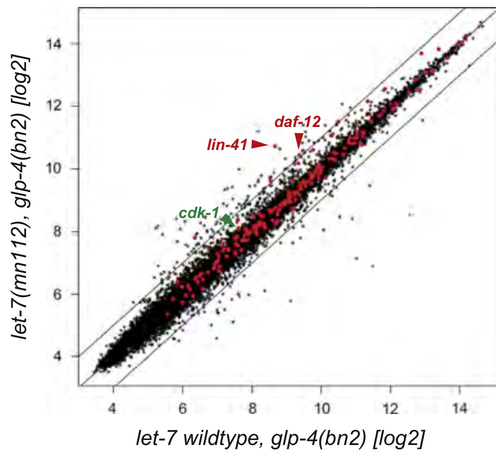

Fig. 4. Expression levels of novel *let-7* suppressors are not affected in *let-7* mutants. Microarray analysis of somatic gene expression in *let-7(mn112)* null mutant in germline-less *glp-4(bn2)* animals shows no changes in mRNA levels for genes identified as suppressors of the *let-7(n2853)* bursting phenotype (marked in red). The known *let-7* targets *lin-41* and *daf-12* are indicated in red for reference, *cdk-1* in green.

We therefore wondered if *cdk-1* functioned further downstream of *let-7* in the heterochronic pathway. We utilized a previously published *cdk-1::gfp* single copy-integrated transgene, which drives expression of a functional fusion protein from the native *cdk-1* promoter (Shirayama et al., 2012), to examine the effect of *let-7* on CDK-1 accumulation. We observed that CDK-1/GFP was present in early L4-stage seam cells, but that its levels declined rapidly upon entry into adulthood (Fig. 6A). However, down-regulation was impaired in *let-7(n2853)* mutant animals where CDK-1/GFP was well visible in the seam cell cytoplasm and, prominently, nucleus. To understand better why CDK-1/GFP protein levels responded so strongly to loss of *let-7* activity although *let-7* did not appear to repress it directly, we tested whether *cdk-1::gfp* expression was modulated by the downstream effector LIN-29. Indeed, knock-down of *lin-29* by RNAi resulted in elevated levels and redistribution of CDK-1/GFP, similar to the effect of *let-7(n2853)* (Fig. 6B). Finally, this was also observed for RNAi of *mab-10* (Fig. 6B), a transcription co-factor that acts in concert with LIN-29 to promote differentiation of the hypodermis (Harris and Horvitz, 2011). Thus, we conclude that *let-7* regulates *cdk-1* indirectly, in a manner that requires the LIN-29 transcription factor.

Conclusion

Using a genome-wide screen, we have identified and characterized here >200 suppressors of *let-7* mutant phenotypes. In combination

Fig. 5. The 3′UTRs of *cdk-1* and *cdc-25.2* do not confer *let-7*-dependent regulation. (A) A hypodermis specific target reporter (*wrt-2* promoter) containing *gfp* fused to the unregulated *unc-54* 3′UTR (*pREP_unc-54*) is expressed both in wild-type and *let-7(n2853)* background at the late L4 stage. (B–D) The reporter containing the *lin-41* 3′UTR (*pREP_lin-41*) is repressed in a *let-7* dependent manner (B) while repression of reporters carrying the *cdk-1* (*pREP_cdk-1*, C) or *cdc-25.2* 3′UTR (*pREP_cdc-25.2*, D) in wild-type worms is less extensive and persists in the *let-7(n2853)* background. Vulvae are marked with asterisks. Scale bar indicates 50 μm.

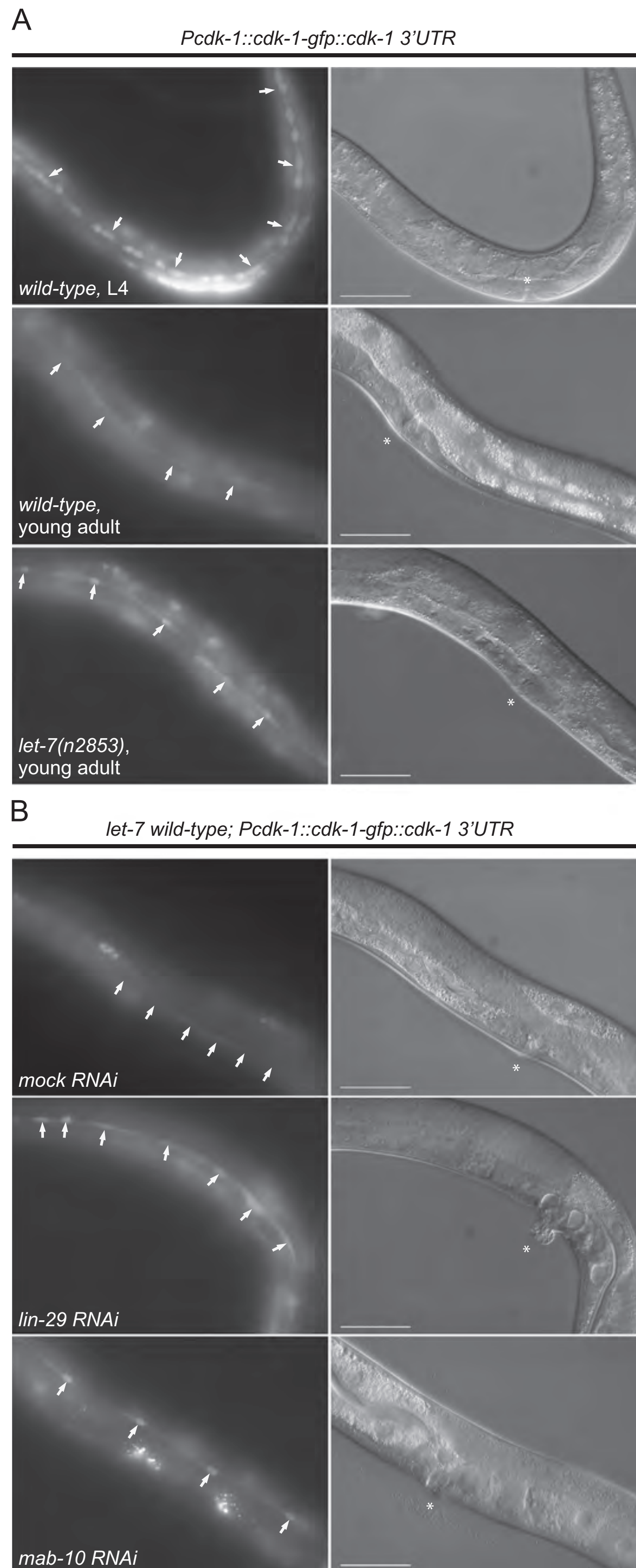

Fig. 6. Repression of *cdk-1::gfp* depends on LIN-29 and MAB-10 (A) Expression of *cdk-1::gfp* from the *cdk-1* promoter can be observed in seam cells (arrows) until the L4 stage. GFP levels decrease during L4 stage in wild-type background. *let-7(n2853)* mutant animals continue to express *cdk-1::gfp* in adult stage. (B) Downregulation of *cdk-1::gfp* in wild-type worms is lost upon RNAi-mediated knockdown of *lin-29* or *mab-10*. Vulvae are marked with asterisks. Scale bar indicates 50 μm.

with previous work using genetic enhancer screening (Parry et al., 2007) and genomics analysis (Hunter et al., 2013) of *let-7* mutant strains, a comprehensive picture of the genetic interactome of *let-7* becomes available, promoting a better understanding of this model miRNA and key developmental regulator. Thus, among the newly identified suppressors, we consider the 'col-19 positive' and the 'double-positive' genes to be of particular interest for studies of the heterochronic pathway and miRNA function and regulation, respectively. Our analysis of CDK-1, which we identified as a putative effector of *let-7* based on its placement in the 'col-19-only' class, illustrates the utility of this approach: whereas CDK-1 was unremarkable in transcriptome analysis, its proficiency in suppressing both *let-7* mutant lethality and hypodermis differentiation defects suggested a functionally relevant interaction with *let-7*, prompting us to test and confirm its regulation by *let-7* and via LIN-29 through more specific means.

As *let-7* controls cell proliferation, it must, at some level, interface with the cell cycle machinery. However, an interaction with the mitotic CDK-1 is unexpected, as the exit of seam cells from proliferation is expected to occur in G1, not G2/M. Therefore, based on the facts that LIN-29 also regulates the cell cycle inhibitor CKI-1 (Hong et al., 1998) and that additional cell cycle genes occur among the 'col-19-only' and the 'double-positive' suppressor genes, we speculate that repression of CDK-1 might be part of a larger program of repression of cell cycle genes during exit of seam cells from proliferation. The observation that CDK functions are plastic such that CDK1 can partially substitute for other CDKs during mouse embryonic development (Santamaria et al., 2007) might explain the need for its repression.

Interestingly, depletion of CDK-1 not only prevents seam cell overproliferation in *let-7* mutant animals, but also promotes hypodermis differentiation by two criteria, expression of *Pcol-19::gfp*, and formation of adult alae. Conceivably, this reflects a tight coupling of cell proliferation and differentiation in the seam so that differentiation ensues when proliferation is blocked. However, we note that *cdk-1(RNAi)* also promotes *Pcol-19::gfp* expression in the postmitotic hyp7, potentially reflecting a more direct role on differentiation. Moreover, we find that even proliferating seam cells can express *Pcol-19::gfp*. For instance, we observed that depletion of *rnr-1*, which codes for the large subunit ribonucleotide reductase, promotes expression of *Pcol-19::gfp* without preventing seam cell overproliferation. Thus, when scored using the seam cell-specific *scm::gfp* marker to visualize seam cells (Koh and Rothman, 2001), *let-7(n2853)* mutant animals exposed to mock or *rnr-1(RNAi)* have a comparable number of seam cells at the young adult stage, i.e., an average of 23.6 cells ($n=22$) and 22.5 ($n=21$), respectively, per side, well above the wild-type 16. Yet *rnr-1(RNAi)* promotes expression of *col-19::gfp* (Table S3). This suggests that a potential coupling between cell cycle exit and differentiation, if it exists, would be unidirectional.

Finally, the observation that the 'double-positive' group of supressors contains a number of genes encoding structural components of chromosomes and cell cycle factors, provides a further illustration of the apparently complex relationship between *let-7* function in the heterochronic pathway and the cell cycle. We propose that our comprehensive genetic screen has thus opened a new door to a deeper understanding of *let-7* and miRNA function more generally.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2015.02.013.

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Supplementary Methods

Primers

primer name	purpose	sequence
pWRT2 GW F attB4	Gateway cloning *wrt-2* promoter	GGGGACAACTTTGTATAGAAAAGTTGTATGACCATGATTACGCCAAG
pWRT2 GW R attB1r	Gateway cloning *wrt-2* promoter	GGGGACTGCTTTTTTGTACAAACTTGCCCGAGAAACAATTGGCA
lin-41_3U F	Gateway cloning *lin-41* 3'UTR	GACACTTTCTTCTTGCTCTTTAC
lin-41_3U R	Gateway cloning *lin-41* 3'UTR	GAAACTCGACTAGGAATTCGAG
cdc-25.2 GW F attB2r	Gateway cloning *cdc-25.2* 3'UTR	GGGGACAGCTTTCTTGTACAAAGTGGAATTATTCCTCCTTGATTTC
cdc-25.2 GW R attB3	Gateway cloning *cdc-25.2* 3'UTR	GGGGACAACTTTGTATAATAAAGTTGCTTTCGCCAAATCACATTAC
cdk-1 GW F attB2r	Gateway cloning *cdk-1* 3'UTR	GGGGACAGCTTTCTTGTACAAAGTGGTGATGTAATTCATTCATCATCA
cdk-1 GW R attB3	Gateway cloning *cdk-1* 3'UTR	GGGGACAACTTTGTATAATAAAGTTGTCTTAATTCCCTATTCTCATTTA
daf-12 3'UTR GW F attB2r	Gateway cloning *daf-12* 3'UTR	GGGG ACA GCT TTC TTG TAC AAA GTG GGACCTACTAGAAATCATCTACC
daf-12 3'UTR GW R attB3	Gateway cloning *daf-12* 3'UTR	GGGG AC AAC TTT GTA TAA TAA AGT TG CCCTTATGGGTTGGCTGAG

Strains

Strain name	genotype
HW769	xeSi10[*Pwrt-2::gfp(PEST)-h2b::lin-41 3'UTR, unc-119 (+)]* II
HW896	xeSi10[Pwrt-2::gfp(PEST)-h2b::lin-41 3'UTR, unc-119 (+)] II, let-7(n2853) X
HW786	xeSi22[*Pwrt-2::GFP(PEST)-H2B::unc-54 3'UTR, unc-119 (+)]* II
HW899	xeSi22[*Pwrt-2::GFP(PEST)-H2B::unc-54 3'UTR, unc-119 (+)]* II, let-7(n2853) X
HW785	xeSi20[Pwrt-2::gfp(pest)-h2b::daf-12 3'UTR] II; let-7(n2853)
WM242	neSi12 [cdk-1::gfp(+), cb-unc-119(+)] II; unc-119(ed3) III
GR1434	wIs54[scm::gfp]; let-7(n2853) V
HW651	let-7(n2853) V; maIs105 [col-19::gfp]
HW1096	*glp-4(bn2); let-7(mn112);* xeEx365[*Ptbb-1::let-7::SL1_operon_GFP, unc-119 (+); Prab-3::mCherry; Pmyo-2::mCherry; Pmyo-3::mCherry*]

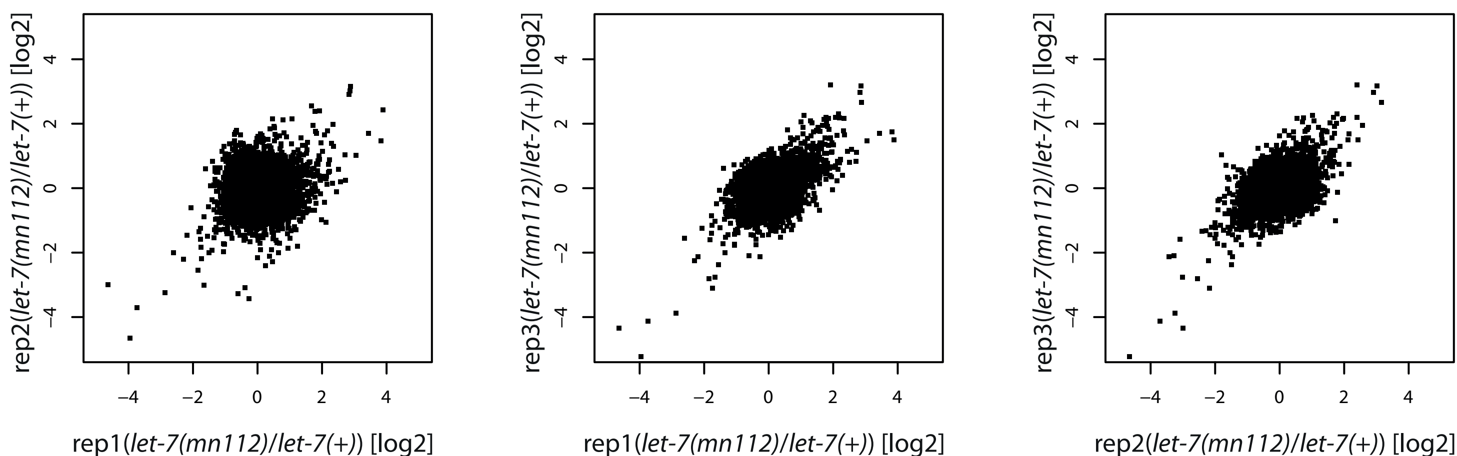

Fig. S1, Replicate correlation of gene expression profiling by microarray

Staged L4 worm populations of *let-7(+)* and *let-7(mn112)* animals were collected in parallel and on three different days to obtain three biological replicates (rep1-3). Both strains were additionally homozygous for the *glp-4(bn2)ts* mutation, and thus germline-less at the temperature used for growth. Gene expression changes in *glp-4(bn2); let-7(mn112)* relative to *glp-4(bn2); let-7(+)* are compared between individual replicate pairs.

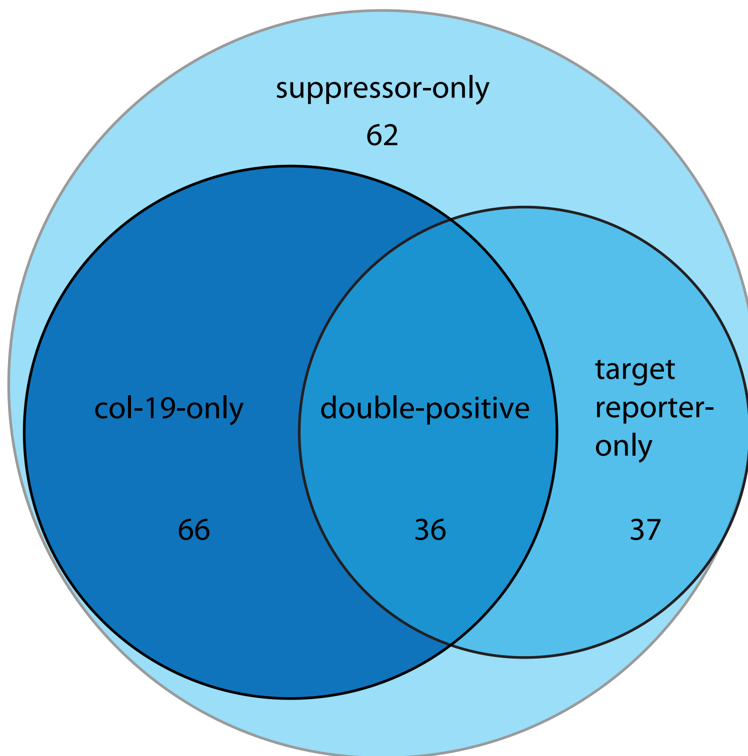

Fig. S2, The suppressors of *let-7(n2853)* can be grouped into four classes

Out of 201 suppressors of *let-7(n2853)* bursting, we find 37 genes that affect repression of a *let-7* target reporter ('target reporter-only') and 66 genes that restore hypodermis differentiation in the *col-19:gfp* assay ('col-19-only'). A group of 36 genes scores positive in both assays ('double-positive'), whereas 62 genes are negative in both assays ('suppressor-only').

Table S2: *let-7* suppressor genes that restore *let-7* target reporter gene repression ('target reporter positives')

Suppressors of *let-7(n2853)* bursting phenotype were analyzed for repression of a destabilized GFP fused to the 3'UTR of the *let-7* targets *lin-41* and *daf-12* (*Pwrt-2::gfp-H2B-PEST::lin-41-3'UTR* or *Pwrt-2::gfp-H2B-PEST::daf-12-3'UTR*) or the unregulated *unc-54* 3'UTR (negative control) in *let-7(n2853)* worms. Shown are genes which upon RNAi restored weak (+), medium (++) or strong (+++) repression in one of the *let-7* target reporters while showing no or minor repression in the negative control.

Predicted Gene	Wormbase ID	target reporter repression: *pREP_lin-41*	target reporter repression: *pREP_daf-12*	target reporter repression: *pREP_unc-54*	
cell cycle/ chromosome maintenance & segregation					
mix-1	WBGene00003367	+++	---	---	condensin II subunit
smc-4	WBGene00004874	++	---	---	condensin II subunit
capg-2	WBGene00010093	++	+	---	condensin II subunit
hcp-6	WBGene00001833	++	+++	+	condensin II subunit
kle-2	WBGene00016202	+	---	---	condensin II subunit
scc-3	WBGene00004738	---	+	---	cohesin subunit
plk-1	WBGene00004042	+	+	---	polo-like kinase
cyb-3	WBGene00000868	++	---	---	cyclin B
him-1	WBGene00001860	+	---	---	structural maintenance of chromosome family
knl-2	WBGene00019432	+++	++	---	kinetochore associated
DNA/replication					
Y47D3A.29	WBGene00012936	---	++	---	DNA polymerase alpha subunit
pri-1	WBGene00004180	+++	++	---	DNA primase
rpa-1	WBGene00017546	---	++	---	replication protein A homolog
lig-1	WBGene00002985	+	---	---	DNA ligase
ruvb-1	WBGene00007784	+	+	---	recombination protein homolog
ruvb-2	WBGene00020687	++	+	---	recombination protein homolog
dbb-1	WBGene00010890	+	+	---	DNA damage binding protein, replication, LET-23 signaling in the vulva
mRNA biogenesis					
rpb-3	WBGene00007971	++	+++	---	RNA Pol II subunit
rpb-7	WBGene00021845	++	+	---	RNA Pol II subunit
rpb-8	WBGene00017830	++	---	---	RNA Pol II subunit
spt-5	WBGene00005015	+++	+++	+	transcription elongation
uaf-1	WBGene00006697	++	++	---	splicing factor related
prp-31	WBGene00022458	---	++	---	spilceosome
prp-21	WBGene00004188	---	+	---	splicing factor
cpsf-2	WBGene00017313	++	++	---	cleavage and polyadenylation
symk-1	WBGene00017797	+	+	---	cleavage and polyadenylation
cel-1	WBGene00000466	+	+	---	mRNA capping
nuclear transport					
npp-6	WBGene00003792	+++	+++	+	nuclear pore protein
npp-9	WBGene00003795	++	++	---	nuclear pore protein
npp-7	WBGene00003793	---	++	---	nuclear pore protein
npp-3	WBGene00003789	---	++	---	nuclear pore protein
xpo-2	WBGene00002079	+	+	---	nuclear export receptor
xpo-2	WBGene00002079	---	++	---	nuclear export receptor
ribosome biogenesis					
rpc-1	WBGene00004411	---	++	---	RNA Pol III subunit
rrbs-1	WBGene00007617	---	+	---	ribosome biogenesis
nst-1	WBGene00003821	---	++	---	ribosome biogenesis?
C37H5.5	WBGene00016508	---	+	---	nucleolar complex protein 3 homolog
C47E12.7	WBGene00008151	---	+	---	rRNA processing?
K12H4.3	WBGene00019678	---	+	---	BRX1 homolog (ribosome biogenesis)
C18A3.3	WBGene00015941	---	++	---	rRNA processing?
translation					
eif-6	WBGene00001234	+	---	---	initiation factor
D2085.3	WBGene00008428	++	+++	+	eIF2B subunit
wars-1	WBGene00006945	---	+	---	tRNA synthetase
vesicle trafficing					
aps-1	WBGene00000159	++	---	---	vesicle trafficing
aps-1	WBGene00000159	+	+	---	
arf-3	WBGene00000183	+	++	---	intracellular traffikcing
dyn-1	WBGene00001130	+	++	---	dynamin related
other					
rnp-7	WBGene00004390	+	---	---	RNA binding
ani-1	WBGene00013038	+	---	---	actin binding protein
pyp-1	WBGene00008149	+	---	---	pyrophosphatase, nucleosome remodelling?
dut-1	WBGene00010609	+	---	---	deoxyUTPase
ril-2	WBGene00007586	+	+	---	RNAi induced longevity
ngp-1	WBGene00003596	---	+	---	GTP-binding protein
aco-2	WBGene00000041	++	---	---	aconitase
apl-1	WBGene00000149	+	---	---	amyloid precursor like
vha-2	WBGene00006911	+	+	---	proton transporting ATPase
hsp-60	WBGene00002025	---	++	---	mitochondial HSP
T09B4.9	WBGene00020383	---	+	---	mitochondrial import
toe-1	WBGene00022739	---	+	---	target of erk kinase
cct-8	WBGene00021934	---	+	---	Chaperonin complex
cacn-1	WBGene00012230	++	+	---	DTC migration vulva morph?
let-607	WBGene00002783	---	+	---	CREB family transcription factor
nhr-25	WBGene00003623	+	+	---	nuclear hormone receptor transcription factor
F44G4.1	WBGene00009711	+	---	---	
C53H9.2	WBGene00016907	+	+	---	
F11A3.2	WBGene00008670	+	+	---	
F11A3.2	WBGene00008670	---	++	---	
C16A3.4	WBGene00015809	---	++	---	
ZK430.7	WBGene00022742	+	---	---	
T11G6.8	WBGene00011722	---	++	---	
F53B7.3	WBGene00009966	---	++	---	
W04A4.5	WBGene00012234	+	++	---	
T06E6.1	WBGene00011538	---	+	---	
T23D8.3	WBGene00011944	---	+	---	
Y48G1A.4	WBGene00021660	---	+	---	
H06I04.3	WBGene00019168	---	+	---	

Significance and open questions

Genetic screens provide an unbiased strategy to identify new players involved in a given biological process. Completion of the primary *let-7* suppressor RNAi screen in a few months demonstrated the feasibility and practicability of this strategy even on a genome-wide scale. Whereas a forward genetic screen yielded about 50 suppressor alleles (Slack et al., 2000), we identified about 200 genes suppressing *let-7* bursting. Probably the major difference between a forward screen using ethylmetane sulfonate (EMS) and the reverse genetic screen by RNAi is the ability to identify lethal or dominant sterile mutations. Indeed, the biggest category of our novel suppressors consists of genes with a role in cell-cycle or cell-division, most of these genes have an embryonic lethal or larval arrest phenotype if deleted. Genetic interaction with cell-cycle genes was partly expected, but the identity of the suppressors was somewhat surprising. Specifically, identification of the condensin II complex, which has a role in chromatin condensation during mitosis, is a novel and unexpected finding. Based on cell-culture experiments, the prevailing hypothesis was that *let-7* would directly target cell-cycle regulators (Johnson et al., 2007). However, we could not validate *cdk-1* and *cdc-25.2* as direct *let-7* targets. In fact, our results reveal a tight connection between differentiation and proliferation in the hypodermis on a level downstream of *let-7*. Knock-down of many cell-cycle genes not only suppresses bursting, but also restored at least one aspect of hypodermal differentiation, namely expression of the adult specific collagen *col-19.* Experiments using *cdk-1(RNAi)* in the *lin-29* mutant background suggest that this effect requires *lin-29.* Block of proliferation might be thus directly linked to differentiation. We wanted to test this idea directly by using chemical inhibition of the cell-cycle, but we were not able to block seam cell division using hydroxyurea or fluorouracil at non-toxic concentrations, although we could suppress bursting at a lower dose of hydroxyurea. We cannot rule out that different tissues are differentially sensitive to cell-cycle inhibition or cell-cycle checkpoints might be not functional in the seam cells. An alternative explanation would imply a novel, cell-cycle unrelated function of at least some of the suppressors identified. On the other hand, we identified that *lin-29* not only induces the cell-cycle inhibitor *cki-1*(Hong et al., 1998), but suppresses also the cyclin-dependent kinase *cdk-1.* Inhibition of proliferation and differentiation are therefore mutually coupled.

