Differential roles of the microRNA *let-7* in *C. elegans* tissue development

Inauguraldissertation

zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät der Universität Basel

von

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aus Mainz, Deutschland

Basel, 2015

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät

auf Antrag von

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Basel, den 11.11.2014

Prof. Dr. Jörg Schibler

Thanks to Jan and Iskra.

I would have never done this without you.

Summary

The organs and tissues of the human body comprise of an astonishing variety of cells as different in morphology and function as muscle cells and neurons. Amazingly, despite their different protein contents, they largely contain the identical genomic information. In order to understand the processes that enable this differentiation, we need to determine the underlying regulatory mechanisms. A very recent discovery in this context was the posttranscriptional regulation of gene expression by microRNAs (miRNAs). miRNAs are small RNA molecules that mediate translational repression and degradation of mRNA transcripts through partial complementarity to their 3' untranslated region (UTR) . Among the first miRNAs to be identified, *let-7* stands out for its high conservation in sequence and developmental functions in development throughout the animal kingdom. During my PhD, I studied the role of *let-7* in *Caenorhabditis elegans* in the context of two distinct processes of tissue development, namely differentiation of the epidermis (called hypodermis), and morphogenesis of the vulva. The functions of the *let-7* miRNA in formation of the adult cuticle have been extensively studied and are well understood. *let-7* controls differentiation of specific, mitotically active epidermal cells by inducing cell cycle exit, fusion, and switch to an adult specific transcriptional program upon repression of targets such as *lin-41*, *daf-12*, *hbl-1* and *let-60/ras*. I set out to identify novel interactors of *let-7* in a genome-wide RNAi screen for suppression of the lethal *let-7* bursting phenotype. Candidates were then verified using fluorescence-based reporter systems for onset of hypodermis differentiation and intensity of repression of a known target. Thereby, I was able to validate a whole set of novel members of the *let-7* network, comprising genes downstream in the pathway as well as potential regulators of *let-7* activity. Notably, both groups of repressors contain factors required for cell cycle progression and mitosis, which indicates an active crosstalk between *let-7* and the cell-cycle machinery. In a second project, I explored the molecular basis for the prominent *let-7* vulval bursting phenotype. Despite the absence of overproliferation or any other obvious phenotype in vulval morphogenesis, I was able to show that *let-7* activity is required in the vulva, and that its major function in this context is repression of a single target, namely *lin-41*. Disruption of *let-7* binding to *lin-41* through modification of the *let-7* complementary sites by CRISPR/Cas9 mediated genome editing suffices to trigger the bursting phenotype, proving that repression of a single target is the key function of the miRNA in this context. In summary, my work shows that while both differentiation of hypodermis as well as vulval integrity are mediated through repression of *lin-41*, the downstream effect of this regulation seem to differ, suggesting that *let-7* can be wired to control distinct processes depending on the cellular

context. With respect to the latest findings both in *C. elegans* as well as in mammals, it will be interesting to determine if this depends on differential molecular functions of LIN-41 in the two tissues.

Contents

Introduction

C. elegans as a classical model organism for development

All multicellular organisms develop different tissues and organs from a single fertilized oocyte. During this process, pluripotent precursor cells have to differentiate into highly specialized cells as different as muscle cells, neuronal cells or germ cells. While these distinct cell types all share the same genomic information, they still differ in cell morphology and function due to individual, cell type-specific gene expression profiles. Numerous studies over the last decades have elucidated that the resulting distinct protein outputs are generated by a delicate interplay of regulatory modules. As a first level, transcription can be regulated by sets of cell type specific transcription factors. The earliest fate choice in mammalian development, the decision between placental trophectoderm and embryonic tissue, is regulated through reciprocal activity of the transcription factors Cdx2 and Oct3/4. Overexpression of Cdx2 can induce trophectoderm formation, while activity of Oct3/4 is required to establish the pluripotent state of the inner cell mass (Niwa et al. 2005). Such transcriptional states can then be enforced by epigenetic modifications of chromatin such as DNA methylation and histone modifications, resulting in closure of genomic regions specific for other downstream cell lineages. An elegant proof of this paradigm was the finding that reprogramming of mouse embryonic fibroblasts (MEFs) into pluripotent stem cells involved a reduction of the repressive histone modification H3K9me2 at the locus of the pluripotency factors Oct3/4 and Nanog (Takahashi and Yamanaka 2006). Differential gene expression can also be established on the level of mRNA. Probably the most drastic developmental decision based on alternative mRNA splicing is the sex determination of Drosophila melanogaster (Hodgkin 1989). More recently, it became apparent that specialized small RNAs themselves can act as regulators of gene expression at the mRNA level. The discovery of posttranscriptional gene repression by microRNAs (miRNAs) and short interfering RNAs (siRNAs) has transformed our view of both RNA biology and gene regulation.