Although we designed several different experiments to identify potential *let-7* regulators, our follow-up assays did not yield good candidates. As the molecular follow-up assay, quantification of targets by qPCR and *let-7* by Northern blot, might be not sensitive enough and *let-7* modulators might have specific functions, e.g. activity restricted to some tissues, we cannot rule out that our lists still contains some factors regulating *let-7.* Identification of targets might depend also on timing and strength of the RNAi, as we could not identify neither *lin-28* nor *xrn-2* in our screen.

let-7 suppressors might be downstream targets of *let-7.* Initial analysis indeed showed enrichment for genes with a predicted *let-7* target site among our positive hits, but microarray analysis did not show upregulation of suppressors in the *let-7* mutant background. Downstream *let-7* targets, if there are any on the list, might be thus regulated only to a small extent or at the protein level, or only in selected tissues.

At the end, the unsolved issues that became apparent during the screen led directly to my two other research projects, elucidation of *let-7's* target specificity and identification of the developmental defect underlying vulva bursting in *let-7* mutant worms.

2. Quantitative imaging of microRNA activity in vivo

Specific aims

The initial motivation to start this project was the desire to follow *let-7* activity in time and space *in vivo*. As *let-7* biogenesis and activity are regulated, *let-7* activity cannot be directly extrapolated from patterns of transcription. Moreover, it is still not clear, where the regulatory elements driving *let-7* expression reside, transcriptional reporters might thus be not adequate to show *let-7's* site of action. Unfortunately, direct visualization of miRNAs by immunofluorescence is not feasible in *C. elegans* and isolation of individual cells from whole worms was at that time an unrealistic goal. I focused therefore on developing further the *let-7* target reporter established in the follow-up stage of the genome-wide RNAi screen. The main goal was to obtain a system that allows quantitative assessment of miRNA activity in different cells of the worm over time.
I wanted to answer two major questions related to *let-7* functions and possible role of *let-7* suppressors. First, it was not clear to what extent *let-7* family members *mir-48/84/241* regulate the same targets as *let-7.* Redundancy between these miRNAs very similar in sequence has significant implications both for our understanding of the heterochronic pathway as well as for the analysis of *let-7* genetic interactors. As this question is also in general poorly understood, an answer to this question would provide important insights how members of a miRNA family or even different miRNAs achieve specific regulation of their targets. Second, available literature and my own results identifying *let-7* suppressors with predicted expression and functions outside the hypodermis suggested that *let-7* acts in many tissues of *C. elegans.* The first step in the elucidation of these functions is the demonstration of *let-7* activity in these cells. Comparison of *let-7* activity in different cells might also provide new insights, how developmental timing works across tissues at the organism levels. Furthermore, resolution of *let-7* activity across different tissues would provide a system to test the contribution of tissue-specific factors modulating *let-7* biogenesis or activity to target repression.

Manuscript: Quantitative imaging reveals target specificity of *let-7* family microRNAs *in vivo*

Quantitative imaging reveals target specificity of *let-7* family microRNAs *in vivo*

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Abstract

To understand functions of microRNAs (miRNAs) in development and pathology, and integrate them with other gene regulatory processes, quantitative measures of miRNA activity *in vivo* are needed. Here, we have developed a microscopy-based assay to quantify the activity of endogenous *let-7* family miRNAs in *C. elegans* under standardized conditions, across different tissues, over time, and for multiple targets. This has yielded a number of unexpected findings. First, targets are silenced extensively, i.e., several fold. Although consistent with a switch-like activity previously postulated, repression, second, occurs gradually over time. Third, despite sharing a seed sequence, members of the *let-7* family differ in their target specificities. Finally, specificity is mediated partially by target sequence, but at the same time context-dependent and variable across tissues. We conclude that individual *let-7* family members exhibit an unexpected degree of intrinsic, but malleable target specificity to facilitate selective and dynamically modulated repression of individual targets. This may provide a rationale for the occurrence of miRNAs in families and emphasizes a need for validation of miRNA-target interactions under physiological conditions.

Introduction

MicroRNAs frequently occur in 'families', characterized by a shared 'seed' sequence, which covers nucleotides two through eight from the 5'-end (Lim et al. 2003). The 'seed' was originally identified computationally, as the sequence yielding the strongest signal in miRNA target predictions (Lewis et al. 2003). Parallel work in flies (Lai 2002) provided evidence for the functionality of these sites, and this was further corroborated by miRNA transfection experiments, where again the predominant shared feature of repressed transcripts is the presence of a seed sequence (Lim et al. 2005). Its structural correlate is provided by the observation that the seed sequence of miRNAs bound to their Argonaute partner protein is pre-organized in a helical conformation (Ma et al. 2005).

Although the miRNA seed concept has been highly successful and influential for our understanding of miRNA targeting, other modes of miRNA target interaction have been identified (Brennecke et al. 2005; Lal et al. 2009; Shin et al. 2010; Chi et al. 2012; Helwak et al. 2013). However, it is less clear whether and how sequence diversity beyond the seed translates into functional differences among individual members of a miRNA family. In particular, although examples have been described where miRNA family members function non-redundantly (e.g. (Chalfie et al. 1981); (Reinhart et al. 2000) (Liu et al. 2008) (Zhao et al. 2011) it remains unresolved whether non-redundancy is caused by intrinsic target specificity or by differences in expression levels in time and/or space. A distinction between these two models requires quantitative knowledge of miRNA activity *in vivo*, which has so far been lacking.

In the heterochronic pathway of *C. elegans*, which directs temporal cell fates during larval development, members of the *let-7* family (Fig 1A) appear to have redundant as well as non-redundant functions (Ambros 2011). Although united in their function to control cell proliferation and differentiation, the four family members *let-7 proper*, miR-48, miR-84, and miR-241 are differentially expressed. Thus, *let-7* is

expressed late in development, with particularly high abundance in larval stage 4 (L4) and adult animals, whereas the sisters are already abundant in L2 (Reinhart et al. 2000; Abbott et al. 2005; Van Wynsberghe et al. 2011). Accordingly, loss of the *let-7* sisters causes defects in transition from L2 to L3 cell fates, whereas loss of *let-7 proper* prevents transition from L4 to adult cell fates (Reinhart et al. 2000; Abbott et al. 2005).

Genetic analysis has revealed three key targets of *let-7* family miRNAs in the heterochronic pathway: the *hbl-1* zinc finger transcription factor (Abrahante et al. 2003; Lin et al. 2003; Abbott et al. 2005); *lin-41*, an RNA binding protein and putative ubiquitin E3 ligase (Reinhart et al. 2000; Slack et al. 2000); and *daf-12*, a nuclear hormone receptor (Grosshans et al. 2005). Available data, in particular epistasis-type analysis of genetic interaction, suggest that all three are targets of both *let-7* and its sisters, but differences in repression patterns suggest the possibility of some specificity. For instance, repression of *hbl-1* in the epidermis occurs already during L2 (Lin et al. 2003), whereas *lin-41* repression occurs during L4 (Slack et al. 2000), when *let-7 proper* accumulates.
The detailed knowledge of genetic interactions, and the fact that sequence-related and unrelated miRNA act as key components, make the heterochronic pathway ideally suited to studying miRNA specificity under physiological conditions. At the same time, a major limitation to studying the heterochronic miRNAs, and miRNA function in *C. elegans* more generally, is the limited knowledge on their spatial expression and activity patterns. In particular, because detection of mature miRNAs has so far not been possible in *C. elegans* in situ, transcriptional reporters, utilizing fusion transgenes of putative miRNA promoters and GFP, have been used as proxies for expression pattern (Esquela-Kerscher et al. 2005). Although these provide indications of the tissues where miRNAs are transcribed, they fail to account for extensive post-transcriptional regulation to which miRNAs in general, and the *let-7* miRNAs in particular, are subject (Krol et al. 2010).

Here, we present a reporter system that has enabled us to quantify systematically the activity of heterochronic miRNAs across different tissues, targets, and developmental stages *in vivo*. Our work reveals not only extensive, several fold, silencing, of several targets and in several tissues, but also a high degree of specificity for individual targets among individual family members that goes beyond differences in miRNA expression patterns. Beyond increasing our understanding of the heterochronic pathway and providing a new tool for quantitative studies of miRNA activity in *C. elegans*, our work thus emphasizes the uniqueness of individual miRNA family members that becomes apparent when studying them under physiological conditions.

Results

The lin-41 3' UTR mediates let-7-dependent repression in multiple tissues

Although *let-7* and its sisters have been studied extensively with regard to their functions in development and disease and as model miRNAs, their spatial and temporal activity patterns in *C. elegans* are largely unknown. In particular, although target reporters have been widely used, these were typically present as integrated or extrachrosomal arrays of unknown copy number and thus variable, and typically strong, expression. To address these problems, we developed a two-color, quantitative imaging reporter system (Fig. 1B). To this end, we used the MosSCI technique (Frøkjaer-Jensen et al. 2008) for single-copy integration of transgenes in defined genomic loci to achieve standardized and physiological expression levels. To permit monitoring of dynamic regulation, we used a destabilized nuclear GFP (GFP/PEST, 'green') with a short (<1hr) half-life (Frand et al. 2005) as a reporter. Expression of the fluorophore from a ubiquitously active *dpy-30* promoter enabled us to survey a broad array of tissues. Finally, to achieve identification and digital segmentation of distinct cells as well as correction for biases arising in the imaging process, and ultimately quantitative analysis, we integrated a second transgene in single copy in a distinct genomic location. This transgene contained the same promoter, an unregulated artificial 3' UTR and encoded mCherry ('red') as a reporter.

To identify the tissues in which the two genomic locations used for integration permitted reproducible and comparable expression, we first fused GFP to the unregulated *unc-54* 3'UTR. Confocal imaging revealed comparable expression of green and red in all somatic tissues of the worm except certain neurons, which were thus not considered in subsequent analyses (Supplemental Movie 1). Differences were also observed in the gonad, where the integration site on chromosome IV (red transgene) is known to be inactive (Frøkjær-Jensen et al. 2012), and this tissue was also excluded from analysis.

Next, we replaced the *unc-54* 3'UTR in the green transgene with that of the *bona fide let-7* target *lin-41* (Fig. S1), to derive the gfp_lin-41 reporter. Repression of *lin-41* by *let-7* had previously been reported for the stem cell compartment of the worm epidermis, i.e., seam cells (Reinhart et al. 2000; Slack et al. 2000), and we could recapitulate this result (Fig. 1C and Supplemental Movie 2). Moreover, our use of a ubiquitously transcribed reporter further revealed that silencing additionally occurred in another, postmitotic epidermal cell type, hyp7, as well as the vulva, and the gut (Fig. 1C and D).

Only two of several partially *let-7* complementary sites, LCS1 and LCS2 (Fig. S1), along with their intervening sequence of 27 nt, are necessary and sufficient for *let-7*-dependent regulation of a *lin-41* 3'UTR reporter gene in seam cells (Vella et al. 2004). Consistent with a general requirement of these sites, deletion of these elements restored *gfp* expression in seam cells as well as the other tissues (Fig. 1C, D, and Supplemental Movie 3). Moreover, silencing depended on *let-7 proper*, as revealed by extensive desilencing of the gfp_lin-41 reporter in the *let-7(n2853ts)* allele at the restrictive temperature, 25°C (Fig. 1C and D). Consistent with the notion that *n2853* allele causes a complete loss of *let-7* activity at 25°C, we saw no further enhancement of desilencing in a *let-7(mn112)* null mutant strain (data not shown). Therefore, we used the temperature-sensitive *n2853* allele in subsequent experiments for ease of technical manipulation.

Finally, because the fluorophores may differ in half-lives and other parameters, it remained formally possible that *let-7* affected *dpy-30* promoter activity without noticeably affecting mCherry levels. We excluded this by verifying that the gfp_unc-54 reporter remained equally unchanged in *let-7* mutant animals (Fig. S2).

We conclude that the reporter system faithfully recapitulates silencing of *lin-41* by *let-7* previously observed by other methods (Slack et al. 2000; Bagga et al. 2005;

Ding and Grosshans 2009) and extends it to additional tissues. Repression in these tissues is likely to reflect physiological regulation of *lin-41*, because *lin-41* is transcribed in these tissues, with comparable levels of activity apparent for *lin-41* and *dpy-30* ((Slack et al. 2000) and Supplemental Fig. S3).

Quantitative monitoring reveals that the lin-41 *3'UTR confers extensive but gradual repression by* let-*7*

Silencing of targets by miRNAs is typically considered to be rather modest, leading to the suggestion that miRNAs mostly 'tune' expression of their targets (Bartel 2009). On the other hand, *lin-41* mRNA is several fold more abundant in *let-7(n2853)* mutant than wild-type animals at the fourth larval (L4) stage (Bagga et al. 2005; Ding and Grosshans 2009), consistent with the switch-like repression inferred from genetic data (Reinhart et al. 2000; Slack et al. 2000). It is possible that initial silencing by *let-7* via the *lin-41* 3'UTR is indeed modest but sufficient to induce further reduction of *lin-41* mRNA levels through feedback mechanisms involving for instance transcriptional repression. However, qualitatively, the confocal images (Fig. 1C and D) appeared to reflect strong repression.

To analyze silencing quantitatively, we computed repression of the gfp_lin-41 reporter relative to the gfp_unc-54 unregulated reporter at the L4 stage (Materials and Methods; Fig. S4). This analysis confirmed the visual impression and revealed extensive downregulation of gfp_lin-41, ranging from ~2- to >5-fold, depending on the tissue investigated (Fig. 2). Repression was particularly pronounced in seam cells, hyp7, and vulva, with a weaker effect in the intestine. We conclude that the *lin-41* 3’UTR makes a major contribution to repression of *lin-41* mRNA

The notion of a switch-like function suggests not only extensive, but also rapid silencing of *lin-41* by *let-7* (Pasquinelli and Ruvkun 2002). Surprisingly, this is not what we observed when we followed repression of gfp_lin-41 over time: Rather, the

extent of repression appeared to increase progressively during larval development, showing a gradual decline of relative signal intensity from the L3 to late L4 stage (Fig. 2B). Because GFP/PEST half-life is short (< 1h) (Frand et al. 2005), this is not an artifact of the reporter system, and mRNA seq revealed that endogenous *lin-41* mRNA levels also declined gradually from L3 to late L4 stage (F Aeschimann, D Gaidatzis and HG, unpublished data). Thus, in contrast to the more switch-like behavior observed by end-point examinations (Bagga et al. 2005; Ding and Grosshans 2009), our data reveal regulation of *lin-41* on a more continuous scale.

Repression of lin-41 relies preferentially but not exclusively on let-7 proper

Qualitative analysis of *lin-41* 3'UTR reporters revealed that LCS1 and LCS2 and their intervening sequence (Fig. S1) are required for *let-7*-dependent repression (Vella et al. 2004). Consistent with this conclusion, quantification of repression reveals complete derepression of a gfp_lin-41ΔLCS reporter in seam cells, where it produced as much GFP signal as the unregulated gfp_unc-54 3' UTR (Fig. 2A). This was also true for hyp7 and vulva (Fig. 2A).

If these sites exclusively mediated repression by *let-7 proper*, loss of this miRNA ought to derepress a reporter carrying the wild-type *lin-41* 3'UTR to the same extent as deletion of the sites. This is indeed what we observed in the vulva of *let-7(n2853)* mutant animals, suggesting that *lin-41* is exclusively or near-exclusively repressed by *let-7* proper in this tissue (Fig. 2C). We confirmed this notion by crossing the *lin-41* reporter into *mir-84* single, *mir-48 mir-241* double, or *mir-48 mir-241; mir-84* triple null mutant animals (in the following simply referred to as "triple mutant"). Little or no derepression was observed in any of these backgrounds (Fig. 2C and data not shown).

Strikingly, however, the situation was different in other tissues: In the seam cells, derepression of the reporter was more extensive when its *let-7* target sites were

deleted than when the wild-type reporter was examined in *let-7(n2853)* mutant animal (Fig. 2 A,C). Accordingly, deletion of the three *let-7* sisters also caused derepression of the *lin-41* reporter in this tissue, although to a lesser extent than loss of *let-7 proper* (Fig. 2C). Similar observations were also made for hyp7 (Fig. 2C). Finally, in the intestine, loss of *let-7* caused partial derepression of the *lin-41* reporter (Fig. 2C), whereas little to no derepression occurred upon loss of the *let-7* sisters (Fig. 2C). Interestingly, the intestine is also the tissue where the ΔLCS deletion may not suffice for full restoration of *gfp* expression (Fig. 2A), suggesting the presence of additional elements in the *lin-41* 3'UTR that can mediate silencing independently of *let-7* family miRNAs. In summary, although repression of *lin-41* seems to be largely restricted to *let-7 proper* in the vulva, this is not true in other tissues where several or all of the *let-7* sisters may contribute to repression, albeit to different extents.

let-7 *sisters, not* let-7 proper*, are the main repressors of* hbl-1

The promoters of all *let-7* family members are active in the vulva, albeit to different degrees (Esquela-Kerscher et al. 2005) and our unpublished data). However, miRNA levels can be extensively regulated post-transcriptionally, and this is particularly true for *let-7* miRNAs (Krol et al. 2010). Hence, individual *let-7* sisters may fail to accumulate in the vulva, thus explaining their inability to repress *lin-41*. We tested this possibility by generating a second *let-7* target reporter, gfp_hbl-1, which contained the 3'UTR of *hbl-1*. This 3'UTR was previously shown to cause repression in the epidermis and neurons (Abrahante et al. 2003; Lin et al. 2003) and loss of *let-7* sisters caused upregulation of an *hbl-1::gfp::hbl-1* reporter in hyp7 (Abbott et al. 2005).

When we quantified gfp_hbl-1 repression, we observed silencing in all four tissues where gfp_lin-41 was also silenced, at an extent of roughly 2-fold that for gfp_lin-41 (Fig. 3A and Supplemental Table S1). Strikingly, however, little or no de-repression was observed for the *hbl-1* reporter in *let-7(n2853)* mutant animals in epidermis,

gut, and vulva (Fig. 3B). By contrast, mutation of the *let-7* sisters caused extensive derepression in all tissues, including the vulva (Fig. 3B). This demonstrates that the *let-7* sisters are active in the vulva. Moreover, these data reveal reveal strikingly distinct miRNA requirements for the silencing of these two targets, namely preferential silencing through *let-7 proper* for *lin-41*, but through *let-7* sisters for *hbl-1*.

The daf-12 *3'UTR confers different levels of* let-7 *family member specificity in different tissues*

To probe the extent of this unexpected target specificity of *let-7* family miRNAs further, we investigated *daf-12*, which is regulated by both *let-7* proper and its sisters in seam cells (Grosshans et al. 2005; Hammell et al. 2009). A *daf-12* 3'UTR reporter, gfp_daf-12, generated a repression pattern that was similar to that of *lin-41* both in the tissues affected and the extent of silencing in each tissue (Fig. 3A). However, the individual contribution of the *let-7* family members to this silencing differed markedly from the pattern observed for either *lin-41* or *hbl-1*. In particular, gfp_daf-12 was almost equally derepressed by mutation of either *let-7 proper* or the three *let-7* sisters in hyp7, intestine and vulva (Fig. 3C). By contrast, in the seam cells, loss of the *let-7* sisters caused much more extensive desilencing than loss of *let-7* (Fig. 3C). Collectively, the data on *daf-12*, *hbl-1*, and *lin-41* thus reveal specificity of *let-7* family members towards individual targets. Moreover, specificity varies across tissues and is thus context-dependent.

lin-4 *but not its sister miR-237 represses lin-28*

Given the striking conservation of *let-7 proper*, where the mature miRNA sequence is identical in species ranging from *C. elegans* to humans, it seemed possible that the specificity observed here was unique to *let-7* family members. To test this possibility,

we examined the regulation of *lin-28* by the *lin-4* family, comprising *lin-4 proper* and miR-237. *lin-28* is an established target of *lin-4* (Moss et al. 1997) whereas it is unknown whether it is repressed by miR-237. *lin-28* may also be a target of *let-7* or its sister, although both the extent and physiological consequences of this repressive event are unclear.

Consistent with the published data, we found a gfp_lin-28 reporter to be strongly repressed in the hypodermis of wild-type animals, but derepressed in *lin-4(e912)* null mutant animals (Figs. 3A, 4). Surprisingly, however, whereas derepression was extensive in the seam cell, substantial silencing still occurred in hyp7. Moreover, in the intestine, where we also observed repression of gfp_lin-28 in wild-type animals, no derepression occurred upon loss of *lin-4* activity. Finally, *lin-4(e912)* animals lack a vulva so that we could not examine gfp_lin-28 derepression in this organ.

A different pattern emerged when we examined *mir-237(n4296)* null mutant animals (Miska et al. 2007): gfp_lin-28 was still fully silenced in all tissues examined. No *bona fide* targets of miR-237 are known so that we cannot formally exclude that miR-237 simply fails to accumulate in these tissues. However, we consider this unlikely because it is transcribed in hypodermis and seam cells (Esquela-Kerscher et al. 2005), and we determined by absolute quantification that it was present in similar abundance as *lin-4* in total worm RNA (Fig. S5). Thus, these data suggest that despite a perfect seed match, *lin-28* reveals preferential regulation by *lin-4*. This finding is fully consistent with the fact that *lin-4*, but not *mir-237* mutant animals exhibit heterochronic phenotypes (Miska et al. 2007).

The lin-41 3'UTR can be reengineered for different miRNA family member specificity

A parsimonious explanation of the specificity of *let-7* and its sisters for individual targets is that basepairing beyond the seed could confer it, consistent with in silico predictions (Fig. S1A). To test this possibility directly, we reengineered the *lin-41* 3'

UTR to increase complementarity to *let-7* sister miRNAs (Fig. 5A; S1B). First, we replaced LCS1 by a site with increased complementarity to miR-84, and LCS2 by a site with increased complementarity to miR-241. Both of the re-engineered sites were modeled on the predicted structure of the *let-7*:LCS2 hybrid (Fig. S1). We examined the silencing capacity of the re-engineered 3'UTR in the vulva as the tissue where activity of the wild-type *lin-41* 3'UTR is fully dependent on *let-7 proper.* We found that the re-engineered transgene continued to be repressed, albeit to a lower extent than the wild-type 3'UTR (Fig. 5B). Notably, however, repression of the novel reporter was no longer dependent on *let-7*, but required the *let-7* sisters (Fig. 5B).