Our understanding of developmental processes in animals has been substantially shaped by countless discoveries made in the nematode *Caenorhabditis elegans*. This roundworm of roughly one millimeter in length can be found in the soil of temperate zones all over the world, where it feeds on microorganisms such as bacteria that decompose organic materials. Adult hermaphrodites lay around 200 eggs from which the larvae hatch after approximately 10 hours of embryonic development at 25°C. Subsequently, the worms go through four larval stages (L1-L4) that each end with a molting event during which the worms

shed off their cuticle. With the fourth and final molt, they reach the sexually mature adult stage and start to produce oocytes, which can be fertilized by sperm from the hermaphrodite's own spermatheca or after mating with rarely occurring male animals. Due to its size and short life cycle, *C. elegans* can be easily cultured in large amounts in the lab using agar plates covered with *E. coli*, and the hermaphrodite's self-fertilization combined with the possibility to use males to cross different mutations facilitates genetics tremendously (Brenner 1974). Moreover, both its small size and the fact that the embryo as well as the worm are transparent facilitate visualization of cell divisions using light microscopy. *C. elegans* has been traditionally used as a model system for forward genetic screens because of its large brood size. Worms can be mutagenized using EMS (ethyl methane sulfonate), and progeny can be screened for obvious phenotypes such as abnormal body morphology (e.g. dumpy, "dpy") or locomotion (uncoordinated, "unc"). These phenotypes can then be mapped to mutations in single genes and gene functions can be determined. Today, researchers have access to a variety of techniques adapted for *C. elegans* that allow to address almost any scientific question possible. One of the most important achievements was the discovery that injection of plasmid DNA into the hermaphrodite gonad results in the establishment of large extrachromosomal DNA arrays (Mello et al. 1991). These arrays can be replicated and propagated during mitosis and inherited to progeny by a mechanism similar to endogenous chromosomes. Furthermore, arrays can be stably integrated into the genome of the worm at a random position by irradiation (Mitani et al. 1993). This means that by using appropriate promoters, *C. elegans* can be engineered to express any transgene of interest, either ubiquitously or in specific tissues or cell types. When this technique was combined with the discovery of GFP (green fluorescent protein) it became possible to visualize the intracellular localization of proteins as well as the expression profiles of gene-specific promoters by fusing the mRNA sequence of gfp to the coding sequence or the promoter of any gene of interest (Chalfie et al. 1994). Since the early nineties, these techniques have evolved to a point where researchers can now integrate a single copy of a transgene at a defined genomic locus using a recombination technique based on Mos1 transposon mobilization, ensuring defined expression levels of the transgene even in the germ line (Frokjaer-Jensen et al. 2008). Major technical advances were also made in the context of gene manipulation. The observation of efficient gene knockdown upon injection of complementary double-stranded RNA molecules into the germ line greatly improved functional gene analysis for the *C. elegans* community (Fire et al. 1998). Moreover, this study on RNA interference (RNAi) thereby lead to the discovery of RNA mediated posttranscriptional gene silencing, which has changed our understanding of gene regulation dramatically. In addition to posttranscriptional knockdown of any gene

of interest, the discovery of the CRISPR/Cas9 system of bacteria and its application for targeted genome editing now allow deletion or modification of genomic loci (Frokjaer-Jensen 2013).