In a second step, we designed two additional variants of the *lin-41* 3'UTR, where we changed both wild-type LCSs to provide complementarity to the 3' end of only a single miRNA, while retaining the specific designs of LCS1 and LCS2 (Fig. S1). Surprisingly, the resulting 3'UTRs were largely devoid of repressive activity (Fig. 5C). We conclude that pairing of at least parts of the 3' end of *let-7* miRNAs is important for effective silencing, but that such pairing alone may not always be sufficient for activity.

Discussion

An understanding of miRNA function at both a molecular-mechanistic and a developmental level requires knowledge of physiologically relevant target genes. Hence, validation of silencing would ideally be performed on endogenous transcripts. However, an inherent disadvantage of such studies is the difficulty to distinguish miRNA-mediated mRNA silencing from secondary effects that might for instance arise when silencing of a transcription factor represses additional genes that are not themselves miRNA targets.

MicroRNA target reporter transgenes help to overcome this problem through use of heterologous promoters. Accordingly, usage of reporter assays has a long history in the miRNA field (Wightman et al. 1993). However, because reporters rely on transgenesis, they may introduce biases. Thus, transcription levels achieved by transient transfection of transgenes in cultured cells or multicopy arrays in *C. elegans* are difficult to standardize and may substantially exceed endogenous promoter activity. Moreover, availability and ease of transgenesis rather than physiological considerations may dictate the choice of cells under investigation, and further promote the use of ectopically expressed miRNAs to investigate regulatory potential.

The new reporter system that we introduce here resolves several of these issues. In particular, the integration of transgenes in single-copy and at defined genomic sites promotes standardized transcription at physiological levels. These improvements not only generate results under more physiological conditions, they facilitate a transition from qualitative to quantitative analysis of miRNA activity *in vivo*. Moreover, the use of confocal microscopy allowed us to broaden the scope of our investigations: rather than using prior knowledge, guessing, or random choice to drive reporter expression at a specific time and in a specific tissue, ubiquitous and continuous expression of a reporter permits investigation of its repression over space and time, followed by focused analysis of acquired images.

The utility of this new system is illustrated by our analysis of *let-7* family miRNAs and their activity towards a number of different targets in the heterochronic pathway. Our observation that *let-7* silences *lin-41* gradually, rather than in a switch-like manner implied by genetic data (Slack et al. 2000), reveals how the ability to follow miRNA activity quantitatively over time can an add a new dimension of knowledge: It implies that *let-7* activity on *lin-41* is integrated with additional layers of *lin-41* regulation or information processing. For instance, transcriptional or post-transcriptional mechanisms might further sharpen the transition in LIN-41 levels. Alternatively, LIN-41 activity may not decline linearly with concentration. Instead, a distinct threshold might separate active from inactive LIN-41.

let-7 sisters have non-redundant functions

We exploited the new assay to determine whether individual members of the *let-7* family exhibit target specificity. The large number of possible permutations of individual targets and mutations, and the likelihood that certain combinations, such as the full *let-7* family deletion, would be lethal, prevents quantification of the full extent of redundancy. However, a focused analysis readily revealed target specificity of individual *let-7* family members, thus refuting a notion of general redundancy. We can exclude a trivial explanation of specificity, i.e., that some family members are generally absent or inactive in a particular tissue, where others thus appear to conduct specific functions. This is because all family members display at least some degree of activity in each of the tissues examined, while differing in the target towards which most activity is directed. Thus, we found that *daf-12* and, in particular, *hbl-1*, preferentially require *let-7* sisters, whereas *lin-41* is preferentially regulated by *let-7 proper*. This target specificity holds true across tissues for *lin-41* and *hbl-1*, but is more variable for *daf-12*. Finally, a general activity trend appears to be superimposed on the specificity effect: deletion of *let-7* sisters affects target silencing more strongly in the epidermis than the vulva, whereas the opposite is

true for *let-7 proper*. Base on these data, we conclude that specialization involves two levels - the target and the tissue where activity occurs. As discussed below, the relevant mechanisms may differ, but jointly, they permit the generation of highly unique and surprisingly dynamic patterns of mRNA regulation.

The role of seed pairing and extensive target complementarity in determining specificity

At the first level, that of the target, specificity of a miRNA is likely to be sequence-based. It had previously been hypothesized that the architectures of the *lin-41* LCSs might render them insensitive to repression by the *let-7* sisters (Brennecke et al. 2005). This is because LCS1 and LCS2 both combine sub-optimal seed matches with extensive complementarity to *let-7*, but not its sisters, outside the seed match, toward the 5' end of the site. Indeed, silencing of *lin-41* by *let-7* requires extensive complementarity as demonstrated experimentally (Vella et al. 2004) and this study). Moreover, our ability to redirect silencing away from *let-7 proper* to a combination of miR-84 and miR-241 through changing sequence in this part of the LCSs provides direct support for its role in driving specificity. Nonetheless, complementarity is not sufficient as illustrated by lack of functionality of LCSs engineered for repression by miR-84 or miR-241 alone. Additional factors, currently unknown, appear to promote activity in a *let-7* specific manner. At the same time, the fact that both *daf-12* and *hbl-1* are preferentially regulated by specific *let-7* family members despite carrying sites with perfect seed complementarity in their 3'UTRs (Lin et al. 2003; Grosshans et al. 2005) demonstrates that suboptimal seed binding does not constitute a general requirement for specificity.

Mechanisms beyond sequence complementarity contribute to, and modulate, miRNA target specificity

At the second level, that of the tissue, mechanisms beyond miRNA:target complementarity must drive miRNA specificity when the target is invariant. A possible mechanism involves alterations in miRNA levels. This is because different miRNA concentrations might not simply distinguish between active versus inactive miRNAs, but instruct a specific degree of activity (Bartel and Chen 2004; Mukherji et al. 2011). Conclusive experimental demonstration of such a mechanism may require methods to quantify miRNA levels in specific tissues of *C. elegans*, which do not currently exist.

Another mechanism to achieve specificity, potentially at both the tissue and target level, would involve distinct, trans-acting factors. Such factors might involve RNA-binding proteins (RBPs) expressed in a tissue-specific manner. For instance, if the binding site of such an RBP on the *lin-41* 3'UTR overlapped with one or both of the LCSs, this might explain why re-engineering the sites towards *let-7* family members would fail to restore full functionality. At this point, however, the fact and identity of such a factor remains hypothetical.

Implications for physiological miRNA functions

Irrespective of the mechanisms by which miRNA family members achieve specificity and by which specificity is modulated, an important implication of our findings is that miRNA activity is highly context-dependent. Hence, even 'functional' sites, in the presence of both the target and the miRNA, may not yield repression. This finding is important when considering not only the developmental function of miRNAs but also the roles of miRNAs in disease. Loss of different *let-7* family members contribute to a different extent to malignant phenotypes of cancer cells (Qian et al. 2011) and this might depend on differences in target repression activity

(Cimadamore et al. 2013). Interestingly, a single nucleotide polymorphism in the Ras 3'UTR residing in a non-seed region alters repression of Ras by *let-7* and increases the risk for several cancers (Chin et al. 2008; Ratner et al. 2010). Moreover, because the same target sites can be either specific for an individual family member or more broadly regulated, regulation of targets can also be more sophisticated and dynamic than previously anticipated. Thus, in some contexts, regulation of a unique family member might be sufficient to repress or derepress a given target, providing sensitivity, whereas in other situations, several family members may function redundantly, providing robustness. More generally then, the occurrence of miRNAs in families may then not simply be driven by a need to facilitate a greater diversity and specificity of expression patterns (Esquela-Kerscher et al. 2005; Liu et al. 2008) through differential regulation (Heo et al. 2012; Vadla et al. 2012), but also provide highly specific targeting of distinct targets expressed across a diversity of tissues.

Materials and methods

Worm handling and strains

Worms were grown using standard methods at 25°C except for the *lin-4(e912)* background which was maintained at 15°C. The genotypes of the strains investigated are provided in Supplemental Table S2. Note that *mir-241* and *mir-84* are divergently transcribed genes that are jointly deleted by nDf51. We have not investigated their individual deletions.

Construction of miRNA target reporters

3'UTRs were amplified using primers indicated in Supplemental Table S3 and inserted into the Multisite Gateway pDONR P2R-P3 vector. The 3' UTR entry vectors obtained were recombined together with a pdpy-30 and a GFP(PEST)-H2B (Wright et al. 2011) or mCherry-H2B plasmids (Supplemental Table S3) into MosSCI compatible destination vectors. All plasmids were verified by sequencing. Transgenic worms were created by Mos-mediated single-copy insertion (Frøkjær-Jensen et al. 2012). All transgenic lines were outcrossed at least three times. Because mCherry and GFP half-lives may differ, we examined expression of *dpy-30p::GFP(PEST)::H2B::unc-54*$_{3'UTR}$ in *mir-48 mir-241Δ* double mutant animals, where seam cell numbers are increased, as well as *let-7(n2853)* single mutant animals to exclude effects on *dpy-30* promoter activity. No differences in expression relative to the wild-type situation were noted (Fig. S2).

Imaging

From the reporter worms, z-stacks of 0.43 µm thickness were acquired in green, red and transmitted light channels at 40x magnification on a Zeiss LSM700 confocal microscope coupled to Zeiss Zen 2010 software equipped with a multi-position tile scan macro (Life Imaging Centre, Freiburg, Germany). The z-stacks were stitched together and compiled into a single image using XUVtools software (Emmenlauer et al. 2009).

Data analysis

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. Signal intensity in the green channel was divided by the red signal intensity for each cell, relative signal intensities were averaged for each tissue in each worm. Finally, the mean signal intensity per group of worms and the corresponding standard error of the mean were calculated. Relative signal intensities and fold changes normalized to *wt* were compared using Student's *t*-test in R. All differences pointed out in the text were significant at the level of $p<0.01$

Acknowledgement

We thank Raphael Thierry for help with image analysis. Some strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). Work on this project was supported by the Novartis Research Foundation through the FMI Foundation. M.E. was an SNF/SAMW Swiss MD/PhD-program fellow supported by an Oncosuisse fellowship.

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Figure legends

Fig. 1: A two-color target reporter system shows *let-7* dependent repression of *lin-41*

A *let-7* family miRNAs. Bold nucleotides indicate the shared seed sequence.

B Schematic depiction of a two-color miRNA target reporter system involving single copy transgene integration at specific sites in the indicated chromosomes.

C Reporter gene expression in the epidermis. Arrows point to seam cell nuclei; larger nuclei are part of the hyp7 syncytium.

D Fluorescent reporters in the vulva and intestine at the late L4 stage. Vulva cells are encircled; asterisks are next to intestinal nuclei.

Fig. 2: Quantitative analysis reveals extent, kinetics, and miRNA specificity of GFP_lin-41 repression

A Repression conferred by the *lin-41* 3'UTR is extensive and depends on presence of LCS1 and 2.

B Repression by the *lin-41* 3'UTR occurs gradually during larval development

C *let-7* is exclusively responsible for *lin-41* repression in the vulva, but *let-7* sisters contribute to silencing in the hypodermis

Error bars show standard error of the mean.

Fig. 3: Distinct target differ in their requirements for individual *let-7* family members

A Heatmap revealing extensive silencing of additional reporters containing 3'UTRs of presumed *let-7* family targets. Black lines separate larval stages as indicated. *unc-54* is an unregulated control 3'UTR.

B, C Elimination of *let-7* or its three sisters has distinct effects on B) *hbl-1* and C) *daf-12* 3'UTR-mediated repression. Effects can also vary across tissues.

Error bars show standard error of the mean.

Fig. 4: *lin-4,* but not its family member *mir-237,* regulates the *lin-28* 3'UTR

Derepression of a gfp_lin-28 reporter was examined in the indicated tissues and genetic backgrounds.

Error bars show standard error of the mean.

Fig. 5: The sequence beyond the seed in the *lin-41* LCSs influences specificity and extent of *lin-41* repression

A Schematic of the constructs used. Nucleotides in the non-seed part of the target site were mutated to bind the indicated *let-7* family members as detailed in Supplemental Fig. S1B

B Repression of the indicated reporter is reduced relative to the wild-type 3'UTR but no longer dependent on *let-7*.

C Repression of the indicated modified reporters is lost.

Error bars show standard error of the mean.

Supplemental Figure Legends

Fig. S1: Schematic depiction of *let-7* complementary sites in the *lin-41* 3'UTR forming hybrids with *let-7* family miRNAs

A Predicted duplexes formed between the two functional LCSs of the *lin-41* 3'UTR and the indicated miRNAs.

B LCSs in the *lin-41* 3'UTR were modified to generate duplexes with *let-7* sisters as indicated.

RNA duplexes were predicted using the RNAhybrid algorithm (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/)

Fig. S2: *let-7* family mutations do not affect *dpy-30* promoter activity

The GFP_unc-54 reporter, containing the unregulated *unc-54* 3'UTR, was crossed into *let-7(n2853)* or *mir-48 mir-241(nDf51)*, which impair differentiation and cause overproliferation of seam cells, respectively. Neither phenotype increased *dpy-30* promoter activity.

Fig. S3: The *lin-41* promoter is active in epidermis, vulva at levels comparable to *dpy-30*

A A *lin-41* promoter fusion (Table S3) transgene was integrated in single copy and found to drive expression in the epidermis (arrows; examples of seam cells), vulva (arrowheads), and intestine (asterisks).

B Animals containing either the *lin-41* or *dpy-30* promoter driven transgene were imaged under identical conditions, revealing comparable activity levels of the two promoters in the indicated tissues.

Fig. S4 Image segmentation for target reporter quantification

Seed points in the indicated tissues are selected. The right panel shows a montage of consecutive *z*-planes with the segmented area chosen by the algorithm outlined in red.

Fig. S5: Absolute quantification reveals comparable levels of *lin-4* and miR-237
RT-qPCR and synthetic standards were used to achieve absolute quantifcation of *lin-4* and miR-237 levels in total RNA from late L4 stage-animals.
Error bars show standard error of the mean from biological replicates (n=3).

Supplemental Tables

Table S1: Summary of the reporter quantification raw data

Table S2: Worm strains used in the study

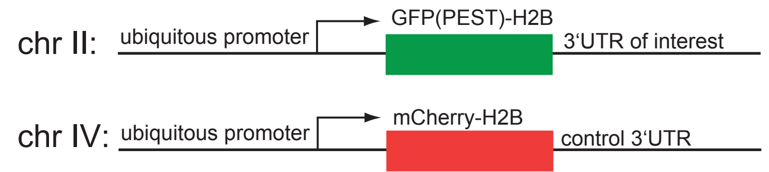
Table S3: Oligonucleotides and plasmids used

Ecsedi_Fig1

A

let-7: 5′ **TGAGGTA**GTAGGTTGTATAGTT
miR-48: 5′ **TGAGGTA**GGCTCAGTAGATGCGA
miR-84: 5′ **TGAGGTA**GTATGTAATATTGTA
miR-241: 5′ **TGAGGTA**GGTGCGAGAAATGA

B



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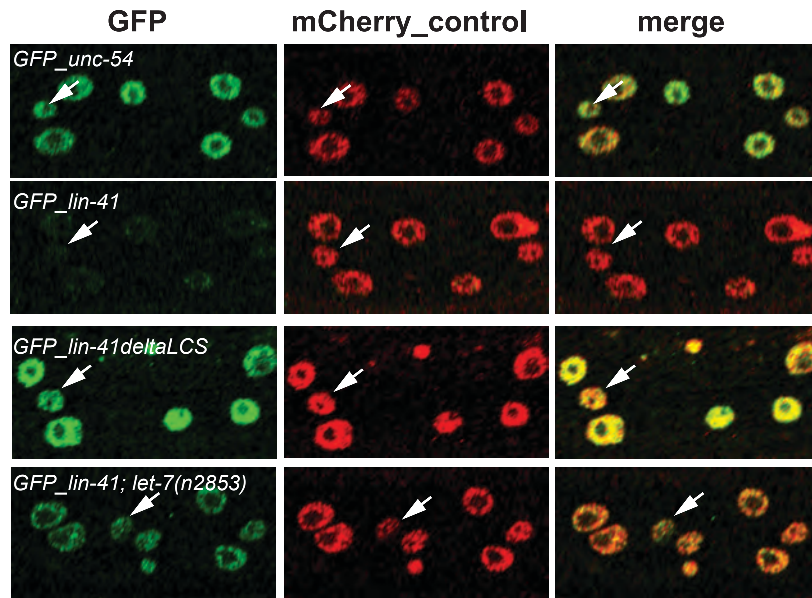

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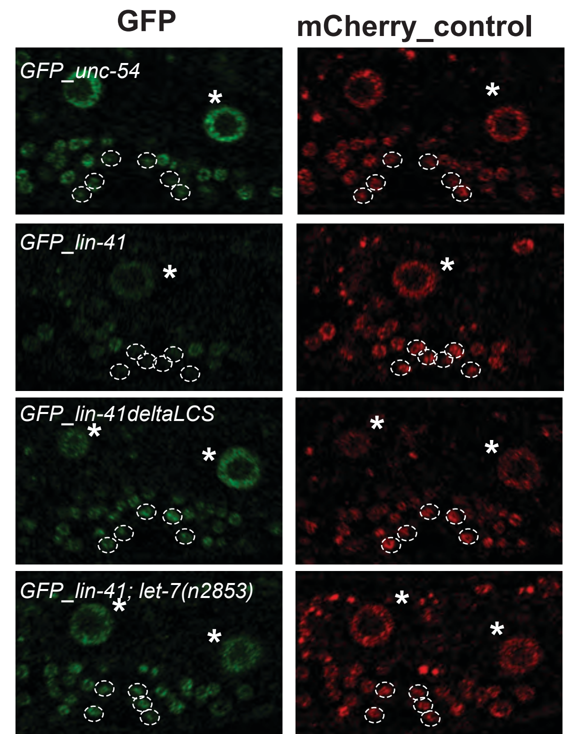
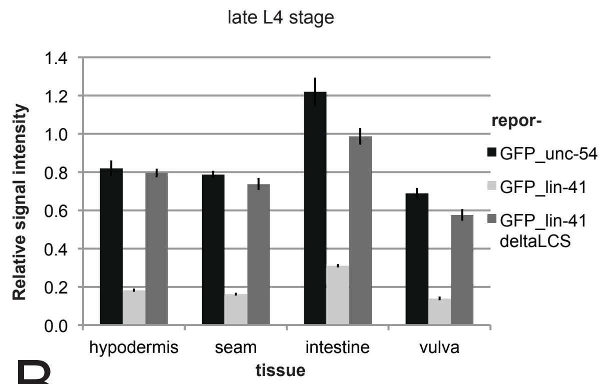


Ecsedi_Fig2

A



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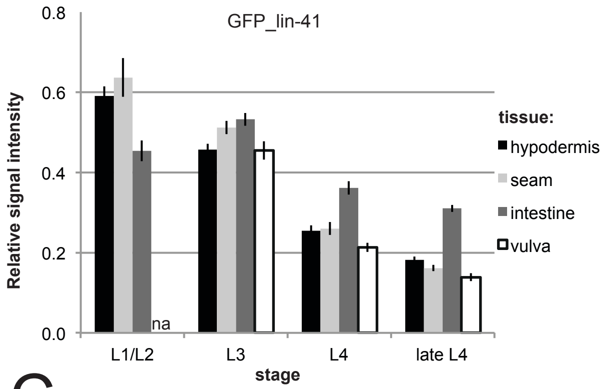

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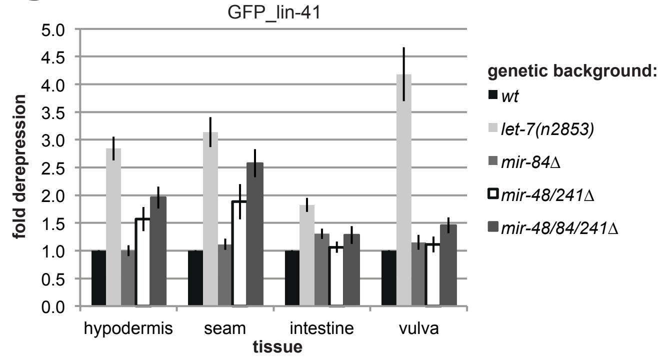

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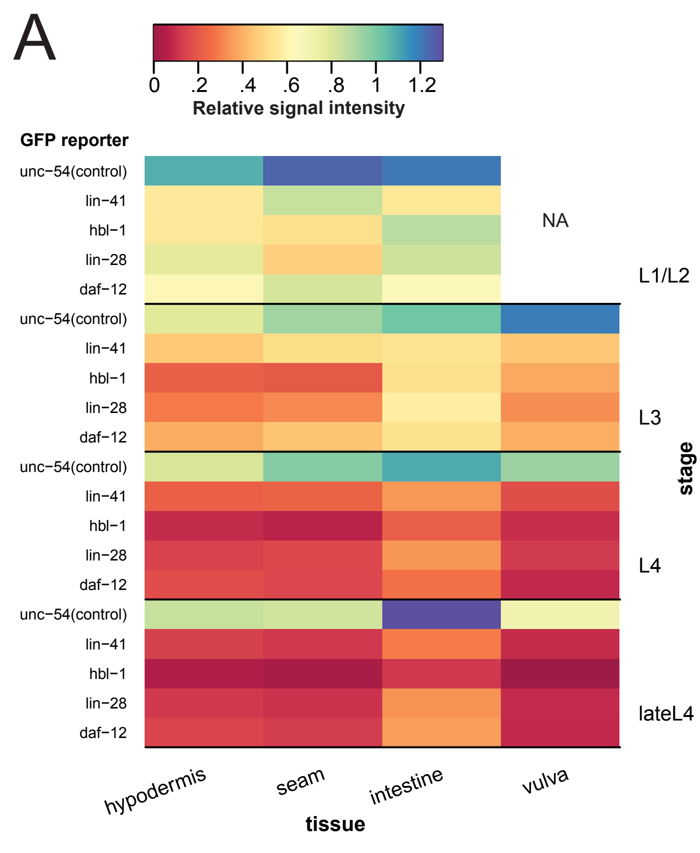
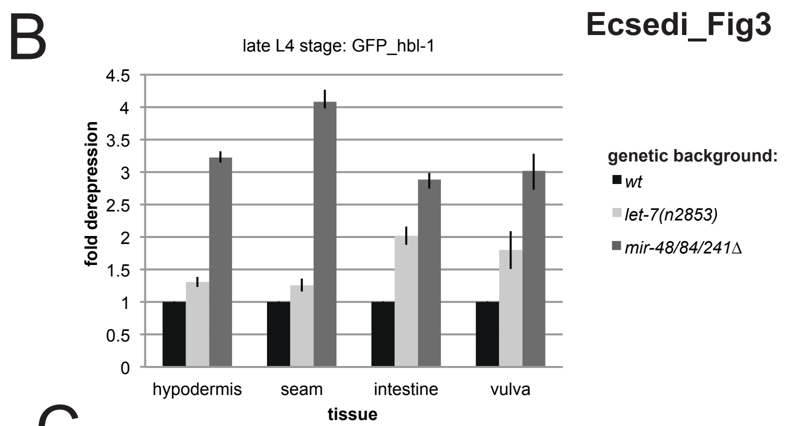
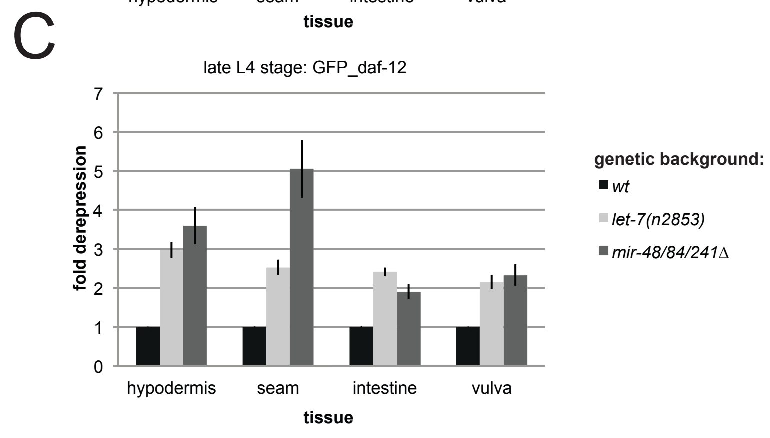
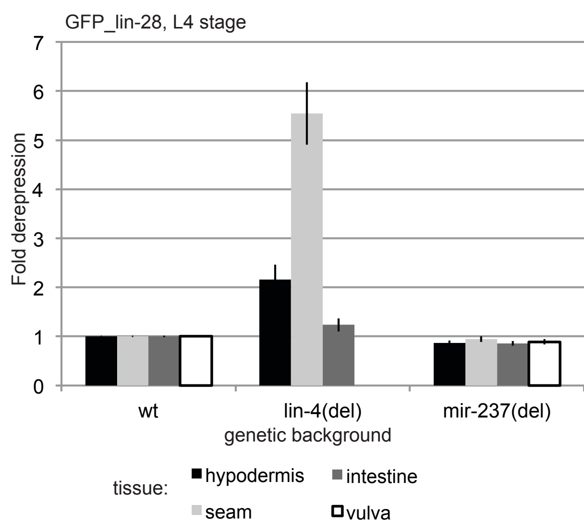


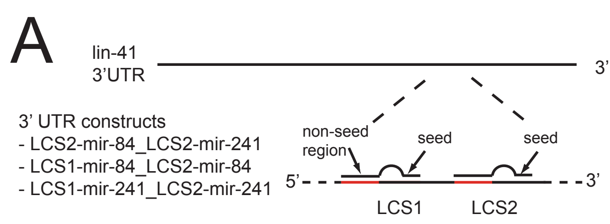


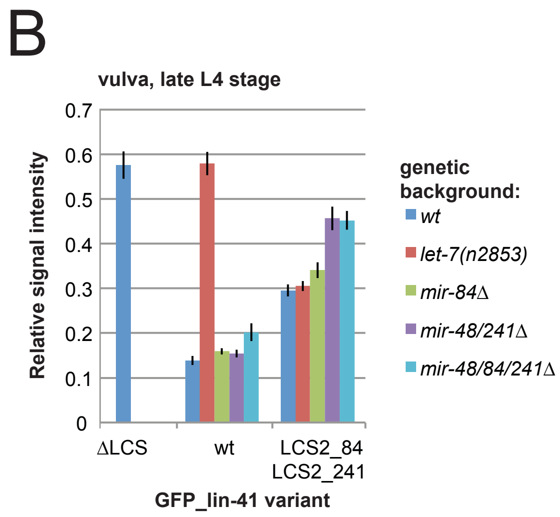


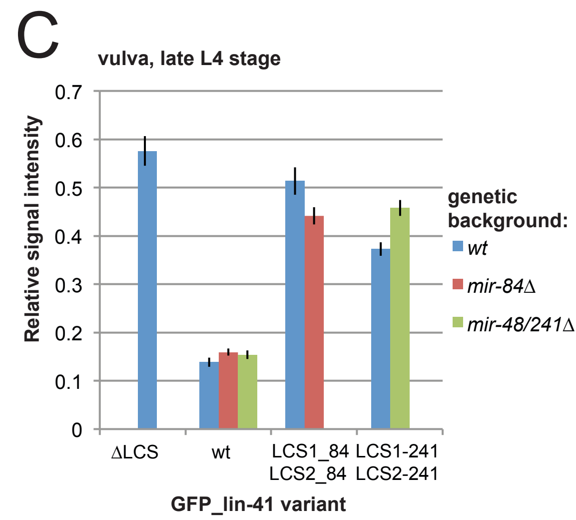
Ecsedi_Fig4



Ecsedi_Fig5







A

C. elegans lin-41 3'UTR

Ecsedi_FigS1

1.1kb

LCS1 position: 687-711 | | LCS2 position: 736-758

LCS1::*let-7*

lin-41 U GUU A A
5' UUAUACAACC **CUAC CUCA**
3' GAUAUGUUGG **GAUG GAGU**
let-7 UU AU

LCS2::*let-7*

lin-41 U AUU U
5' UUAUACAACC **CUGCCUC**
3' GAUAUGUUGG **GAUGGAG**
let-7 UU AU U

LCS1::*mir-48*

lin-41 A AACC U A A
5' UAC G **UCUAC CUCA**
3' AUG C **GGAUG GAGU**
mir-48 AGCGUAG ACU

LCS2::*mir-48*

lin-41 UUUAUACAACCAUU U
5' **CUGCCUC**
3' **GAUGGAG**
mir-48 AGCGUAGAUGACUC U

LCS1::*mir-84*

lin-41 A C U A A
5' UACA A CGU **CUAC CUCA**
3' AUGU U GUA **GAUG GAGU**
mir-84 AG UA AAU U

LCS2::*mir-84*

lin-41 A CC U U
5' UACAA AU **CUGCCUC**
3' AUGUU UG **GAUGGAG**
mir-84 AG AUAA UAU U

LCS1::*mir-241*

lin-41 C A A
5' CGU **UCUAC CUCA**
3' GCG **GGAUG GAGU**
mir-241 AGUAAAGA U

LCS2::*mir-241*

lin-41 A U U
5' C CA **UCUGCCUC**
3' G GU **GGAUGGAG**
mir-241 AGUAAAGA C U

B

Modified LCS constructs:

LCS2-mir-84_LCS2-mir-241

LCS2::*mir-84*

3'UTR UU AUU
5' CAAUAUUACA **CUGCCUCA**
3' GUUAUAAUGU **GAUGGAGU**
mir-84 AGAU AU

LCS2::*mir-241*

3'UTR A UGU U
5' CAUUUCUCGC **CUGCCUC**
3' GUAAAGAGCG **GAUGGAG**
mir-241 A UG U

LCS1-mir-84_LCS2-mir-84

LCS1::*mir-84*

3'UTR UU GUU A A
5' CAAUAUUACA **CUAC CUCA**
3' GUUAUAAUGU **GAUG GAGU**
mir-84 AGAU AU

LCS2::*mir-84*

3'UTR UU AUU U
5' CAAUAUUACA **CUGCCUC**
3' GUUAUAAUGU **GAUGGAG**
mir-84 AGAU AU U

LCS1-mir-241_LCS2-mir-241

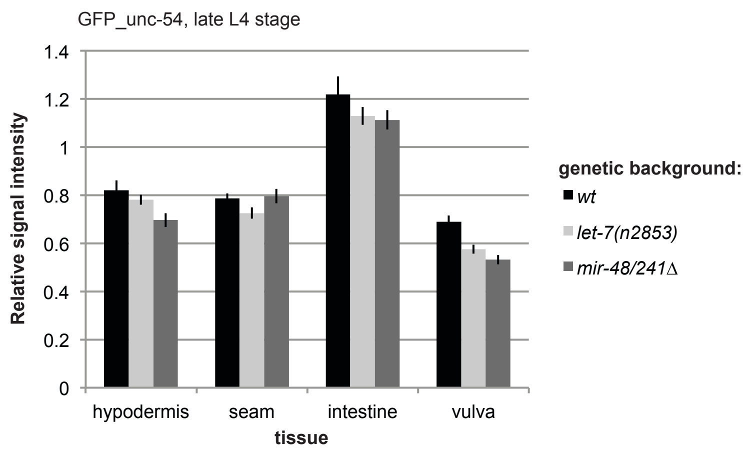
LCS1::*mir-241*

A GUU A A
3'UTR 5' CAUUUCUCGC **CUAC CUCA**
3' GUAAAGAGCG **GAUG GAGU**
A UG
mir-241

LCS2::*mir-241*

3'UTR A UGU U
5' CAUUUCUCGC **CUGCCUC**
3' GUAAAGAGCG **GAUGGAG**
mir-241 A UG U

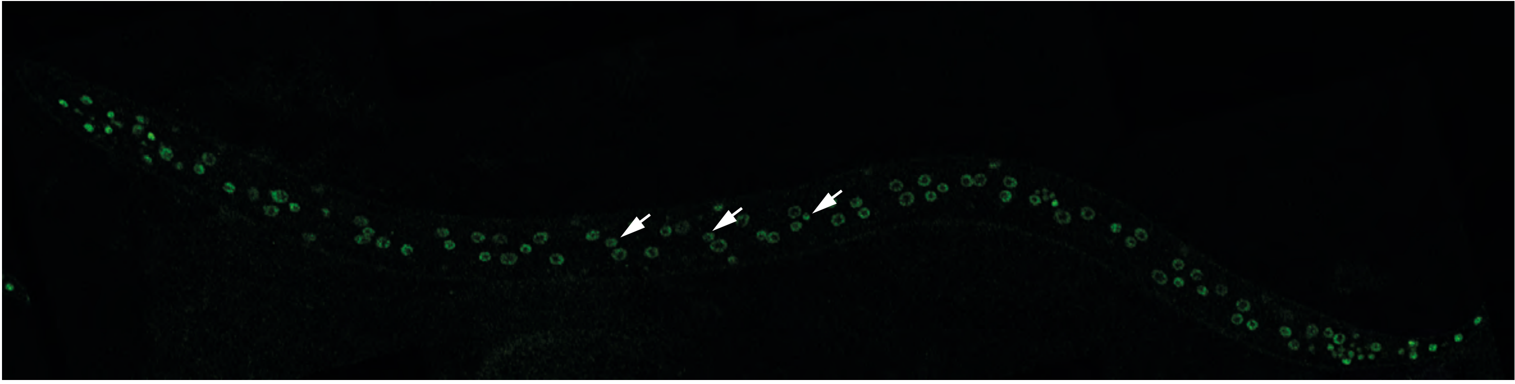
Ecsedi_FigS2

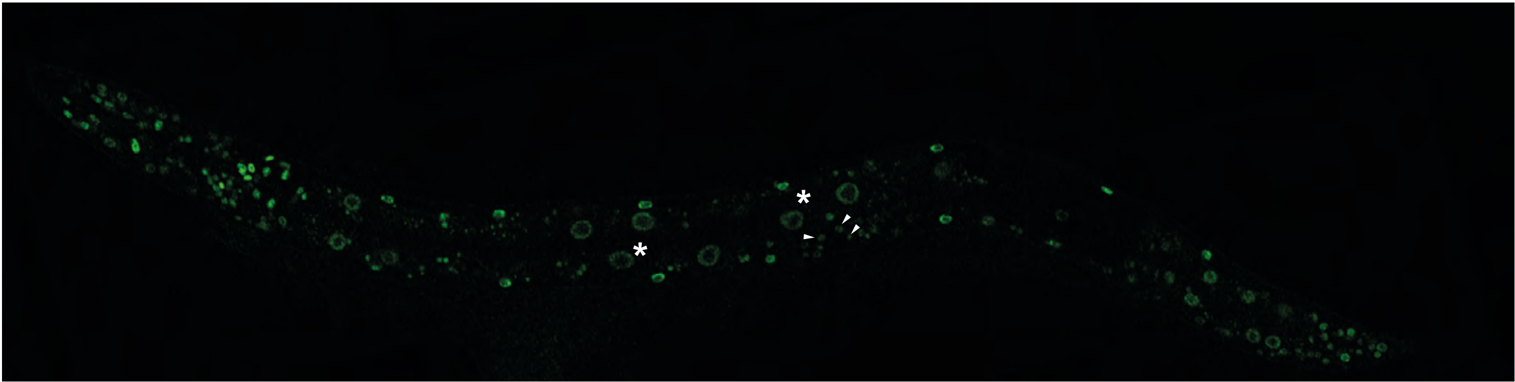


A

Ecsedi_FigS3

lin-41::GFP::let-858; L4 stage

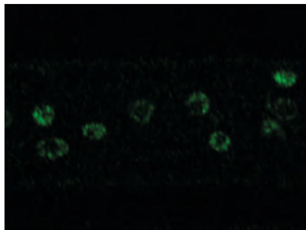


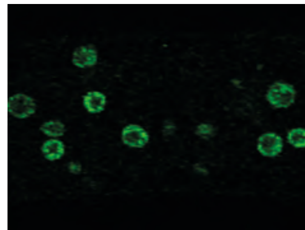


B

lin-41::GFP::let-858 *dpy-30::GFP::unc-54*

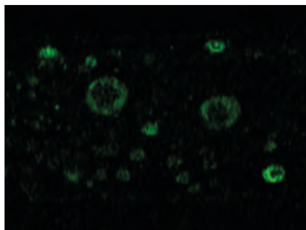
hypodermis

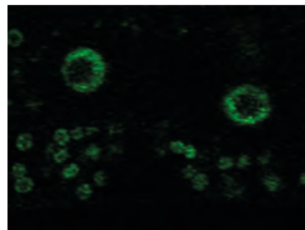




L4 stage

intestine
vulva



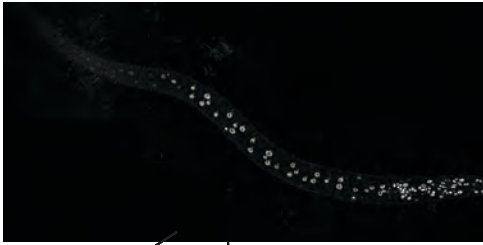


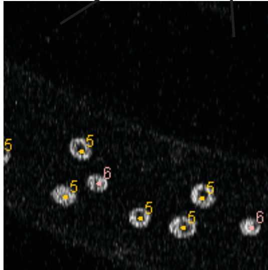
L4 stage

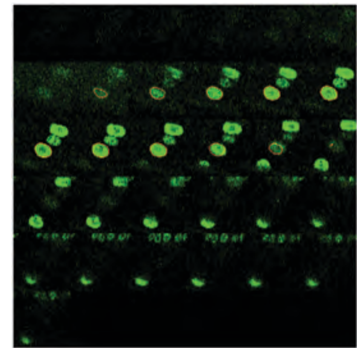
selection of cells of interest

segmentaton in 3D

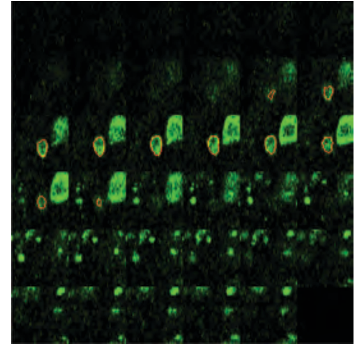
hypodermis



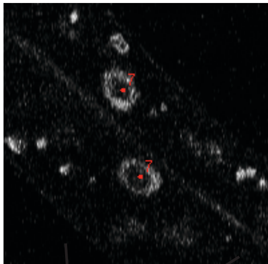




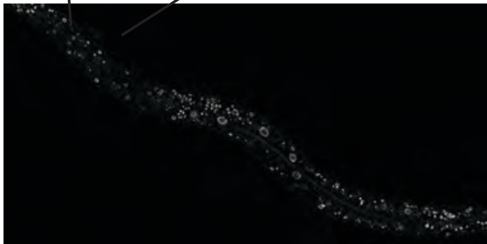
hyp7

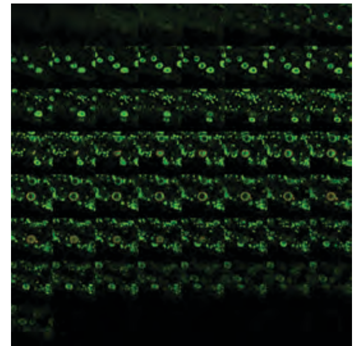


seam

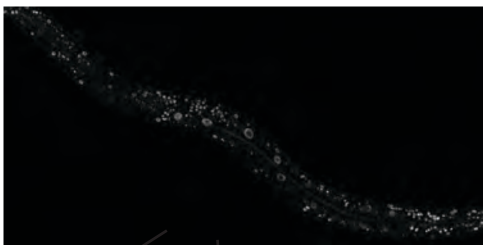


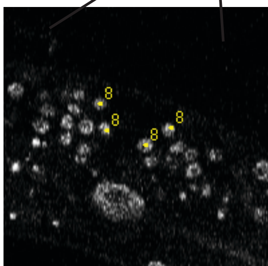
intestine

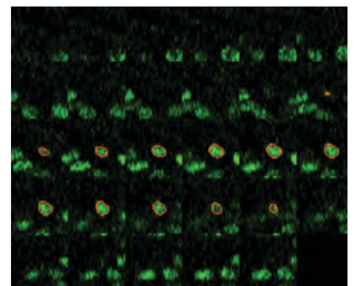


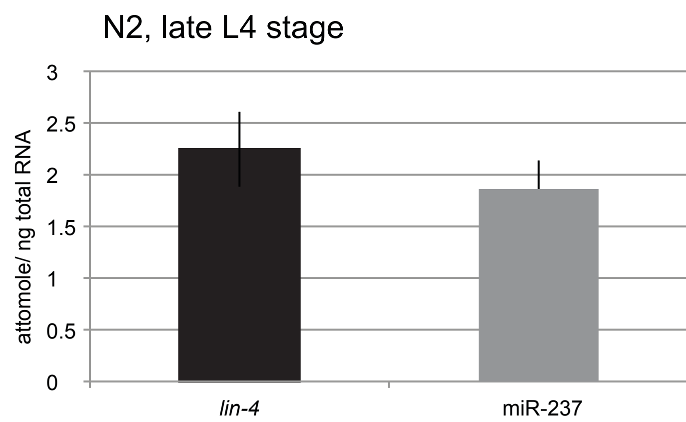


vulva








N2, late L4 stage
attomole/ ng total RNA
3
2.5
2
1.5
1
0.5
0
lin-4
miR-237

Reporter; genetic background	relative signal intensity(nr of worms quantified/standard error of the mean)			
GFP_unc-54; wt	**L1/L2**	**L3**	**L4**	**late L4**
hypodermis	0.82(n=21/SEM=0.064)	0.73(n=22/SEM=0.057)	0.76(n=27/SEM=0.044)	0.82(n=15/SEM=0.041)
seam	0.6(n=12/SEM=0.097)	0.87(n=22/SEM=0.056)	0.91(n=27/SEM=0.043)	0.79(n=15/SEM=0.02)
intestine	0.77(n=21/SEM=0.095)	0.9(n=22/SEM=0.085)	1(n=27/SEM=0.066)	1.22(n=14/SEM=0.074)
vulva		1.05(n=16/SEM=0.107)	0.86(n=23/SEM=0.068)	0.69(n=14/SEM=0.027)
GFP_unc-54; let-7(n2853)	**L1/L2**	**L3**	**L4**	**late L4**
hypodermis				0.78(n=16/SEM=0.021)
seam				0.73(n=16/SEM=0.024)
intestine				1.13(n=16/SEM=0.037)
vulva				0.58(n=16/SEM=0.018)
GFP_unc-54; mir-48/241Δ	**L1/L2**	**L3**	**L4**	**late L4**
hypodermis				0.7(n=19/SEM=0.029)
seam				0.8(n=19/SEM=0.03)
intestine				1.11(n=19/SEM=0.04)
vulva				0.53(n=19/SEM=0.019)
GFP_lin-41; wt	**L1/L2**	**L3**	**L4**	**late L4**
hypodermis	0.59(n=22/SEM=0.024)	0.46(n=29/SEM=0.014)	0.25(n=16/SEM=0.014)	0.18(n=22/SEM=0.008)
seam	0.64(n=16/SEM=0.048)	0.51(n=29/SEM=0.017)	0.26(n=16/SEM=0.016)	0.16(n=22/SEM=0.008)
intestine	0.45(n=16/SEM=0.026)	0.53(n=26/SEM=0.016)	0.36(n=16/SEM=0.017)	0.31(n=21/SEM=0.009)
vulva		0.45(n=15/SEM=0.023)	0.21(n=13/SEM=0.012)	0.14(n=22/SEM=0.01)
GFP_lin-41_ΔLCS	**L1/L2**	**L3**	**L4**	**late L4**
hypodermis	0.57(n=10/SEM=0.036)	0.53(n=6/SEM=0.054)	0.74(n=30/SEM=0.013)	0.79(n=16/SEM=0.022)
seam	0.45(n=6/SEM=0.051)	0.64(n=6/SEM=0.051)	0.92(n=30/SEM=0.02)	0.74(n=16/SEM=0.032)
intestine	0.56(n=10/SEM=0.035)	0.58(n=6/SEM=0.051)	0.68(n=25/SEM=0.018)	0.99(n=16/SEM=0.044)
vulva		0.84(n=3/SEM=0.062)	0.7(n=21/SEM=0.034)	0.58(n=15/SEM=0.03)
GFP_lin-41_ΔLCS; mir-48/241Δ	**L1/L2**	**L3**	**L4**	**late L4**
hypodermis				0.73(n=12/SEM=0.028)
seam				0.7(n=12/SEM=0.035)
intestine				0.93(n=12/SEM=0.051)
vulva				0.54(n=12/SEM=0.027)
GFP_lin-41; let-7(n2853)	**L1/L2**	**L3**	**L4**	**late L4**
hypodermis		0.41(n=7/SEM=0.042)	0.56(n=20/SEM=0.033)	0.52(n=37/SEM=0.017)
seam		0.48(n=7/SEM=0.069)	0.78(n=20/SEM=0.058)	0.51(n=37/SEM=0.019)
intestine		0.4(n=7/SEM=0.064)	0.57(n=20/SEM=0.037)	0.57(n=31/SEM=0.024)
vulva		0.54(n=7/SEM=0.123)	0.77(n=18/SEM=0.065)	0.58(n=30/SEM=0.026)
GFP_lin-41; mir-84delΔ	**L1/L2**	**L3**	**L4**	**late L4**
hypodermis			0.32(n=4/SEM=0.033)	0.18(n=21/SEM=0.01)
seam			0.4(n=4/SEM=0.073)	0.18(n=21/SEM=0.008)
intestine			0.45(n=4/SEM=0.038)	0.41(n=22/SEM=0.017)
vulva			0.35(n=4/SEM=0.04)	0.16(n=21/SEM=0.007)
GFP_lin-41; mir-48/241Δ	**L1/L2**	**L3**	**L4**	**late L4**
hypodermis			0.33(n=12/SEM=0.027)	0.29(n=15/SEM=0.028)
seam			0.39(n=12/SEM=0.051)	0.3(n=15/SEM=0.036)
intestine			0.31(n=12/SEM=0.012)	0.33(n=15/SEM=0.022)
vulva			0.24(n=10/SEM=0.021)	0.15(n=15/SEM=0.009)
GFP_lin-41; mir-48/84/241Δ	**L1/L2**	**L3**	**L4**	**late L4**
hypodermis			0.42(n=10/SEM=0.021)	0.36(n=13/SEM=0.019)
seam			0.47(n=10/SEM=0.036)	0.42(n=15/SEM=0.029)
intestine			0.39(n=10/SEM=0.029)	0.4(n=14/SEM=0.021)
vulva			0.23(n=10/SEM=0.016)	0.2(n=14/SEM=0.02)
GFP_lin-41_LCS2-mir-84_LCS2-mir-241	**L1/L2**	**L3**	**L4**	**late L4**
hypodermis			0.37(n=10/SEM=0.032)	0.38(n=24/SEM=0.019)
seam			0.49(n=10/SEM=0.029)	0.34(n=25/SEM=0.018)
intestine			0.44(n=10/SEM=0.042)	0.65(n=25/SEM=0.028)
vulva			0.36(n=10/SEM=0.024)	0.3(n=24/SEM=0.013)
GFP_lin-41_LCS2-mir-84_LCS2-mir-241; let-7(n2853)	**L1/L2**	**L3**	**L4**	**late L4**
hypodermis			0.32(n=8/SEM=0.012)	0.28(n=18/SEM=0.007)
seam			0.32(n=8/SEM=0.01)	0.3(n=19/SEM=0.008)
intestine			0.32(n=8/SEM=0.014)	0.37(n=17/SEM=0.019)
vulva			0.3(n=8/SEM=0.02)	0.31(n=18/SEM=0.011)
GFP_lin-41_LCS2-mir-84_LCS2-mir-241; mir-84Δ	**L1/L2**	**L3**	**L4**	**late L4**
hypodermis			0.5(n=4/SEM=0.025)	0.38(n=17/SEM=0.021)
seam			0.56(n=4/SEM=0.018)	0.36(n=17/SEM=0.019)
intestine			0.69(n=4/SEM=0.017)	0.74(n=17/SEM=0.031)
vulva			0.41(n=4/SEM=0.02)	0.34(n=17/SEM=0.018)
GFP_lin-41_LCS2-mir-84_LCS2-mir-241; mir-48/241Δ	**L1/L2**	**L3**	**L4**	**late L4**
hypodermis				0.63(n=16/SEM=0.035)
seam				0.67(n=16/SEM=0.038)
intestine				0.73(n=16/SEM=0.05)
vulva				0.46(n=16/SEM=0.026)
GFP_lin-41_LCS2-mir-84_LCS2-mir-241; mir-48/84/241Δ	**L1/L2**	**L3**	**L4**	**late L4**
hypodermis			0.61(n=15/SEM=0.019)	0.51(n=20/SEM=0.019)
seam			0.73(n=15/SEM=0.028)	0.59(n=19/SEM=0.016)
intestine			0.61(n=15/SEM=0.026)	0.64(n=19/SEM=0.032)
vulva			0.57(n=15/SEM=0.02)	0.45(n=20/SEM=0.021)
GFP_lin-41_LCS1-mir-84_LCS2-mir-84	**L1/L2**	**L3**	**L4**	**late L4**
hypodermis			0.69(n=8/SEM=0.021)	0.59(n=18/SEM=0.019)
seam			0.8(n=8/SEM=0.046)	0.57(n=18/SEM=0.028)
intestine			0.77(n=8/SEM=0.041)	0.83(n=17/SEM=0.025)
vulva			0.63(n=8/SEM=0.035)	0.51(n=18/SEM=0.029)
GFP_lin-41_LCS1-mir-84_LCS2-mir-84; let-7(n2853)	**L1/L2**	**L3**	**L4**	**late L4**
hypodermis			0.47(n=11/SEM=0.023)	0.46(n=11/SEM=0.017)
seam			0.52(n=10/SEM=0.032)	0.46(n=12/SEM=0.015)
intestine			0.41(n=9/SEM=0.027)	0.45(n=12/SEM=0.022)
vulva			0.51(n=9/SEM=0.028)	0.55(n=13/SEM=0.015)
GFP_lin-41_LCS1-mir-84_LCS2-mir-84; mir-84Δ	**L1/L2**	**L3**	**L4**	**late L4**
hypodermis			0.67(n=4/SEM=0.022)	0.52(n=11/SEM=0.029)