Because of the numerous advantages in handling and the technical tools available *C. elegans* has become essential in various fields of biological research such as cell biology, neuroscience or aging. However, the worm originally began its career as a model organism for development. The simple anatomy and total life cycle of only about 50 hours at 25°C make it an ideal system to follow development over the course of time. In a laborious study John E. Sulston and coworkers were able to map the cell divisions of each single cell from the oocyte to its final position in the adult worm. This pioneering work resulted in a complete cell lineage of the developing animal which revealed that the tiny worm shows a strikingly invariant pattern of cell division, apoptosis and tissue specification events during development (Sulston and Horvitz 1977, Kimble and Hirsh 1979, Sulston et al. 1983, Hedgecock et al. 1983). This always gives rise to a final 959 nuclei in wild-type hermaphrodites and 1031 nuclei in male animals. Subsequent to this finding, mutants were identified that show abnormalities in the cell division pattern, leading to cell fate specification or tissue differentiation defects (Horvitz and Sulston 1980, Sulston and Horvitz 1981, Chalfie et al. 1981). Looking at the cell lineage of *C. elegans*, a hallmark of development becomes apparent: Developmental control has to be exerted on two different, interconnected levels. Pathways must exist to ensure that a cell division gives rise to a pair of daughter cells with defined properties. But in order to result in a functional organ, cell divisions of a given type have to occur at a precisely controlled time point. The invariant cell lineage of *C. elegans* has allowed researchers to ask fundamental questions regarding the genetic and molecular regulation of development in time and space. One important contribution to our understanding of developmental control was made by the discovery of the heterochronic pathway in *Caenorhabditis elegans*.

The heterochronic pathway of *C. elegans* controls developmental timing

At the beginning of the first larval stage a hermaphrodite *C. elegans* larva consists of 558 cells. The division events of the remaining profiferative blast cells that allow growth and result in the final 959 somatic cells of the adult have been carefully mapped and are relatively easy to follow over larval development (Sulston and Horvitz 1977, Kimble and Hirsh 1979, Sulston et al. 1983). Soon after the initial mapping of postembryonic cell divisions, mutants were identified that showed alterations in the normally invariant pattern (Horvitz and Sulston 1980). It became apparent that some of these mutations lead to reiteration

of parental or even grandparental division patterns (Chalfie et al. 1981). This observation prompted Victor Ambros and Robert Horvitz to adapt the concept of heterochrony for *C. elegans* development (Ambros and Horvitz 1984). They defined heterochronic defects as cell division or differentiation events occurring in the correct cell lineage but at the wrong time point. As a consequence, mutant animals would display altered developmental timing in one cell type or tissue relative to the rest of the animal. Indeed, they were able to identify both precocious mutants expressing a given fate too early due to skipping of a developmental event, as well as retarded mutants that reiterated the previous fate. These findings proved that *C. elegans* could be used to study the timing mechanisms that underlie development and ultimately lead to the discovery of the heterochronic pathway as a consecutive cascade of gene products regulating developmental decisions from the L1 larva to the adult worm (Rougvie 2001).

The heterochronic pathway controls timing of vulva development

One of the first major developmental processes found to be affected by the heterochronic pathway is the development of the hermaphrodite vulva. Morphogenetic events as well as the underlying events of signal transduction and transcriptional activation have been intensively studied and serve as a general model for organogenesis, and tissue remodeling (Sternberg 2005). The *C. elegans* vulva is formed postembryonically by a complex sequence of highly orchestrated cell divisions and morphogenesis events, which are governed by several conserved signaling pathways (Horvitz and Sternberg 1991). Six multipotent vulval precursor cells (VPCs) are generated during early larval development from a set of ventral hypodermal blast cells of the P lineage (Sulston and Horvitz 1977). Three of these cells, P5.p to P7.p, respond to a graded EGF (LIN-3) signal from the anchor cell, a specialized cell of the somatic gonad, during the early L3 stage (Kimble 1981, Hill and Sternberg 1992). This signal initiates vulva development and coordinates vulva morphogenesis with the beginning development of the germ line (Figure 1). The P6.p cell, located closest to the anchor cell, receives the highest dose of LIN-3 signal, which initiates signal transduction from the EGF receptor LET-23 through the Ras homolog LET-60 to the MAP kinase MPK-1 (Han et al. 1990, Beitel et al. 1990, Han and Sternberg 1990, Lackner et al. 1994). This in turn activates transcriptional programs characteristic for the primary vulva cell fate (1° VPC) (Tan et al. 1998). One important change in gene expression is the upregulation of Notch ligands and downregulation of the Notch receptor LIN-12 on the surface of the P6.p cell, which dampens the Notch signaling in the 1° cell (Greenwald et al. 1983, Levitan and Greenwald 1998, Chen and Greenwald 2004). Weaker EGF signal from