seam			0.7(n=4/SEM=0.033)	0.44(n=11/SEM=0.019)
intestine			0.67(n=4/SEM=0.039)	0.76(n=11/SEM=0.03)
vulva			0.5(n=3/SEM=0.016)	0.44(n=10/SEM=0.018)
GFP_lin-41_LCS1-mir-84_LCS2-mir-84; mir-48/241∆	**L1/L2**	**L3**	**L4**	**late L4**
hypodermis			0.7(n=3/SEM=0.053)	0.61(n=6/SEM=0.055)
seam			0.72(n=3/SEM=0.032)	0.59(n=6/SEM=0.052)
intestine			0.78(n=3/SEM=0.089)	0.76(n=6/SEM=0.061)
vulva			0.63(n=3/SEM=0.037)	0.44(n=6/SEM=0.039)
GFP_lin-41_LCS1-mir-241_LCS2-mir-241	**L1/L2**	**L3**	**L4**	**late L4**
hypodermis			0.49(n=16/SEM=0.012)	0.47(n=19/SEM=0.014)
seam			0.61(n=16/SEM=0.018)	0.47(n=19/SEM=0.018)
intestine			0.53(n=14/SEM=0.02)	0.61(n=19/SEM=0.029)
vulva			0.46(n=13/SEM=0.013)	0.37(n=19/SEM=0.014)
GFP_lin-41_LCS1-mir-241_LCS2-mir-241; mir-48/241∆	**L1/L2**	**L3**	**L4**	**late L4**
hypodermis			0.59(n=10/SEM=0.03)	0.6(n=16/SEM=0.024)
seam			0.67(n=9/SEM=0.04)	0.57(n=16/SEM=0.024)
intestine			0.53(n=11/SEM=0.041)	0.76(n=17/SEM=0.039)
vulva			0.46(n=10/SEM=0.03)	0.46(n=15/SEM=0.017)
GFP_hbl-1	**L1/L2**	**L3**	**L4**	**late L4**
hypodermis	0.56(n=20/SEM=0.061)	0.25(n=15/SEM=0.014)	0.14(n=26/SEM=0.005)	0.1(n=19/SEM=0.003)
seam	0.36(n=13/SEM=0.072)	0.24(n=15/SEM=0.013)	0.12(n=26/SEM=0.005)	0.08(n=18/SEM=0.004)
intestine	0.79(n=19/SEM=0.065)	0.51(n=15/SEM=0.019)	0.25(n=26/SEM=0.016)	0.16(n=18/SEM=0.006)
vulva		0.4(n=13/SEM=0.036)	0.15(n=26/SEM=0.011)	0.06(n=21/SEM=0.006)
GFP_hbl-1; let-7(n2853)	**L1/L2**	**L3**	**L4**	**late L4**
hypodermis			0.17(n=12/SEM=0.006)	0.13(n=20/SEM=0.004)
seam			0.14(n=12/SEM=0.006)	0.1(n=20/SEM=0.003)
intestine			0.36(n=12/SEM=0.013)	0.33(n=20/SEM=0.012)
vulva			0.15(n=12/SEM=0.012)	0.12(n=19/SEM=0.009)
GFP_hbl-1; mir-84del∆	**L1/L2**	**L3**	**L4**	**late L4**
hypodermis			0.15(n=9/SEM=0.009)	0.11(n=18/SEM=0.004)
seam			0.12(n=9/SEM=0.008)	0.09(n=18/SEM=0.003)
intestine			0.4(n=9/SEM=0.023)	0.36(n=18/SEM=0.014)
vulva			0.16(n=9/SEM=0.017)	0.1(n=18/SEM=0.005)
GFP_hbl-1; mir-48/241del∆	**L1/L2**	**L3**	**L4**	**late L4**
hypodermis			0.25(n=8/SEM=0.034)	0.21(n=20/SEM=0.01)
seam			0.22(n=7/SEM=0.037)	0.17(n=19/SEM=0.01)
intestine			0.29(n=8/SEM=0.037)	0.27(n=19/SEM=0.01)
vulva			0.22(n=7/SEM=0.025)	0.12(n=19/SEM=0.006)
GFP_hbl-1; mir-48/84/241del∆	**L1/L2**	**L3**	**L4**	**late L4**
hypodermis			0.4(n=14/SEM=0.021)	0.31(n=16/SEM=0.022)
seam			0.51(n=14/SEM=0.049)	0.32(n=16/SEM=0.026)
intestine			0.62(n=14/SEM=0.042)	0.47(n=16/SEM=0.031)
vulva			0.37(n=13/SEM=0.047)	0.2(n=16/SEM=0.015)
GFP_daf-12	**L1/L2**	**L3**	**L4**	**late L4**
hypodermis	0.68(n=15/SEM=0.048)	0.36(n=19/SEM=0.021)	0.2(n=20/SEM=0.013)	0.13(n=20/SEM=0.006)
seam	0.6(n=12/SEM=0.068)	0.33(n=19/SEM=0.022)	0.17(n=20/SEM=0.013)	0.1(n=20/SEM=0.004)
intestine	0.68(n=15/SEM=0.042)	0.44(n=19/SEM=0.021)	0.28(n=20/SEM=0.014)	0.25(n=20/SEM=0.007)
vulva		0.31(n=11/SEM=0.032)	0.14(n=19/SEM=0.011)	0.09(n=20/SEM=0.004)
GFP_daf-12; let-7(n2853)	**L1/L2**	**L3**	**L4**	**late L4**
hypodermis			0.4(n=11/SEM=0.011)	0.39(n=17/SEM=0.01)
seam			0.33(n=11/SEM=0.016)	0.26(n=17/SEM=0.01)
intestine			0.64(n=11/SEM=0.017)	0.6(n=17/SEM=0.01)
vulva			0.31(n=11/SEM=0.02)	0.2(n=17/SEM=0.008)
GFP_daf-12; mir-84∆	**L1/L2**	**L3**	**L4**	**late L4**
hypodermis			0.27(n=9/SEM=0.032)	0.15(n=27/SEM=0.006)
seam			0.24(n=9/SEM=0.035)	0.13(n=27/SEM=0.005)
intestine			0.42(n=9/SEM=0.03)	0.3(n=27/SEM=0.011)
vulva			0.27(n=9/SEM=0.062)	0.12(n=27/SEM=0.006)
GFP_daf-12; mir-48/241del∆	**L1/L2**	**L3**	**L4**	**late L4**
hypodermis			0.48(n=6/SEM=0.028)	0.41(n=5/SEM=0.017)
seam			0.61(n=6/SEM=0.046)	0.46(n=5/SEM=0.056)
intestine			0.44(n=6/SEM=0.028)	0.39(n=6/SEM=0.024)
vulva			0.28(n=5/SEM=0.02)	0.2(n=6/SEM=0.016)
GFP_daf-12; mir-48/84/241del∆	**L1/L2**	**L3**	**L4**	**late L4**
hypodermis			0.66(n=9/SEM=0.025)	0.47(n=19/SEM=0.043)
seam			1(n=9/SEM=0.089)	0.52(n=19/SEM=0.055)
intestine			0.61(n=9/SEM=0.031)	0.47(n=19/SEM=0.034)
vulva			0.43(n=9/SEM=0.045)	0.22(n=18/SEM=0.016)
GFP_lin-28	**L1/L2**	**L3**	**L4**	**late L4**
hypodermis	0.73(n=35/SEM=0.063)	0.31(n=16/SEM=0.019)	0.18(n=16/SEM=0.007)	0.17(n=16/SEM=0.007)
seam	0.25(n=18/SEM=0.046)	0.33(n=16/SEM=0.026)	0.2(n=16/SEM=0.009)	0.15(n=16/SEM=0.006)
intestine	0.7(n=33/SEM=0.031)	0.57(n=15/SEM=0.032)	0.36(n=16/SEM=0.009)	0.35(n=16/SEM=0.012)
vulva		0.35(n=15/SEM=0.011)	0.17(n=16/SEM=0.005)	0.14(n=16/SEM=0.004)
GFP_lin-28; let-7(n2853)	**L1/L2**	**L3**	**L4**	**late L4**
hypodermis			0.18(n=4/SEM=0.001)	0.18(n=17/SEM=0.009)
seam			0.19(n=4/SEM=0.009)	0.16(n=17/SEM=0.005)
intestine			0.32(n=4/SEM=0.009)	0.35(n=17/SEM=0.017)
vulva			0.16(n=4/SEM=0.004)	0.15(n=17/SEM=0.011)
GFP_lin-28; mir-84∆	**L1/L2**	**L3**	**L4**	**late L4**
hypodermis			0.17(n=2/SEM=0.004)	0.15(n=17/SEM=0.007)
seam			0.18(n=2/SEM=0.001)	0.16(n=17/SEM=0.012)
intestine			0.34(n=2/SEM=0.017)	0.34(n=17/SEM=0.013)
vulva			0.16(n=2/SEM=0.036)	0.13(n=17/SEM=0.006)
GFP_lin-28; mir-48/241del∆	**L1/L2**	**L3**	**L4**	**late L4**
hypodermis			0.34(n=5/SEM=0.026)	0.25(n=20/SEM=0.012)
seam			0.47(n=5/SEM=0.041)	0.27(n=20/SEM=0.021)
intestine			0.5(n=5/SEM=0.014)	0.44(n=20/SEM=0.018)
vulva			0.25(n=5/SEM=0.033)	0.17(n=19/SEM=0.011)
GFP_lin-28; mir-48/84/241del∆	**L1/L2**	**L3**	**L4**	**late L4**
hypodermis			0.37(n=14/SEM=0.015)	0.29(n=21/SEM=0.01)

seam			0.46(n=14/SEM=0.028)	0.32(n=21/SEM=0.017)
intestine			0.44(n=14/SEM=0.014)	0.41(n=21/SEM=0.011)
vulva			0.23(n=14/SEM=0.016)	0.18(n=21/SEM=0.008)
GFP_lin-28; lin-4(e912)	**L1/L2**	**L3**	**L4**	**late L4**
hypodermis			0.38(n=15/SEM=0.042)	
seam			0.98(n=15/SEM=0.076)	
intestine			0.44(n=14/SEM=0.039)	
vulva			na	
GFP_lin-28; mir-237Δ	**L1/L2**	**L3**	**L4**	
hypodermis			0.15(n=22/SEM=0.004)	
seam			0.15(n=22/SEM=0.003)	
intestine			0.31(n=22/SEM=0.006)	
vulva			0.13(n=22/SEM=0.004)	

Strain number	Genotype
HW1120	*xeSi100[Pdpy-30::GFP(PEST)-H2B::unc-54 3'UTR, unc-119 (+)] II, xeSi36[Pdpy-30::mCherry::H2B::6xmir-35mut, unc-119(+)] IV*
HW1180	*xeSi100[Pdpy-30::GFP(PEST)-H2B::unc-54 3'UTR, unc-119 (+)] II, xeSi36[Pdpy-30::mCherry::H2B::6xmir-35mut, unc-119(+)] IV; let-7(n2853) X*
HW1181	*xeSi100[Pdpy-30::GFP(PEST)-H2B::unc-54 3'UTR, unc-119 (+)] II, xeSi36[Pdpy-30::mCherry::H2B::6xmir-35mut, unc-119(+)] IV; mir-48/241(nDf51) V*
HW1113	*xeSi78 [Pdpy-30::GFP(PEST)-H2B::lin-41 3'UTR, unc-119 (+)] II, xeSi36[Pdpy-30::mCherry::H2B::6xmir-35mut, unc-119(+)] IV*
HW1114	*xeSi78 [Pdpy-30::GFP(PEST)-H2B::lin-41 3'UTR, unc-119 (+)] II, xeSi36[Pdpy-30::mCherry::H2B::6xmir-35mut, unc-119(+)] IV; let-7(n2853) X*
HW1115	*xeSi78 [Pdpy-30::GFP(PEST)-H2B::lin-41 3'UTR, unc-119 (+)] II, xeSi36[Pdpy-30::mCherry::H2B::6xmir-35mut, unc-119(+)] IV, mir-84(n4037) X*
HW1116	*xeSi78[Pdpy-30::GFP(PEST)-H2B::lin-41 3'UTR, unc-119 (+)] II, xeSi36[Pdpy-30::mCherry::H2B::6xmir-35mut, unc-119(+)] IV; mir-48/241(nDf51) V*
HW1117	*xeSi78[Pdpy-30::GFP(PEST)-H2B::lin-41 3'UTR, unc-119 (+)] II, xeSi36[Pdpy-30::mCherry::H2B::6xmir-35mut, unc-119(+)] IV, mir-48/241(nDf51) V; mir-84(n4037) X*
HW1159	*xeSi87[Pdpy-30::GFP(PEST)-H2B::lin-41 deltaLCS 3'UTR, unc-119 (+)] II, xeSi36[Pdpy-30::mCherry::H2B::6xmir-35mut, unc-119(+)] IV*
HW1162	*xeSi87[Pdpy-30::GFP(PEST)-H2B::lin-41 deltaLCS 3'UTR, unc-119 (+)] II, xeSi36[Pdpy-30::mCherry::H2B::6xmir-35mut, unc-119(+)] IV; mir-48/241(nDf51) V*
HW1121	*xeSi79 [Pdpy-30::GFP(PEST)-H2B::daf-12 3'UTR, unc-119 (+)] II, xeSi36[Pdpy-30::mCherry::H2B::6xmir-35mut, unc-119(+)] IV*
HW1122	*xeSi79.[Pdpy-30::GFP(PEST)-H2B::daf-12 3'UTR, unc-119 (+)] II, xeSi36[Pdpy-30::mCherry::H2B::6xmir-35mut, unc-119(+)] IV; let-7(n2853) X*
HW1123	*xeSi79.[Pdpy-30::GFP(PEST)-H2B::daf-12 3'UTR, unc-119 (+)] II, xeSi36[Pdpy-30::mCherry::H2B::6xmir-35mut, unc-119(+)] IV, mir-84(n4037) X*
HW1125	*xeSi79[Pdpy-30::GFP(PEST)-H2B::daf-12 3'UTR, unc-119 (+)] II, xeSi36[Pdpy-30::mCherry::H2B::6xmir-35mut, unc-119(+)] IV, mir-48/241(nDf51) V; mir-84(n4037) X*
HW1140	*xeSi82[Pdpy-30::GFP(PEST)-H2B::hbl-1 3'UTR, unc-119 (+)] II, xeSi36[Pdpy-30::mCherry::H2B::6xmir-35mut, unc-119(+)] IV*
HW1141	*xeSi82[Pdpy-30::GFP(PEST)-H2B::hbl-1 3'UTR, unc-119 (+)] II, xeSi36[Pdpy-30::mCherry::H2B::6xmir-35mut, unc-119(+)] IV; let-7(n2853) X*
HW1142	*xeSi82[Pdpy-30::GFP(PEST)-H2B::hbl-1 3'UTR, unc-119 (+)] II, xeSi36[Pdpy-30::mCherry::H2B::6xmir-35mut, unc-119(+)] IV, mir-84(n4037) X*
HW1143	*xeSi82[Pdpy-30::GFP(PEST)-H2B::hbl-1 3'UTR, unc-119 (+)] II, xeSi36[Pdpy-30::mCherry::H2B::6xmir-35mut, unc-119(+)] IV; mir-48/241(nDf51) V*
HW1144	*xeSi82[Pdpy-30::GFP(PEST)-H2B::hbl-1 3'UTR, unc-119 (+)] II, xeSi36[Pdpy-30::mCherry::H2B::6xmir-35mut, unc-119(+)] IV, mir-48/241(nDf51) V; mir-84(n4037) X*
HW1133	*xeSi81[Pdpy-30::GFP(PEST)-H2B::lin-28 3'UTR, unc-119 (+)] II, xeSi36[Pdpy-30::mCherry::H2B::6xmir-35mut, unc-119(+)] IV*
HW1183	*xeSi81[Pdpy-30::GFP(PEST)-H2B::lin-28 3'UTR, unc-119 (+)], lin-4(e912) II, xeSi36[Pdpy-30::mCherry::H2B::6xmir-35mut, unc-119(+)] IV*
HW1139	*xeSi81[Pdpy-30::GFP(PEST)-H2B::lin-28 3'UTR, unc-119 (+)] II, xeSi36[Pdpy-30::mCherry::H2B::6xmir-35mut, unc-119(+)] IV; mir-237(n4296) X*
HW1154	*xeSi85[Pdpy-30::GFP(PEST)-H2B::lin-41 LCS2_mir-84-LCS2_mir-241 3'UTR, unc-119 (+)] II, xeSi36[Pdpy-30::mCherry::H2B::6xmir-35mut, unc-119(+)] IV*
HW1155	*xeSi85[Pdpy-30::GFP(PEST)-H2B::lin-41 LCS2_mir-84-LCS2_mir-241 3'UTR, unc-119 (+)] II, xeSi36[Pdpy-30::mCherry::H2B::6xmir-35mut, unc-119(+)] IV; let-7(n2853) X*
HW1156	*xeSi85[Pdpy-30::GFP(PEST)-H2B::lin-41 LCS2_mir-84-LCS2_mir-241 3'UTR, unc-119 (+)] II, xeSi36[Pdpy-30::mCherry::H2B::6xmir-35mut, unc-119(+)] IV, mir-84(n4037) X*
HW1157	*xeSi85[Pdpy-30::GFP(PEST)-H2B::lin-41 LCS2_mir-84-LCS2_mir-241 3'UTR, unc-119 (+)] II, xeSi36[Pdpy-30::mCherry::H2B::6xmir-35mut, unc-119(+)] IV; mir-48/241(nDf51) V*
HW1189	*xeSi85[Pdpy-30::GFP(PEST)-H2B::lin-41 LCS2_mir-84-LCS2_mir-241 3'UTR, unc-119 (+)] II, xeSi36[Pdpy-30::mCherry::H2B::6xmir-35mut, unc-119(+)] IV; mir-48/241(nDf51) V, mir-84(n4037) X*
HW1146	*xeSi83.[Pdpy-30::GFP(PEST)-H2B::lin-41 LCS LCS1_mir-84-LCS2_mir-84 3'UTR, unc-119 (+)] II, xeSi36[Pdpy-30::mCherry::H2B::6xmir-35mut, unc-119(+)] IV*
HW1147	*xeSi83[Pdpy-30::GFP(PEST)-H2B::lin-41 LCS1_mir-84-LCS2_mir-84 3'UTR, unc-119 (+)] II, xeSi36[Pdpy-30::mCherry::H2B::6xmir-35mut, unc-119(+)] IV; let-7(n2853) X*
HW1148	*xeSi83[Pdpy-30::GFP(PEST)-H2B::lin-41 LCS1_mir-84-LCS2_mir-84 3'UTR, unc-119 (+)] II, xeSi36[Pdpy-30::mCherry::H2B::6xmir-35mut, unc-119(+)] IV, mir-84(n4037) X*
HW1149	*xeSi83[Pdpy-30::GFP(PEST)-H2B::lin-41 LCS1_mir-84-LCS2_mir-84 3'UTR, unc-119 (+)] II, xeSi36[Pdpy-30::mCherry::H2B::6xmir-35mut, unc-119(+)] IV; mir-48/241(nDf51) V*
HW1150	*xeSi84[Pdpy-30::GFP(PEST)-H2B::lin-41 LCS LCS1_mir-241-LCS2_mir-241 3'UTR, unc-119 (+)] II, xeSi36[Pdpy-30::mCherry::H2B::6xmir-35mut, unc-119(+)] IV*
HW1153	*xeSi84[Pdpy-30::GFP(PEST)-H2B::lin-41 LCS1_mir-241-LCS2_mir-241 3'UTR, unc-119 (+)] II, xeSi36[Pdpy-30::mCherry::H2B::6xmir-35mut, unc-119(+)] IV; mir-48/241(nDf51) V*

Ecsedi_TableS3

Cloning

Primer name	sequence	Use	Reference
lin-41 3'UTR GW f	ggggacagctttcttgtacaaagtggACACTTTCTTCTTGCTCTTTACCC	Gateway primer to create 3'UTR entry clone; genomic sequence indicated in uppercase	Slack et al, 2000, to create lin-41 ΔLCS, pFS1031 lacking LCS1and 2 was used as a template (Vella et al,
lin-41 3'UTR GWr	ggggacaactttgtataataaagttgTTTATTCCAATTATGTTATCAGC	Gateway primer to create 3'UTR entry clone; genomic sequence indicated in uppercase	
hbl-1 3'UTR GW f	ggggacagctttcttgtacaaagtggatAGCCAGACACCAATAATGAGGAC	Gateway primer to create 3'UTR entry clone; genomic sequence indicated in uppercase	Lin et al, 2003
hbl-1 3'UTR GWr	ggggacaactttgtataataaagttgaGTGGTAAAACAAGATGCTTCAAG	Gateway primer to create 3'UTR entry clone; genomic sequence indicated in uppercase	
daf-12 3'UTR GW f	ggggacagctttcttgtacaaagtggGACCTACTAGAAATCATCTACC	Gateway primer to create 3'UTR entry clone; genomic sequence indicated in uppercase	Grosshans et al, 2005
daf-12 3'UTR GW f	ggggacaactttgtataataaagttgCCCTTATGGGTTGGCTGAG	Gateway primer to create 3'UTR entry clone; genomic sequence indicated in uppercase	
lin-28 3'UTR GW f	ggggacagctttcttgtacaaagtggaaCCTCTGATGAATAGAATCATCTAGAC	Gateway primer to create 3'UTR entry clone; genomic sequence indicated in uppercase	3'UTRome http://asparagus.bio.nyu.edu/cgi-bin/UTRome/utrome.cgi
lin-28 3'UTR GWr	ggggacaactttgtataataaagttgtGCCAACTTGTTGAGGATTG	Gateway primer to create 3'UTR entry clone; genomic sequence indicated in uppercase	
lin-41 p GW fwd	ggggacagctttcttgtacaaagtggtaCCACGCAGACAAGGAGCTAC	Gateway primer to create promoter entry clone; genomic sequence indicated in uppercase	
lin-41 p GW rev	ggggacaactttgtataataaagttgtCACTTTTTCCAAGTCTGAAAAGG	Gateway primer to create promoter entry clone; genomic sequence indicated in uppercase	
LCS2mir84andmir241ized inf	CACCAACTCAAGTATACCTTTCAATATTACAATTCTGCCTCACGCGATGTAAATATCGCAATCCCTTACATTTCTCGCTGTCTGCCTCTGAACCATTGAAACACTTCTCCCGTACTC	oligo to perform an infusion®(Clonetech) reaction on the lin-41 3'UTR GW entry clone after digestion with AccI and XmnI (site created by site directed mutagenesis at position 795) to replace LCS1 and 2	
LCS1/2mir241ized inf	CACCAACTCAAGTATACCTACATTTCTCGCGTTCTACACTCAACGCGATGTAAATATCGCAATCCCTTACATTTCTCGCTGTCTGCCTCTGAACCATTGAAACACTTCTCCCGTACTC	oligo to perform an infusion®(Clonetech) reaction on the lin-41 3'UTR GW entry clone after digestion with AccI and XmnI (site created by site directed mutagenesis at position 795) to replace LCS1 and 2	
LCS1/2mir84ized inf	CACCAACTCAAGTATACCTTTTCAATATTACAGTTCTACACTCAACGCGATGTAAATATCGCAATCCCTTTTTCAATATTACAATTCTGCCTCTGAACCATTGAAACACTTCTCCCGTACTC	oligo to perform an infusion®(Clonetech) reaction on the lin-41 3'UTR GW entry clone after digestion with AccI and XmnI (site created by site directed mutagenesis at position 795) to replace LCS1 and 2	

Plasmids from other sources

pENTRL4-R1p_dpy-30	dpy-30 promoter GW entry clone covering the V:12189538-12191540 genomic region
pBMF2.7	Gfp(PEST)-H2b GW enty clone, Wright et al, 2011
pCM5.37	unc-54 3'UTR GW entry clone (Seydoux lab)
pCM1.151	mCherry-H2b GW enty clone, Merrit et al, 2008
control 3'UTR (6xmir-35mut)	3'UTR GW enty clone, adapted from Wu et al, 2012

Significance and open question

At the technological level, I successfully established a quantitative miRNA target reporter system in *C. elegans.* The use of confocal microscopy resulted in very high spatial resolution and accurate visualization of the fluorescent reporter in virtually all cells of the worm. Application of single copy-integrated transgenes allowed for the first time direct comparison between different 3'UTRs. With the help of the Imaging Facility at the Friedrich Miescher Institute, I set up an image-processing pipeline to reconstruct worms in 3D, select cells of interest and quantify signal intensity in these cells. The wealth of data obtained during this project demonstrated that this method is robust, quantitative and easy to adapt to examine new research questions. E.g. imaging of the vulva in isolation and in higher resolution allowed quantification of different individual cell types even within the same organ. The reporter assay can be used in principle to image worms over time, i.e. in live, time-lapse imaging, for the dissection of miRNA activity over time, e.g. in relation to events during a specific larval stage.

Using this new tool, I could at least partially answer the question of *let-7* redundancy. Despite having an identical seed, *let-7* and its sisters clearly regulate different targets. *lin-41* expression is mainly controlled by *let-7, hbl-1* by *mir-48/84/241* and *lin-4,* whereas *daf-12* is affected by the loss of both *let-7* and its sisters. Unfortunately, the molecular mechanism(s) responsible tor target specificity among members of the same miRNA family remain largely unknown. I explored the most obvious hypothesis, specificity through non-seed base-pairing to target mRNAs. As the presence of contextual factors, and obviously of other miRNA binding sites, might alter miRNA activity, I used the two LCSs in the *lin-41* 3'UTR as a model to analyze this question. By examining different designed LCS architectures, I could show that for *let-7* base-pairing through the non-seed region is indeed necessary for *lin-41* regulation. On the other hand, adopting the non-seed base-pairing to *mir-84* or *mir-241* was not sufficient for robust repression. Alternative explanations for specificity include the binding of specificity factors near the binding site or specific local secondary structure required for regulation.

Quantitative analysis of heterochronic pathway was very informative about its principles. Heterochronic miRNA targets were, unlike in cell culture, robustly repressed, typically by 5-10 fold. I could not detect any switch-like repression of a miRNA target, target repression occurred gradually from earlier to later larval stages. Together with the finding that miRNAs often cooperatively repress targets, this suggests threshold effects for the function of miRNA target genes such as *lin-41.* Regarding *let-7* activity in different tissues, I could for the first time directly demonstrate *let-7* dependent 3'UTR regulation in the intestine and vulva.

3. Novel role of the *let-7* microRNA in vulva development

Specific aims

Although *let-7* is probably the most studied miRNA in *C. elegans*, its functions have been mainly characterized in the hypodermis. Expression studies suggest that *let-7* is active in a wide variety of tissues and the most obvious *let-7* phenotype is lethal vulva bursting at the young adult stage. The developmental defects responsible for bursting have not been known so far. Based on literature, two explanations for vulva bursting were most plausible. One model predicted that defects of developmental timing in the seam cells would somehow compromise the vulva-seam cell connection and would lead to vulva bursting. Alternatively, *let-7* might be involved in some aspects of vulva development. This was suggested by the finding that *let-7* could regulate *let-60,* a gene with a very prominent role in vulva cell fate specification and probably morphogenesis.
I wanted to differentiate between these two possibilities and was in fact very excited about a connection between cell signaling pathways and *let-7* activity*.* I focused therefore first on *let-7's* role in VPC specification and regulation of *let-60.* As it became immediately clear that this stage of vulva development is not affected by loss of *let-7,* I systematically assessed the later steps and examined the involvement of different *let-7* effectors in *let-7* vulva phenotypes.
Another motivation for analyzing vulva development in *let-7* mutant worms, was the identification of several novel *let-7* suppressors with predicted functions in the vulva. Typically, knock-down of these genes did not affect the hypodermal defect of *let-7,* as examined by expression of *col-19::Gfp.* It was therefore plausible that *let-7* might have tissue-specific functions, e.g. in the vulva or uterus. More generally speaking, I wanted to test whether *let-7's* role in two different tissues can be attribute to a similar functional principle, e.g. timing of proliferation vs. differentiation.
Concerning *let-7*'s possible functions in the vulva, another key question was whether *let-7* would regulate the same target or set of targets in different tissues. In addition to classical, mostly correlative, experiments such as analysis of target regulation in different tissues, observation of vulva phenotypes upon target level manipulation, I leveraged a new genome editing technique called based on CRISPR/Cas) to directly prove the importance of *lin-41* regulation by *let-7* in the *C. elegans* vulva.

Publication 3:

The *let-7* microRNA directs vulval development through a single target

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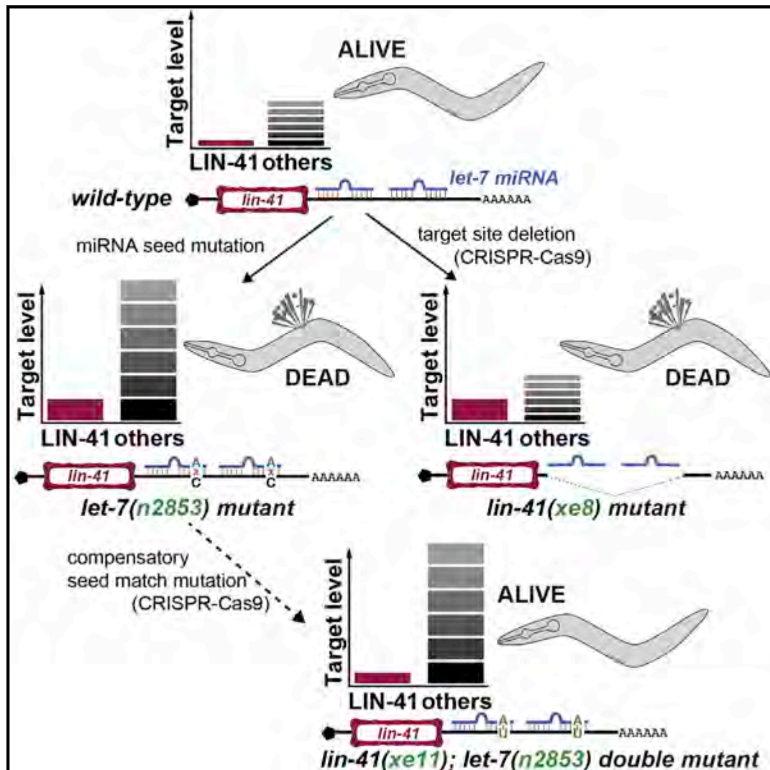
Article

Developmental Cell

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

Graphical Abstract



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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.

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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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

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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.

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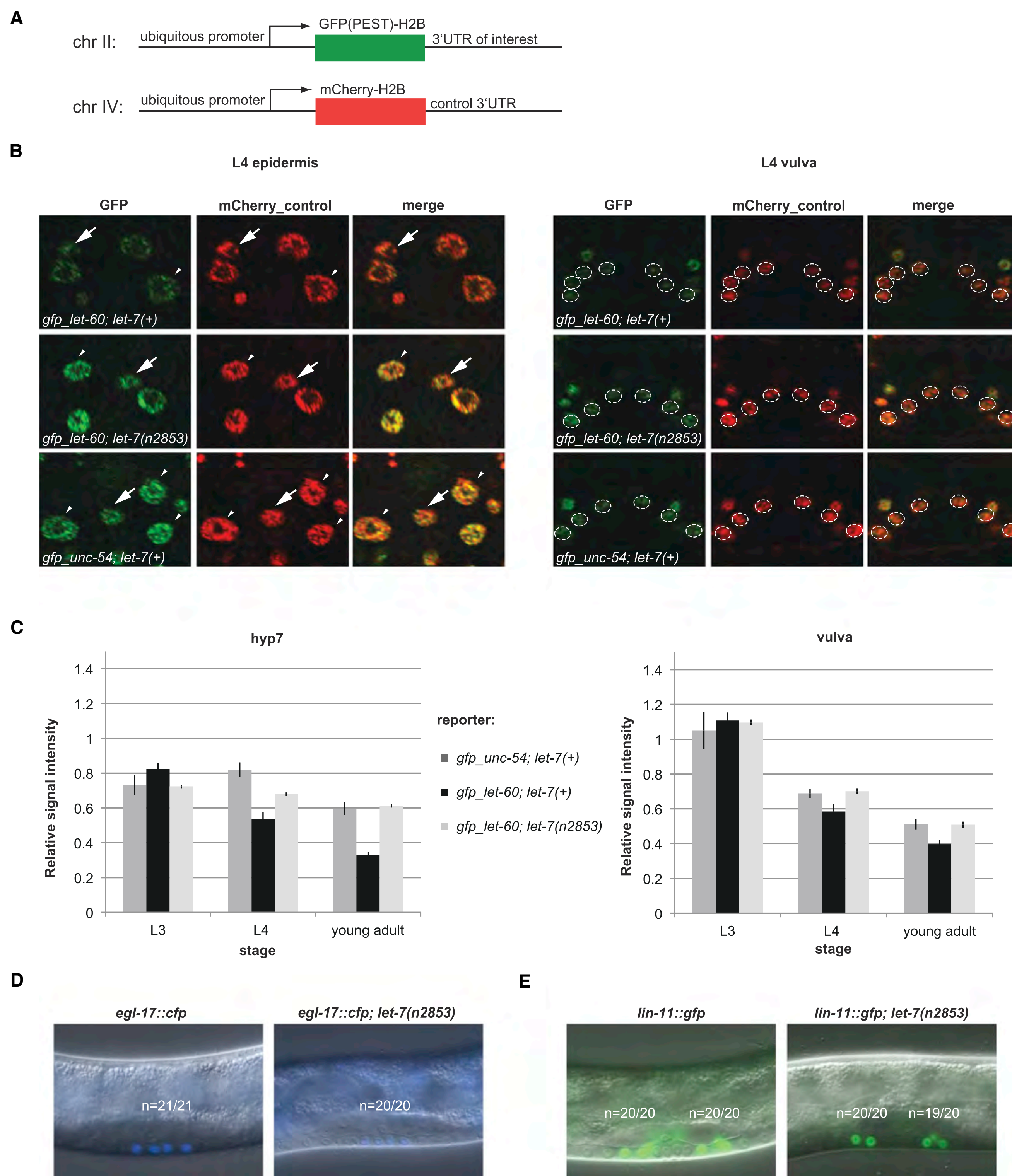

Figure 1. *let-7* and Its Regulation of *let-60* Are Dispensable for VPC Specification

(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.

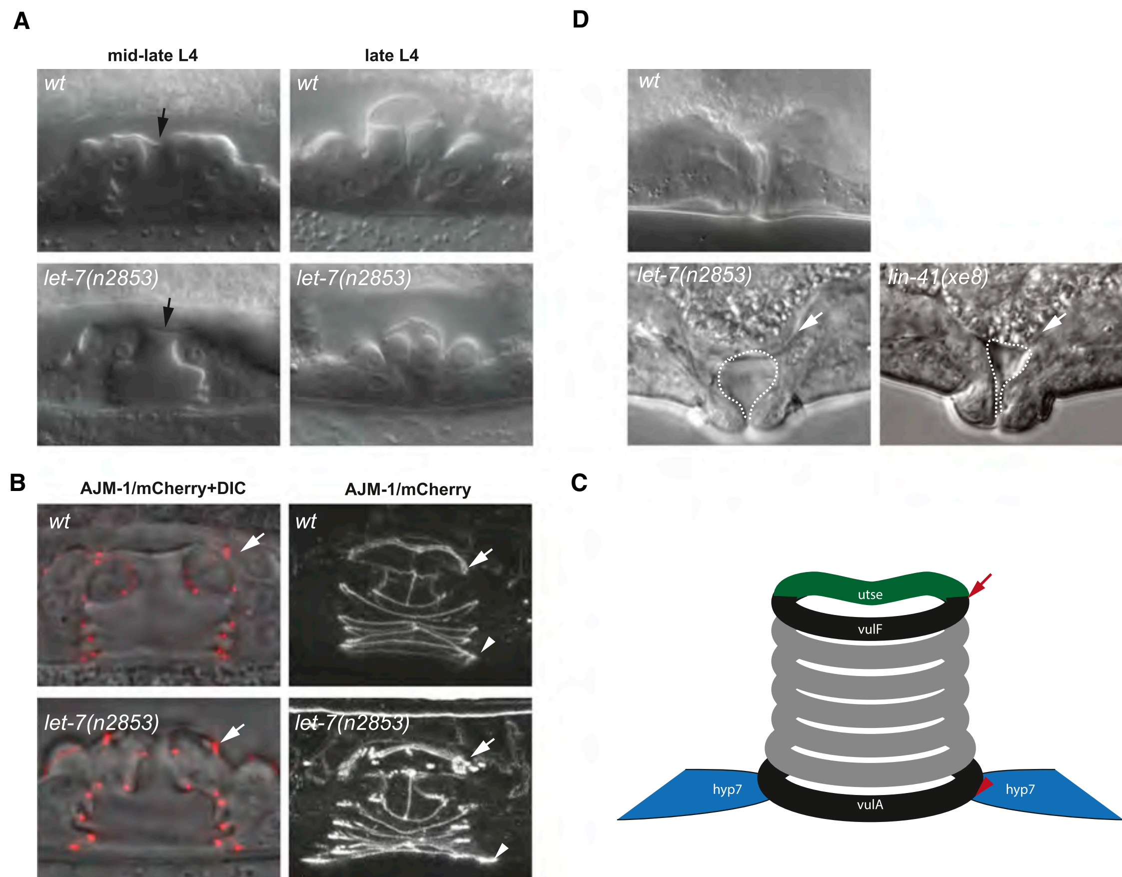

Figure 2. Loss of *let-7* Leads to Vulva Morphogenesis Defects at the Young-Adult Stage

(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

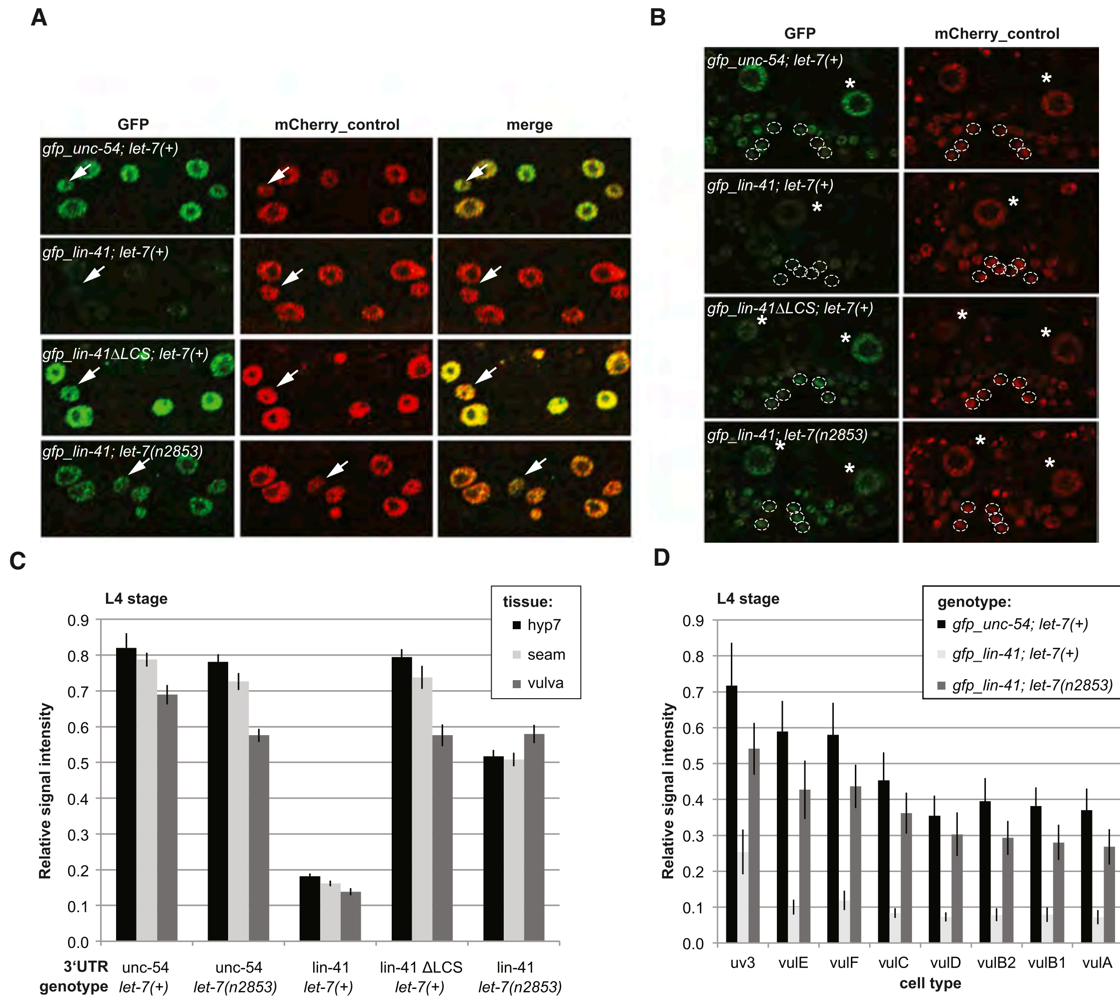

Figure 3. *let-7* Mediates Extensive Repression of *lin-41* in the Vulva

(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-41ΔLCS* 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

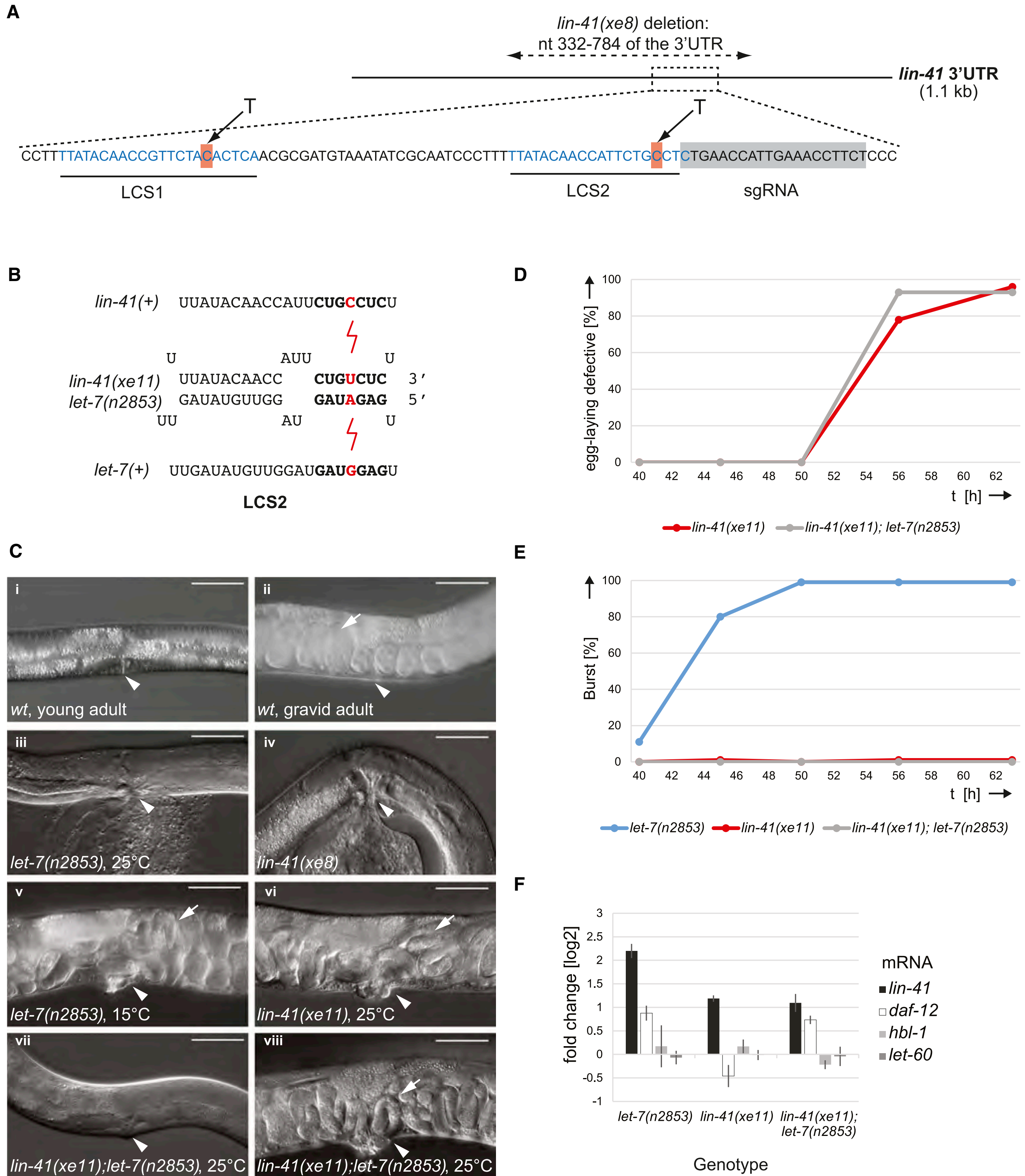

Figure 4. *lin-41* Is the Key *let-7* Target

(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)

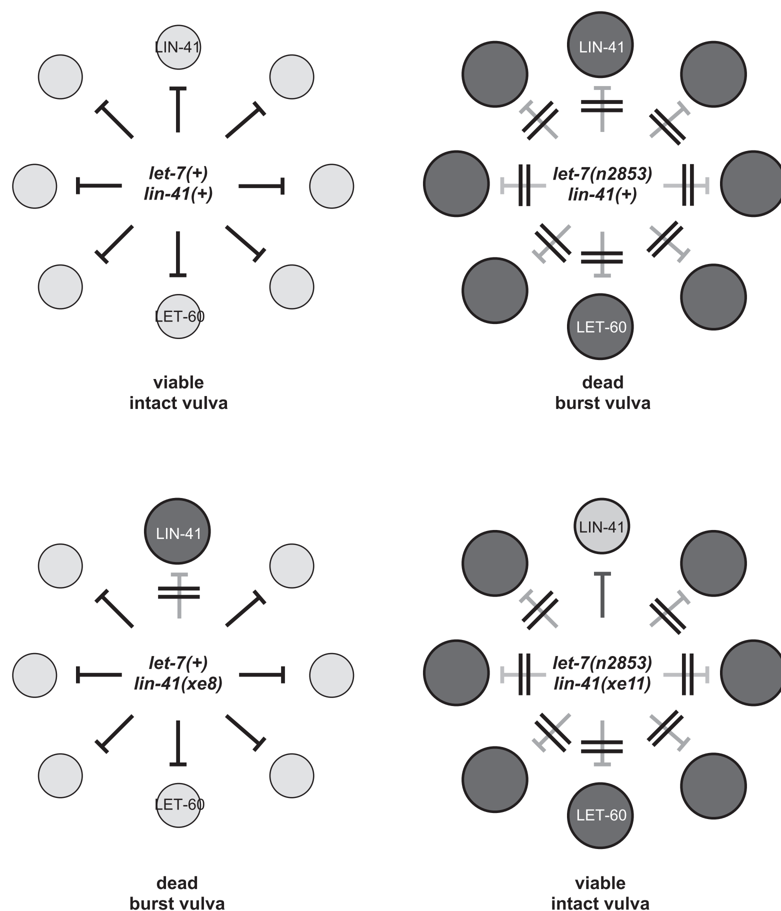

Figure 5. Schematic Depiction of the Effects of *let-7* and *lin-41* Alleles on *let-7* Target Expression and Phenotypes