the anchor cell and the lateral Notch signal coming from the 1° VPC drive the adjacent P5.p and P7.p cells to downregulate LET-23/MAPK signaling.

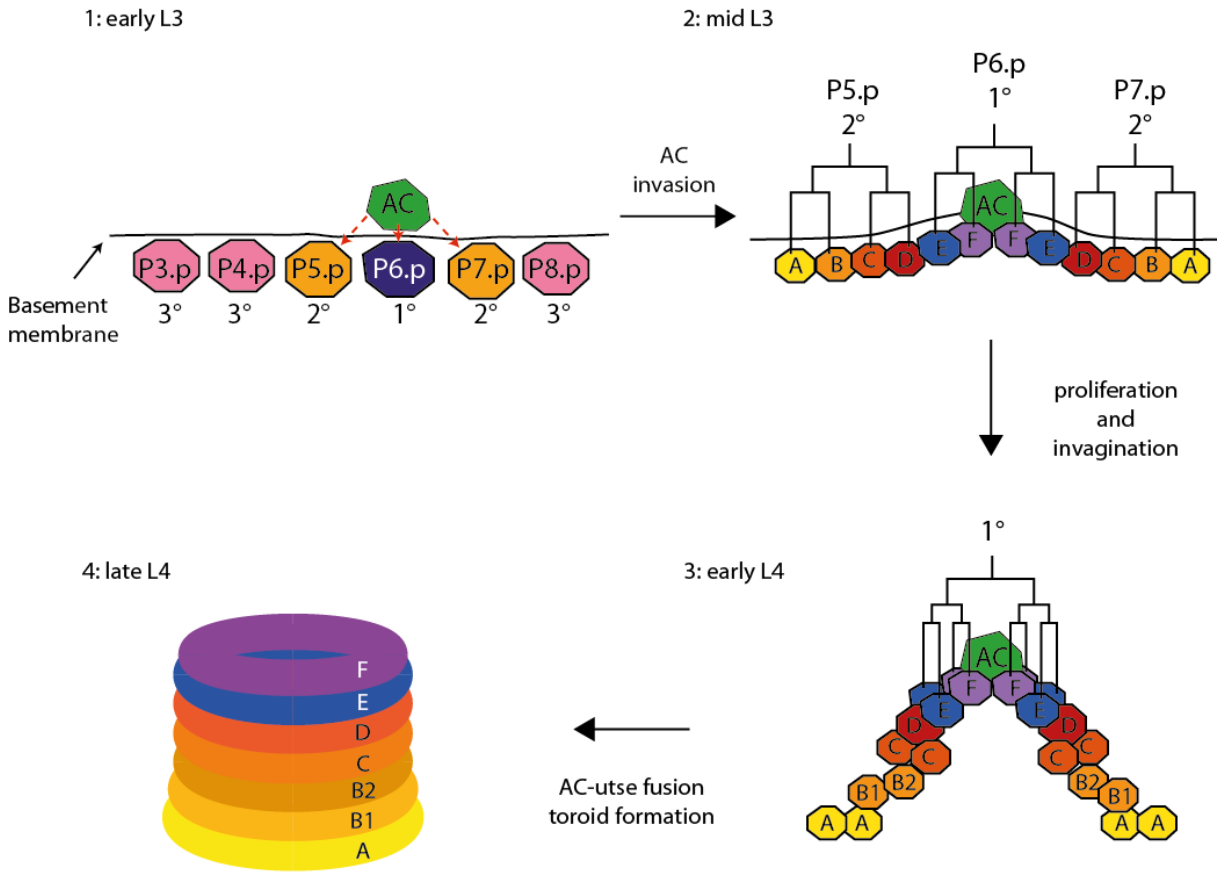

Figure 1 Vulva morphogenesis

The six vulval progenitor cells (VPCs) P3.p to P8.p derive from hypodermal blast cells of the P lineage during L1 stage. During L3 stage, an EGF signal from the anchor cell (AC), a specialized cell of the gonad, induces the P6.p cell and the P5.p and P7.p to adopt 1° and 2° vulval cell fates, respectively (1). Subsequently, these cells produce 22 progeny, which differentiate to the final vulval cell types A to F, while the remaining vulval precursor cells give rise to daughter cells that fuse with hyp7. Parallel to the proliferation, the vulval cells move towards each other and start to invaginate at the end of L3 stage (3). This morphogenesis event establishes the tubular shape of the vulva and finally gives rise to seven toroids, which are generated through fusion of specific vulva cell subsets (4). Following VPC specification, the anchor cell breaks the basal membrane that separates uterus and hypodermis and invades between the 1° lineage daughters, contributing to their specification (2). During mid L4 stage, it withdraws from the vulF cells and fuses with the uterine utse cell, leaving only a thin process of cytoplasm to cover the connection between vulva and uterus. Figure modified from wormbook (Gupta et al. 2012).

The therefore dominant Notch signaling pathway drives these cells to adopt a 2° VPC fate (Greenwald et al. 1983, Berset et al. 2001, Yoo et al. 2004). The remaining three uninduced VPCs automatically adopt a 3° fate and subsequently produce daughter cells that become part of the hypodermis. Notably, all six VPCs have the capacity to adopt a vulval cell fate, and ectopic activation of EGF signaling can induce additional 1° VPCs, resulting in multivulva phenotypes (Aroian and Sternberg 1991). Following cell fate specification