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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Developmental Cell

Supplemental Information

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

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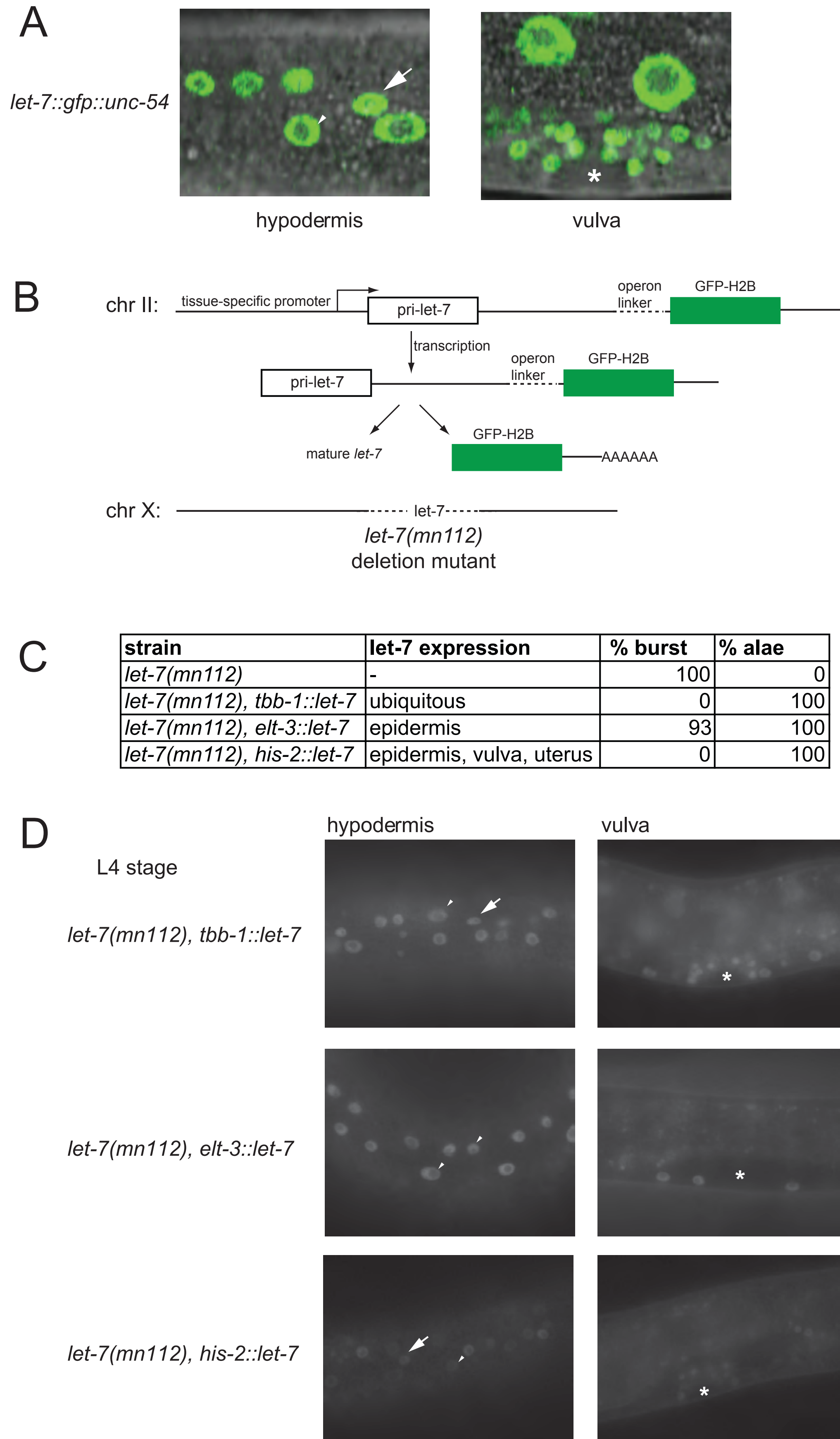
Legend to Movie S1: Representative time-lapse movie of a young adult *let-7(n2853)* worm grown at 25 °C. Related to Figure 2.



strain	let-7 expression	% burst	% alae
let-7(mn112)	-	100	0
let-7(mn112), tbb-1::let-7	ubiquitous	0	100
let-7(mn112), elt-3::let-7	epidermis	93	100
let-7(mn112), his-2::let-7	epidermis, vulva, uterus	0	100

Fig. S1: *let-7* is active in the vulva. Related to Fig. 3.
A, *let-7* is transcribed in both hypodermis and vulva. Arrow: seam cell; arrowhead: hyp7, asterisk indicates vulva lumen.
B, Schematic of tissue-specific *let-7* expression. *Pri-let-7* is expressed in a tissue-specific manner in worms lacking endogenous *let-7* in all cells. A *gfp* marker is transcriptionally linked to *pri-let-7* through use of an operon linker, permitting visualization of promoter activity.
C, Bursting (n>100) and alae (n>25) in animals expressing let-7 in a tissue-specific manner.
D, Expression patterns of tissue-specific *let-7* rescue constructs as visualized by the co-transcribed *gfp* marker. Arrows indicate seam cells, arrowheads point to hyp7 cells, asterisks show the vulva lumen.

Supplemental Experimental Procedures

Worm handling and strains

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

Construction of miRNA target reporters

3'UTRs were amplified using primers indicated in the Supplemental Table below and inserted into the Multisite Gateway pDONR P2R-P3 vector. The 3' UTR entry vectors obtained were recombined together with a *pdpy-30* and a GFP(PEST)-H2B (Wright et al., 2011) or mCherry-H2B plasmids (Supplemental Table below) into MosSCI compatible destination vectors. All plasmids, listed in the Supplemental Table below, were verified by sequencing. Transgenic worms were created by Mos1-mediated single-copy insertion (Frøkjær-Jensen et al., 2012) (MosSCI) in position ttTi5605 or cxTi10882 (mCherry control reporter). All transgenic lines were outcrossed at least three times.

qPCR primers

Name	**Sequence**
lin-41 R2 qPCR	aagcgttgacgtgtgtatcg
act-1 F1 qPCR	gttgcccagaggctatgttc
act-1 R1 qPCR	caagagcggtgatttccttc
pgk-1 qPCR F2	ctcctactttagcaaggccctcg
pgk-1 qPCR R2	ttgactccctgggcaactttc
daf-12 qPCR F2	gatcctccgatgaacgaaaa
daf-12 qPCR R2	ctcttcggcttcaccagaac
let-60 qPCR F1	ttggagatggaggagttggt
let-60 qPCR R1	agaaatccttcgcctgtcct
hbl-1 qPCR F1	actgcacatatgccaccaaa
hbl-1 qPCR R1	tgatgtaaccggctcaactg

DNA cloning primers

Name	Sequence (genomic sequence indicated in uppercase)	Use	Reference
his-2 GW fwd	ggggacaactttgtatagaaaagttgcaTTGCGACGACTTTGGGAG	GW primer to create promoter entry clone	
his-2 GW rev	ggggactgctttttgtacaaacttggAATCCGATAAGGACTGTG	GW primer to create promoter entry clone	
elt-3 GW fwd	ggggacaactttgtatagaaaagttgaaCGCTGATGGGGGTACGGTC	GW primer to create promoter entry clone	
elt-3 GW rev	ggggactgctttttgtacaaacttgtGAAGTTTGAAATACCAGGTAGCCG	GW primer to create promoter entry clone	
let-60 promoter GW fwd	ggggacaactttgtatagaaaagttgcgCAGTCAGTAGAATACAAAATTTTAG	GW primer to create promoter entry clone	
let-60 promter GW rev	ggggactgctttttgtacaaacttgcTACCCTTTTCTGAAAAAAGACGC	GW primer to create promoter entry clone	
lin-41 p GW fwd	ggggacagctttcttgtacaaagtggtaCCACGCAGACAAGGAGCTAC	GW primer to create promoter entry clone	
lin-41 p GW rev	ggggacaactttgtataataaagttgtCACTTTTTCCAAGTCTGAAAAGG	GW primer to create promoter entry clone	
pri-let-7 GW f	ggggacaagtttgtacaaaaaagcaggctTCGCGGGTTTCTGTTCATATA	GW primer to create pri-let-7 entry clone	
pri-let-7GW r	ggggaccactttgtacaagaaagctgggtTATTTCCTGCTCGTTCTTCAC	GW primer to create pri-let-7 entry clone	
let-60 CDS Gibson f	ATGACGGAGTACAAGCTTGTG	PCR primer for cloning of let-60 CDS::unc-54 3'UTR	
let-60 CDS Gibson r	TCACATTATTTGACACTTCTTCTTC	PCR primer for cloning of let-60 CDS::unc-54 3'UTR	
unc-54 Gibson f	AGAAGTGTCAAATAATGTGAgtccaattactcttcaacatccc	PCR primer for cloning of let-60 CDS::unc-54 3'UTR, sequence complementary to let-60 CDS indicated in uppercase	
unc-54 Gibson r	accccatagacactactccac	PCR primer for cloning of let-60 CDS::unc-54 3'UTR	
unc-54 Gibson r attB2	ggggaccactttgtacaagaaagctgggtaACCCCATAGACACTACTCCAC	GW primer to create let-60 CDS::unc-543'UTR entry clone	
let-60 CDS GW fwd	ggggacaagtttgtacaaaaaagcaggctaaATGACGGAGTACAAGCTTGTGGTAG	GW primer to create let-60 CDS::unc-54 3'UTR entry clone	
lin-41 3'UTR GW f	ggggacagctttcttgtacaaagtggACACTTTCTTCTTGCTCTTTACCC	GW primer to create 3'UTR entry clone	Slack et al, 2000. To create lin-41 ΔLCS, pFS1031 lacking
lin-41 3'UTR GWr	ggggacaactttgtataataaagttgTTTATTCCAATTATGTTATCAGC	GW primer to create 3'UTR entry clone	LCS1and 2 was used as a template (Vella et al, 2004)

Plasmids

Name	Use, reference
pENTR_L4-R1_Pdpy-30	dpy-30 promoter GW entry clone covering the V:12189538-12191540 genomic region
pENTR_L4-R1_Phis-2	his-2 promoter GW entry clone
pENTR_L4-R1_Pelt-3s	elt-3 promoter GW entry clone
pENTR_L4-R1_Ptbb-1	tbb-1 promoter GW entry clone
pENTRL4-R1_Plet-60	let-60 promoter GW entry clone
pBMF2.7	Gfp(PEST)-H2b GW enty clone, Wright et al, 2011
pCM1.151	mCherry-H2b GW enty clone, Merrit et al, 2008
pENTRL1-L2_let-7 rescue fragment	pri-let-7 GW entry clone
pENTR_L1-L2_let-60-unc54-3'UTR	let-60::unc-54 3'UTR GW entry clone
pENTR_R2-L3_operon-GFP-H2b	gpd-2/gpd-3 intergenic region:GFP:Histone H2B:tbb-2 3'UTR GW entry clone, contains 25 nucleotides of the gpd-2 3'UTR followed by the gpd-2 polyadenylation signal; based on Merritt et al, 2008
pCM5.37	unc-54 3'UTR GW entry clone (Seydoux lab)
pENTR_R2-L3_ (6xmir-35mut)l3'UTR	artificial (control) 3'UTR GW entry clone containing 6xmir-35 scrambled sites, adapted from Wu et al, 2012
pENTR_R2-L3_lin-41 3'UTR	lin-41 3'UTR GW entry clone
pENTR_R2-L3_lin-41 3'UTR ΔLCS	lin-41 3'UTR GW entry clone lacking LCS1 and 2
pENTR_R2-L3_lin-41(n2853) 3'UTR	lin-41(n2853) 3'UTR GW entry clone containing compensatory mutations to *let-7(n2853)* in LCS 1 and 2, derived from pENTR_R2-L3_lin-41 3'UTR using site-directed mutagenesis
pIK82	*peft-3::Cas9::2xNLS::tbb-2*
plin-41sgRNA	*pU6::lin-41sgRNA*
pCFJ104	*pmyo-2::mCherry*

***C. elegans* strains**

Strain number	Genotype
HW1120	xeSi104[Pdpy-30::GFP(PEST)-H2B::unc-54 3'UTR, unc-119 (+)] II, xeSi36[Pdpy-30::mCherry::H2B::artificial 3'UTR, unc-119(+)] IV
HW1169	xeSi104[Pdpy-30::GFP(PEST)-H2B::unc-54 3'UTR, unc-119 (+)] II, xeSi36[Pdpy-30::mCherry::H2B::artificial 3'UTR, unc-119(+)] IV; let-7(n2853) X
HW1113	xeSi78 [Pdpy-30::GFP(PEST)-H2B::lin-41 3'UTR, unc-119 (+)] II, xeSi36[Pdpy-30::mCherry::H2B::artificial 3'UTR, unc-119(+)] IV
HW1114	xeSi78 [Pdpy-30::GFP(PEST)-H2B::lin-41 3'UTR, unc-119 (+)] II, xeSi36[Pdpy-30::mCherry::H2B::artificial 3'UTR, unc-119(+)] IV; let-7(n2853) X
HW1159	xeSi87[Pdpy-30::GFP(PEST)-H2B::lin-41 deltaLCS 3'UTR, unc-119 (+)] II, xeSi36[Pdpy-30::mCherry::H2B::artificial 3'UTR, unc-119(+)] IV
HW1128	xeSi80[Pdpy-30::GFP(PEST)-H2B::let-60 3'UTR, unc-119 (+)] II, xeSi36[Pdpy-30::mCherry::H2B::artificial 3'UTR, unc-119(+)] IV
HW1129	xeSi80[Pdpy-30::GFP(PEST)-H2B::let-60 3'UTR, unc-119 (+)] II, xeSi36[Pdpy-30::mCherry::H2B::artificial 3'UTR, unc-119(+)] IV; let-7(n2853) X
HW1191	xeSi117[Plet-7::GFP(PEST)-H2B::unc-54 3'UTR, unc-119 (+)] II
HW1097	let-7(mn112) X; xeEx365[Ptbb-1::let-7::SL1_operon_GFP , unc-119 (+); Prab-3::mCherry; Pmyo-2::mCherry; Pmyo-3::mCherry]
HW1175	xeSi34[Ptbb-1::let-7::SL1_operon_GFP] , unc-119 (+)] II; let-7(mn112) X,
HW1186	xeSi95.[Phis-2::let-7::SL1_operon_GFP] , unc-119 (+)] II, let-7(mn112) X

HW1207	xeSi97.[Pelt-3s::let-7::SL1_operon_GFP] , unc-119 (+)] II; let-7(mn112) X, xeEx365[Ptbb-1::let-7::SL1_operon_GFP , unc-119 (+); Prab-3::mCherry; Pmyo-2::mCherry; Pmyo-3::mCherry]
HW 1187	syIs103[unc-119(+) + pPGF11.13(lin-11::GFP)], outcrossed from PS4198
HW1188	syIs103[unc-119(+) + pPGF11.13(lin-11::GFP)], let-7(n2853) X
HW1192	arIs92[egl-17p::NLS-CFP-LacZ + unc-4(+) + ttx-3::GFP], from the CGC strain GS3582
HW1193	arIs92[egl-17p::NLS-CFP-LacZ + unc-4(+) + ttx-3::GFP], let-7(n2853) X
HW1230	mjIs15[ajm-1::mCherry]
HW1277	mjIs15[ajm-1::mCherry]; let-7(n2853) X
HW1320	lin-41(xe8) I
HW1329	lin-41(xe11) I
HW1330	lin-41(xe11) I, let-7(n2853) X
HW1413	xeSi145[Plet-60::let-60CDSw/intr-unc-54 3'UTR::gfp operon] II
HW1594	xeSi145[Plet-60::let-60CDSw/intr-unc-54 3'UTR::gfp operon] II, let-60(ok1932) IV

Supplemental References

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Significance and open questions

With my work, I could show that *let-7* regulates some aspects of late vulva development. *let-7* expression in the vulva is required to prevent vulva bursting. Unfortunately, my results are not conclusive whether *let-7* activity in the vulva would be also sufficient for survival in a *let-7* mutant background. Although I was not able to assign a very specific function to *let-7* in vulva morphogenesis, I could rule out many different possibilities and alternative hypotheses. In contrary to previous models proposed, *let-7* does not affect early stages of VPC specification. In this regard, I could demonstrate that *let-60* is not a relevant *let-7* target in the vulva. By characterizing vulva morphology in *let-7* mutant worms, I narrowed down the *let-7* vulva defect to the young adult stage. This is in accordance with the peak of *let-7* activity in the hypodermis. I examined *let-7* expression and activity in the different vulva cell types and found uniform *let-7* activity among these cells. *let-7* thus coordinates gene expression in all vulva cells in the same way rather than acting in only a subset of cells and generating spatial patterns of gene expression. The results of the CRISPR experiments editing the *let-7-lin-41* 3'UTR interface unequivocally show that *lin-41* is the key *let-7* target in the vulva and provide an example for a microRNA exerting its effects in a given biological process through regulation of a single target. Interestingly, *let-7* and *lin-41* do not regulate *lin-29* in the vulva, suggesting that these two core components of the heterochronic pathway have different effectors in different tissues.

My results provide in addition a framework to test candidate *let-7* suppressors for their role in vulva development and strains to look at *let-7* functions in the intestine and neurons in isolation.

Discussion

What is a microRNA target?

As miRNAs exert their biological effects through repression of target mRNAs, the identification of miRNA targets and elucidation of principles underlying target regulation both at the sequence and the functional level is a key goal in miRNA research. My thesis work investigated miRNA-target relationships at both levels. The *in vivo* miRNA target reporter assay that I had established investigated quantitative aspects and sequence requirements for target regulation. These studies revealed that miRNAs are highly flexible in target regulation. Some targets such as *hbl-1* or *daf-12* are almost completely repressed (5-10 fold) through their 3'UTRs. On the other hand, *let-60* is repressed in the hypodermis only modestly, by a factor of two. Importantly, my data suggest that this difference in extent of regulation is inherent to the 3'UTR as both *lin-41* and *let-60* are almost exclusively targeted by *let-7.* As in the reporters assay, the only experimental variable is the 3'UTR, difference in expression levels cannot account for the different extent of regulation. The sequence and architecture of the miRNA target site not only determines whether a miRNA regulates a target, but influences also the extent of repression. Tissue specific factors also play a role in determining target repression. *let-7* clearly represses *let-60* in the hypodermis, barely affects it in the intestine or the vulva, although *let-7* is highly expressed and very active on other targets in these tissues. It is at this point not clear, whether this difference is determined by the target site or by some additional features in one of the 3'UTRs, e.g. by the presence of binding sites for RNA-binding proteins present in only a subset of tissues. *lin-41* is very strongly repressed in most tissues, but repression is clearly weaker in uterine cells. This indicates, that even a single miRNA can achieve a quantitatively graded repression, which can be different in different cells.
For the reporter assay, I used a destabilized GFP with a reported half-life of less than 1 hr (Frand et al., 2005). I could thus in principle detect quite fast changes in gene expression mediated by miRNA activity. Previously, *let-7* has been predicted to act as a switch in regulating *lin-41.* Appearance of *let-7* would downregulate *lin-41* to allow *lin-29* expression required for the larval-to-adult transition (Reinhart et al., 2000; Slack et al., 2000). Surprisingly, regulation of the *lin-41* 3'UTR shows continuous decline from the L3 stage on until reaching about five-fold repression at the young adult stage. It is unclear at this point, whether this reflects relatively slow accumulation of *let-7* or miRNA-mediated target regulation is inherently slow in *C. elegans.* Further kinetic analysis of miRNA-mediated target regulation is clearly warranted to answer this question.
Assignment of 3'UTR repression to a miRNA is highly complicated by the presence of multiple binding sites for different miRNAs as well as the presence of miRNAs of the same family with potentially overlapping target specificity. Absence of change in reporter regulation upon deletion of a miRNA can either indicate absence of a regulatory relationship or redundancy with another factor. Complete derepression upon loss of a given miRNA can either mean exclusivity of the regulatory interaction or alternatively a cooperative regulation requiring the presence of all factors involved in the process. Moreover, the miRNAs or other regulatory factors can regulate each other, further complicating the

interpretation of single experiments. With the 3'UTR reporters examined, I could analyze the interaction between *lin-4* and the *let-7* family on the *hbl-1* and *lin-28 3'UTRs*. Both 3'UTRs are affected by the loss of either *lin-4* or *mir-48/84/241,* but there are striking differences. In the seam cells, the *lin-28* 3'UTR is completely derepressed upon loss of *lin-4* and is slightly upregulated upon loss of *mir-48/84/241.* In *hyp7*, I observed only modest deregulation in either case, suggesting redundancy in *hyp7,* but dominant activity of *lin-4* in the seam. Repression of the *hbl-1* 3'UTR in *wt* worms was the strongest observed so far. It is partially alleviated both by loss of *lin-4* and the *let-7* sisters, but not *let-7.* This suggests additive regulation by *lin-4* and the *let-7* sisters. In these two examples, two distinct miRNAs might simply use two different target sites two repress together a common target. This shows, how the use of different target sites, which can have very different characteristics regarding extent of regulation, can create not only complicated patterns of gene expression, but possibly also serve as information processing devices. This is analogous to the role of phosphorylation sites in intracellular signal transduction or post-translational histone modifications in epigenetic gene regulation. In the case of the 3'UTR, various binding sites for miRNAs and RNA-binding proteins could interact to define logical operations. E.g. the output, repression of a target, would require the presence of two different inputs, binding of two different miRNAs. In this way, a 3'UTR would integrate information about different variables such as developmental time, temperature, availability of food, etc. and regulate the expression of the upstream gene product accordingly. Given the possibility of crosstalk between mRNAs *in trans*, the "miRNA-code", or more broadly the "3'UTR-code" create a highly sophisticated cellular language.
The existence of miRNA families with several members is quite a remarkable phenomenon. According to the principles of evolution, variation and natural selection, an unnecessary cellular product should be selected against and eliminated over time. In fact, the opposite seems to happen with miRNA families, they tend to expand with the complexity of an organism. It has been proposed that the complexity of multicellular organisms, especially the complexity of neuronal structures such as the human brain, is largely due to expansion of post-transcriptional regulation (Berezikov et al., 2006). If this is the case, expansion of miRNA families might contribute to the increased sophistication of gene regulation. By analogy to the evolution of protein coding genes, a gene duplication event allows decreases the selection pressure on a miRNA gene enabling sequence variation. In an equilibrium state, the new gene would have adopted a new function or have been eliminated. In the case of miRNAs, the co-evolution of miRNA targets have to be considered as well, being a miRNA target can be advantageous or have detrimental effects. At any rate, the divergence of miRNA family members should lead to separation or at least divergence of function. This could be on the level of target specificity and/or regulation of the miRNA, e.g. difference in spatial expression. Actually, the concept of miRNA families, grouping miRNAs together based on a common seed, is based on the assumption, that members of a given family have the same target specificity. Using the *in vivo* target reporter assay, I have examined target specificity within the *lin-4* and *let-7* miRNA families. My results clearly show that miRNAs of the same family differ in their ability to regulate a given miRNA target. This is depends exclusively on the identity of the target, as differences in targeting

activity between two miRNAs can be reversed in the same tissue at the same time in the case of another target. Specificity is not absolute, as e.g. the three *let-7* sisters are at least partially redundant in their target specificity and even *lin-41* is modestly regulated by *mir-48/84/241* in the hypodermis. For *lin-41,* loss of LCS and 2 in the 3'UTR completely abrogates repression, indicating that even these sites with specific base pairing to *let-7* in the non-seed part can be regulated by the *let-7* sisters through seed-only binding. In the case of other 3'UTRs examined containing multiple miRNA binding sites, it is currently unclear whether target specificity can be attributed to and is inherent to a single target site or it is due to distinct features of the whole 3'UTR such as binding of an RNA-binding protein, which could serve as a specificity factor. A miRNA target site possibly represents a continuum between a promiscuous site with a broad spectrum of potentially relevant miRNAs and specific site that can be regulated by only a handful of miRNAs. The simplest model would predict that a minimal binding through the seed explains the promiscuous type of regulation and additional hybridization at the 3' end of the miRNA would make a target site more and more effective and specific. I tested this hypothesis for the two LCSs of *lin-41.* I predicted that altering the target site in a way that they are predicted to bind *mir-84* or *mir-241* could rewire specificity. However using in total four different architectures, I observed, first of all, decline of regulation. The residual repression did not depend on *let*-7, although I could not rule out that the constructs are regulated redundantly by the whole *let-7* family. My most successful attempt was the expression of a LCS corresponding to *mir-84* followed by a second for *mir-241* separated by the linker of *wt* sequence and length. This construct was repressed to some extent and repression was abrogated by deletion of *mir-48/84/241.* The *lin-41* 3'UTR might have some very special features that are altered by these modifications and theses experiments might not be representative end generalizable to other miRNA targets. According to the thermodynamic model, specificity would depend on miRNA levels, more of a non-optimal miRNA would compensate for less affinity and regulate a non-optimal target. Although I cannot conclusively reject this hypothesis, I have no indication for overriding of target specificity by miRNA overexpression. An alternative or additional component of the rules governing target specificity might be the presence of negative sequence elements preventing binding of the miRNA not intended to bind to the target site. Arguably, the mechanisms of such repulsion are elusive. Without a clear hypothesis and without the ability to control or at least predict secondary effects of target site manipulation, testing of different target site mutations would only lead to meaningful results when performed systematically with a number of constructs that is not realistic using the current techniques.

Taken together, the first definition of a miRNA target is the existence of regulation *in vivo* and in a particular context. This interaction is not absolute, but highly context dependent and is influenced by many factors that likely have important implications for the functional of target regulation. Verifying the miRNA-mediated repression of an mRNA is thus only the first step in the understanding of this regulatory relationship. The most important aspects to characterize are the magnitude of regulation, description of the timing of repression and the tissues where the interaction occurs.

Being a miRNA target is also not absolute in functional terms. Loss of regulation of some targets might be well tolerated under some circumstances, for others even a slight change in the extent or timing of repression might be detrimental. This aspect is also clearly context dependent. Defining the importance of a miRNA-target interaction and categorization of mRNA targets based upon function should be the next step after target identification. My work contributed in several ways to this goal. First, descriptive analysis of *let-7* activity in time and space provided new hypotheses on how and where *let-7* might act. Second, characterization of *let-7's* function in the vulva provides a new paradigm to test the importance of *let-7* targets in a context other than the hypodermis. Finally, misexpression of *let-60* or *lin-41* shows how direct manipulation of a miRNA target gene helps to define the functionality of the targeting relationship. What determines the importance of a miRNA-target interaction besides the strength of regulation as assessed by a reporter assay? Obviously, the miRNA and its target have to be expressed in the same tissue. The miRNA target must have a functional role in the process examined and possible redundancy with other factors have to be taken in account. Additionally, miRNA-mediated regulation is only one layer influencing gene expression. The architecture of further transcriptional and posttranscriptional regulatory mechanism can significantly influence the outcome of miRNA-mediated regulation. Feed-back loops between miRNAs and its targets create bistable switches, as shown for *let-7* and *lin-28* in mammalian stem cells (Rybak et al., 2008) or *lsy-6* in *C. elegans* (Johnston et al., 2005). In this case, miRNA activity can shift the balance towards two mutually exclusive states. In other cases, expression of mRNA targets is already low and miRNA-mediated gene regulation acts as a fail-safe mechanism ensuring mutual exclusion of target expression (Stark et al., 2005). Autoregulation of genes at the protein level in turn would counteract miRNA-mediated repression. These network effects clearly depend on quantitative factors, but it is evident from individual examples, that the functional consequences of miRNA activity range from zero to critical.

In the case of *let-7* activity in *C. elegans* these differences are readily apparent. Regulation of *lin-41* has at any rate major consequences for the development of the animal. Whether this is enforced by additional layers of *lin-41* regulation or even counteracted by *lin-41* autoregulation as suggested by genetic evidence (Del Rio-Albrechtsen et al., 2006) and by autoubuiqitylation activity of LIN-41 in other systems (Rybak et al., 2009) remains to be defined. It is also not clear whether *lin-41* function requires a certain threshold in expression level or different functions even have different thresholds. In contrast to *lin-41*, the relevance of *let-60* repression is under laboratory conditions not evident. *let-60* is clearly a *let-7* target, but loss of regulation does not cause any obvious phenotype. As *let-60* is a small GTPase under tight control at the protein level, changes in expression might be not relevant for its function. Alternatively, regulation of *let-60* and of other *let-7* targets without a clear role in *let-7* functions might become relevant under some kind of "stress" such as changes in temperature, attack of pathogens or exposure to environmental toxins.

A family business: functional consequences of target regulation by the *let-7* family

The *let-7* family plays a key role in regulating developmental processes in the hypodermis and in the vulva.
Hypodermal functions of these miRNAs are well described by the concept of heterochrony: activity of the *let-7* family gives hypodermal cells temporal identity and thereby instructs execution of developmental events at the right time. The open questions to define in this context are in addition to targets of miRNA activity and interaction between the four *let-7* family members, the time and the tissue compartment of miRNA activity. Traditionally, the activity of *let-7* and its sisters has been subdivided in an early timer consisting of *let-7* sisters and a late timer represented by *let-7.* Genetically, lack of *let-7* sisters causes reiteration of the L2-like seam cell divisions at the L3 stage. These miRNAs could therefore act in the L3 stage to prevent execution of earlier fates. This is in line with the observed appearance of *mir-48/84/241* at the L3 stage. On the other hand loss of *mir-48* alone leads to reiteration of the adult molt after the larval-to-adult transition, thus two stages after the described *mir-48/84/241* seam cell phenotype. *mir-48* acts therefore at least at two different timepoints during *C. elegans* larval development. *let-7* expression starts clearly later than that of the sisters and this is probably largely due to regulation of *let-7* biogenesis by *lin-28 (Van Wynsberghe et al., 2011).* Loss of *let-7* leads to reiteration of the last larval seam cell division and postponement of the larval-to-adult transition by one stage. My results on the timing of *let-7* family activity partially support this model, but also raise new questions. Compared to *lin-41,* the *hbl-1* and *daf-12* 3'UTRs are controlled in the hypodermis more by *let-7* than by its sisters. They are also repressed stronger in the L3 stage than *lin-41,* although it is difficult to rule out that this is a consequence of generally stronger repression or of *lin-4* activity towards *hbl-1.* Intriguingly, the *let-60* 3'UTR is repressed in the hypodermis by *let-7* and this repression starts only at the late L4 stage. This data suggests that *let-7* indeed acts later than its sisters. Interestingly, loss of *mir-48/84/241*, the proposed early timer, led to derepression of targets also at a later timepoint, at the late L4 stage. Similar results were obtained with *lin-4,* the earliest timer. Loss of *lin-4* expression derepressed targets even at the L4 stage. Heterochronic miRNA targets frequently possess target sites for both the *lin-4* and the *let-7* families. One model of their action would be that as expression of these miRNAs comes in waves, repression of already repressed targets would be handed over to the next miRNA. This is clearly not the case, miRNAs keep their targets even at later larval stages. Of note, this applies to the reporter assay situation with constitutive transcription. It is very possible, that initial repression of miRNA targets is reinforced by other layers of gene regulation, e.g. transcriptionally in the case of endogenous targets. In the hypodermis, specificity of *let-7* towards *lin-41* is not absolute, loss of *mir-48/84/241* leads to upregulation of the *lin-41* reporter in the hypodermis. An intriguing hypothesis to test is that the *let-7* sisters repress *lin-41* at least to some extent already at the L3 stage, which is then increased by the appearance of *let-7* at the L4 stage. Indeed, loss of *lin-41* causes execution of the larval-to-adult transition already at the L3 molt (Slack et al., 2000) in about half of the worms examined, two stages earlier than normal. How *lin-41* could function in promoting both L3 and L4 fates

is currently not known, although the genetic evidence in the L1/L2 and L2/L3 transitions where *lin-14* and *lin-28* have partially overlapping functions provides an example for overlap of effector functions. In this model, temporal cell-fates could be defined not by the presence of one master factor, but by a quantitatively defined expression pattern of at least two factors with functions at more than one stage. The L1 fate is e.g. defined by high *lin-14* and *lin-28,* L2 by low *lin-4* and high *lin-28* (Moss et al., 1997). *hbl-1* and *lin-41* could cooperate in a similar way to define the L3 and L4 stages. Alternatively, gradual decline of *lin-41* could provide a temporal *lin-41* gradient, which in conjunction with distinct thresholds of *lin-41* activity could specify L4 and adult cell fates.
The lateral hypodermis consists of clearly separable anatomical compartments, the seam cells and the *hyp7* syncytium. As the seam cells divide asymmetrically and produce a daughter cell fusing to *hyp7,* seam cells can be regarded as stem cells in this tissue. Beyond this, little is known about the contribution of the two compartments to hypodermal functions such as molting and alae synthesis. Although alae synthesis has been mostly assigned to the seam and molting to *hyp7,* genes required for molting are often expressed in the seam (Frand et al., 2005). Furthermore, almost nothing is known about signaling between these two tissues. Signaling from the seam to *hyp*7 can be achieved by the contribution of signaling molecules such as miRNAs to *hyp7* upon fusion of seam daughter cells. In other stem cell models, signaling from the microenvironment, the so-called stem-cell niche, has an instructive role in maintaining the balance of proliferation vs. differentiation in stem cells. In many cases, the niche inhibits the default differentiation program of stem cells. As a contrast, in the *C. elegans* epidermis, seam cells have to be protected from the differentiating action of *hyp7 (Brabin et al., 2011).* What is the relevance of *let-7* miRNA activity in this context? Transcriptional reporters show that *let-7* is expressed in both compartments. For the sisters, expression is more variable, but *mir-48* is expressed predominantly in seam cells whereas *mir-84* and *241* are mostly present in *hyp7.* In addition to uncertainty associated with a more or less arbitrarily defined promoter region and regulated biogenesis of *let-7* miRNAs, the fate of mature miRNAs after biogenesis is not known. Even if e.g. *mir-48* is transcribed in the seam, the mature miRNA could be rapidly degraded and thus never reach *hyp7* or can be on the contrary used as an intercellular signaling molecule when specifically enriched in the anterior daughter cell. Association of the miRNA machinery with membrane bound compartments (Gibbings et al., 2009; Stalder et al., 2013) and the potential use of miRNAs in cell-to-cell communication in other systems (Vickers et al., 2011) make such a scenario realistic. Genetic evidence suggests that *let-7* miRNAs are upstream of both *hyp-7* and seam cell functions, as mutant phenotypes include both reiteration of molting and seam cell fates. Interestingly, *mir-48* single and *mir-48/84* mutants have specific defects exclusively in *hyp7* but not in the seam (Abbott et al., 2005). This result suggests at least some degree of compartmentalization of *let-7* family activity. On the target regulation level, I observed repression of *let-7* family targets both in the seam and *hyp7,* indicating that *let-7* miRNAs are active in both compartments. The extent of repression was similar in both tissues and I could not observe any clear difference in target specificity, targets were derepressed in both cells to similar extent upon loss of a given miRNA, with a slight tendency towards higher *mir-48/241* activity in the seam than in the hypodermis. This can

also reflect more redundant factors in *hyp7.* At the end, it is therefore not entirely clear, whether seam cell proliferation is regulated cell autonomously or through activity in *hyp7.* Assuming cell autonomous miRNA activity in the seam, an interesting question is at which time during a larval stage and compared to cell division miRNA activity is required. Specifically, distinct temporal cell fates of the seam cells are characterized by features that could be specified at different phases before or after cell division. Execution or absence of seam cell division could be either promoted or inhibited before its occurrence. Similarly, the asymmetric or symmetric, proliferative, nature of the division is probably determined by miRNA activity before the division. On the other hand, the fate of the two daughter cells could be set also later. Whether the anterior daughter cell remains in the cell-cycle and thus a seam cell could depend on, or at least reinforced by, miRNA-mediated gene repression after the division. At the larval-to-adult transition, the posterior seam daughter cell exits the cell cycle and differentiates. Again, to define this feature miRNA, specifically *let-7* activity, might be required before or after the last seam cell division.
The data generated in this work lacks the temporal resolution required to answer these questions. Continuous increase in repression favors the first model: determination of temporal identity already at the beginning of a larval stage. Although mean repression in both seam and *hyp7* was highly robust and reproducible, individual nuclei differed remarkably in the extent of target repression. At this point, this can be either noise or have a functional correlate of distinct cell fate. Further studies are required to define the principles of compartmentalization among *let-7* family members.
In addition to contributing to the characterization of *let-7* activity in the hypodermis, I established a novel *let-7* function in the vulva. Although the early heterochronic genes *lin-4, lin-14* and *lin-28* have clear roles in VPC development (Euling and Ambros, 1996), not much was known about *let-7's* role in the vulva. I could demonstrate *let-7* expression and activity in the L4 vulva and showed that this expression is necessary to suppress the vulva bursting phenotype. In contrast to previous hypotheses, *let-7* is not required for VPC specification at the L3 stage and does not regulate *let-60* in this context. *let-7* is active in all vulva cells to the same extent and to a lesser degree in uterine cells at the L4 stage. This is similar to *lin-41* transcription, but in sharp contrast to LIN-29 expression that is strongly induced in specific vulva cells at the late L4 stage. On the level of morphology, *let-7* mutant worms execute most L4 events in the vulva correctly, they have the right number of cells forming seven toroids and a lumen. Uterine cells are also specified in the normal way, the anchor cell invades the vulva basal membrane and fuses to uterine cell forming the utse cell, just like in *wt* worms. Nevertheless, all *let-7* worms burst and die at young adults. Bursting occurs after vulva eversion and starts with apparent weakening of the vulva itself. How gene expression defines complex morphogenetic processes is poorly understood. One important aspect is certainly the patterning of cells in space, induction of specialized cell types in a defined spatial arrangement to each other. We have no evidence for such a function of *let-7.* Temporal identity is equally important, as execution of normal cell fates at the wrong time can have detrimental effects. *let-7* phenotypes might reflect such a defect. Unfortunately, precise definition would require showing that a certain event is delayed or occurs too early relative to one another. At this point, it is neither clear which event would be delayed nor

known to which event the occurrence of a candidate process should be related. An alternative hypothesis, equally difficult to prove, is that *let-7* has a more specific effect on cell motility, cell-cell contacts or the cytoskeleton. Such functions for *let-7* have been described in cancer cells (Yang et al., 2012; Hu et al., 2013). Although for reasons of time, I was not able to characterize the vulva defects of the *let-7* sisters, tose miRNAs definitely have a similar if not more important role in the vulva. *mir-48/84/241* mutant worms also burst, although the penetrance of this phenotype is not complete. Similar to *let-7,* VPC specification is carried out normally in these worms (Li, 2011). Morphologically, the defects caused by loss of the three *let-7* family members is more severe than the *let-7* phenotype. Regulating some aspects of vulva morphogenesis might be a common, but not redundant, function of the *let-7* family. This is well supported by very high expression of *mir-48* and *mir-84* in the vulva. Readily apparent vulva phenotypes in *let-7* single and *mir-48/241* double mutants indicating non-redundancy in the *let-7* family despite presumably similar expression levels supports the notion of non-overlapping targets of these miRNAs.
Concerning downstream targets, *lin-41* is a good candidate for mediating *let-7* activity in the vulva. *lin-41* knock-down completely suppresses vulva bursting and *lin-41* overexpression has a similar phenotype as *let-7* loss. Interestingly, *lin-41* is thought to regulate mostly proliferation of cells. In the context of vulva, *lin-41* likely has a completely different role. Moreover, in contrast to the hypodermis, where *lin-29* is probably the key target of *lin-41, lin-29* expression is not influenced neither by *let-7* nor *lin-41.* It is a very exciting finding that the heterochronic pathway can have different effectors in different tissues. My data shows that the *let-7* sisters do not regulate *lin-41.* Thus deregulation of another gene must be thus responsible for the vulva defects of *mir-48/84/241* mutants. The identity of this gene is not known, but loss of *hbl-1* has strong defects in vulva development (Fay et al., 1999). It is therefore quite likely that similar to *let-7, mir-48/84/241* have only one key target both in the hypodermis and the vulva. The list of *let-7* suppressors identified in our RNAi suppressor screen provides promising candidates to test for involvement both in hypodermal and vulval *let-7* functions and probably downstream *lin-41* effectors.

Future directions

The results of my work can serve as a basis to explore many different questions in miRNA- and developmental biology.

The quantitative *in vivo* reporter assay established during this project represents a first indication of what is possible using current techniques in genetics, microscopy and image processing rather than the end of the possibilities. In its current state, it can be easily used as tool to examine the effects of factors on miRNA target regulation, validate miRNA targets or dissect miRNA-mediated gene regulation in time and space. Further development of this method should aim to increase the resolution in time. In a typical reporter experiment, synchronized worms or worms of mixed stage are imaged and individual worm are further assigned to stages or substages based on morphology. This classification has limited accuracy and is especially problematic in mutant backgrounds interfering with normal development. A first step towards data of higher temporal resolution would be analysis of miRNA-mediated target repression in a time-course experiment, as recently proved to be informative for analysis of gene expression (Gert-Jan Hendriks, Dimos Gaidatzis and Helge Grosshans, unpublished). Currently, manual annotation of cells of interest limits the speed of image analysis, an issue potentially circumvented by limited depth of imaging or use of fluorescent markers of a third color in cells of interest. The ultimate experiment would be time-lapse imaging of reporter worms. Besides some technical challenges, such as phototoxicity, bleaching of the fluorophores and sample movements, analysis of such 4D datasets is not trivial. At the end all these issues could be certainly solved. Application of these techniques could help to define the role of miRNA activity in regulating the fate of the stem cell-like seam cells. As most stem cell models are not amenable to live imaging, data assessing miRNA-mediated gene regulation before, during and after asymmetric cell division would be very informative. Recent identification of oscillatory gene expression patterns in *C. elegans* (Kim et al., 2013) (G-J. H., D.G and H.G, unpublished) raises the question how these oscillations are created and how miRNA contribute to or counteract them. Visualization of transcriptional patterns and repression in real time would certainly very helpful to answer this question.

One of the main findings of my project is the demonstration of target specificity within a miRNA family. As the 3'UTRs examined so far are complex, they possess different miRNA target sites, it is not clear at this point to which extent specificity depends on the target site sequence and what are the sequence requirements for target site specificity. To answer this question, an individual miRNA target sites should be transplanted in a non-regulated 3'UTR and target specificity assessed using different permutations of the target site sequence. Another gap in our understanding of miRNA-mediated target repression *in vivo* is the contribution of quantitative aspects to extent and specificity of target regulation. Using the reporter assay, I could start examining quantitative aspects on the side of the miRNA target. Currently, miRNA levels cannot be measured in individual cells and not be easily manipulated, two major technical obstacles achieve quantitative understanding of miRNA activity. Quantification of miRNA (and mRNA) levels could be achieved by isolation of individual cell-types using fluorescence-activated cell-sorting (FACS), an approach actively investigated in the Grosshans lab. Experimental manipulation of miRNA levels is even more

challenging. Expression of miRNA precursors from heterologous promoters is limited by the availability of well-characterized promoters. The same limitations apply for the use of a Cre/lox system or expression of miRNA inhibitors such as sponges. The only small molecule inducible expression system in *C. elegans* is not well characterized regarding kinetics of expression (Wei et al., 2012). At least for functional studies, a defined and relatively simple alternative would be the use of miRNA expression constructs driven by the heat-shock promoter that can be induced tissue-specifically by a laser-beam (Stringham and Candido, 1993).
Open questions in developmental biology are literally unlimited. In my opinion, it is crucial to assess the relevance and possible implications of a given question in terms of general concepts. Development in a model organism should serve indeed as model to understand a given process that is important in other organisms and contexts as well. At the same time, caution should be taken not to overgeneralize findings and blur differences between organisms and contexts.
The heterochronic pathway in the *C. elegans* hypodermis is an established and conserved model of different aspects of stem-cell biology and developmental timing. Classic genetic experiments identified the main players in this system and provided model to test with other methods. The elucidation of miRNA-target relationships including kinetic and spatial aspects would help to better understand this system. In addition to the principles underlying developmental timing, more downstream layers of gene regulation are similarly interesting and relevant. Differentiation and proliferation are generally separated either temporally or spatially. How gene expression programs underlying these two states are connected is not known. Whereas block of proliferation is well describe by the concept of the cell-cycle, the hierarchy of gene regulation in epidermal differentiation are less well defined. In *C. elegans,* differentiation has different dimensions such as cell-cell fusions and various steps of molting. Further characterization of genes revealed in the *let-7* suppressor screen might provide insights how these processes are regulated.

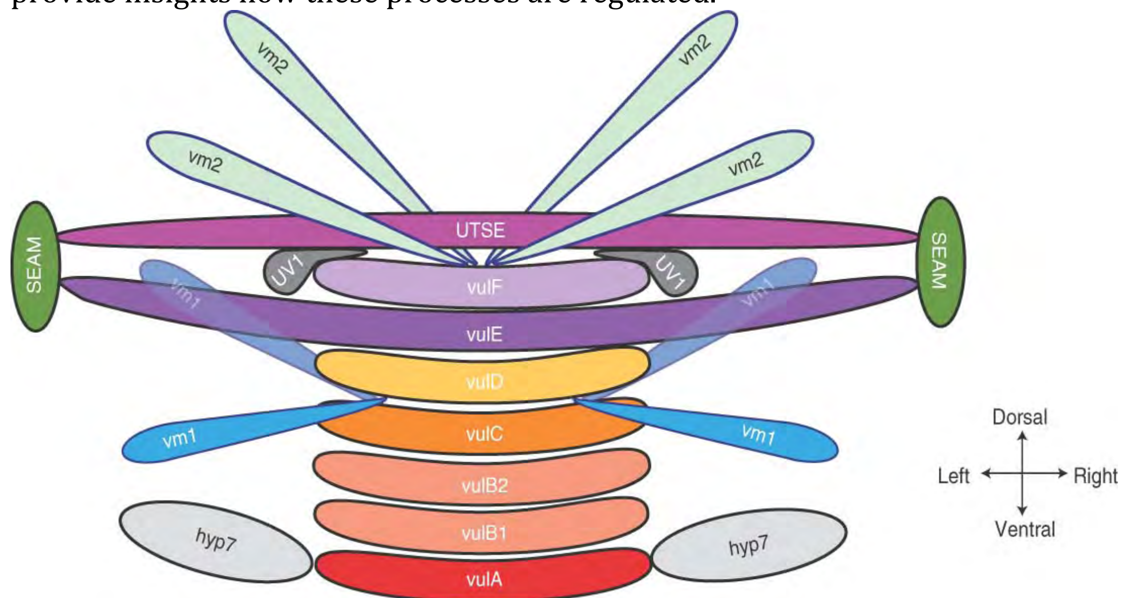

Fig. 6. Schematic model of the late L4 vulva and its attachments.
vulA-vulF designates the seven ring-like toroids. vm1 are vulva muscles, uv1 are uterine cells. Figure used with permission from doi: 10.1002/wdev.87.

Morphogenesis of organs is a complicated process. The *C. elegans* vulva with its 22 cells might represent a minimal model to study such events including cell movements, attachments, fusions, interaction with different cell-types (Fig. 6). As the same processes take place in the development of higher organisms and in many pathological states, e.g. in cancer metastasis, vulva development is a very valuable experimental model. Accordingly, the focus of research in vulva development slowly shifts from the early specification events towards the later morphogenesis steps. So far, miRNAs were not appreciated players in vulva morphogenesis. The *let-7* functions described here might lead to further studies examining the role of temporal identity in vulva morphogenesis. As experimental study of this model system requires a high degree of expertise, such studies are beyond the scope of miRNA-focused research group. Furthermore, the lack of information about the latest stages of vulva morphogenesis at the morphological, molecular and even more at the genetic level, currently prohibits to assign *let-7* a specific role in vulva development.

Finally, with the description of two tissue-specific *let-7* functions, hypodermis and vulva, the question of *let-7* activity across tissues becomes important. Is developmental time synchronous between different tissues? If yes, is it in each cell cell-autonomous or somehow orchestrated by external signals? The identification of several suppressors with a role in energy metabolism or with a function in the intestine provides support for such a signal originating in the intestine. These candidates merit further characterization. Moreover, if improper coordination of developmental events is the key defect in *let-7* mutant worms, knock-down of genes with differential effects on developmental speed in individual tissues might suppress *let-7* bursting.

Answers to the outstanding questions on the developmental functions of *let-7* and on the principles underlying the regulation of its targets will be obtained only by careful study of worm development at the genetic, molecular and perhaps biochemical level. As exemplified by my work, results of such studies can contribute important insights about the functions and molecular mechanisms of miRNA-mediated target repression in general and prove the utility of *C. elegans* as a model system for miRNA biology.

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