during early L3 stage, the 1° and 2° cells begin to divide to produce a distinct set of 22 daughter cells that eventually differentiate into the seven different vulval cell types (vulA to vulF). Even before proliferation has finished, the vulval cells start to migrate towards each other, which results in an invagination of the vulval tissue (Herman et al. 1999). Parallel to these morphogenetic changes the anchor cell breaks the basal membrane that separates gonad and body wall and invades between the 1° lineage cells at mid L3 stage (Sherwood and Sternberg 2003). This event facilitates the establishment of a connection between uterus and vulva to form a functional egg-laying apparatus (Estes and Hanna-Rose 2009). Final morphogenetic events of vulva development occur during the late L4 stage, when the cells of the seven vulval cell types fuse with their sisters to form the seven vulva toroids, giving the vulva its final tubular shape (Sharma-Kishore et al. 1999), while the anchor cell fuses with the utse, a syncytium of uterine cells (Newman et al. 1996).

A link between the heterochronic genes and vulva development became apparent even before the existence of a heterochronic pathway had been postulated. In addition to their inability to produce an adult specific cuticle, *lin-4(e912)* worms show a fully penetrant vulvaless phenotype (Horvitz and Sulston 1980) due to complex deviations from the wild-type VPC division pattern (Chalfie et al. 1981). *lin-14* and *lin-28* loss-of-function mutants in contrast show premature proliferation of the VPCs during L2 stage due to a shortened G1 phase, resulting in a protruding vulva (pvl) phenotype (Euling and Ambros 1996) . A direct link between heterochronic genes and the cell cycle was established when *lin-14* was shown to activate expression of *cki-1*, a cyclin-dependent kinase inhibitor of the CIP/KIP family (Hong et al. 1998) , pausing progression to S phase until mid L3 stage.

Heterochronic regulation of hypodermis development

A second process regulated by the heterochronic pathway is the postembryonic development of the hypodermis of C. elegans, an epidermal cell layer forming the outer body wall of the worm. Main function of the hypodermis is the secretion of the cuticle, a collagenous structure that protects the animal from

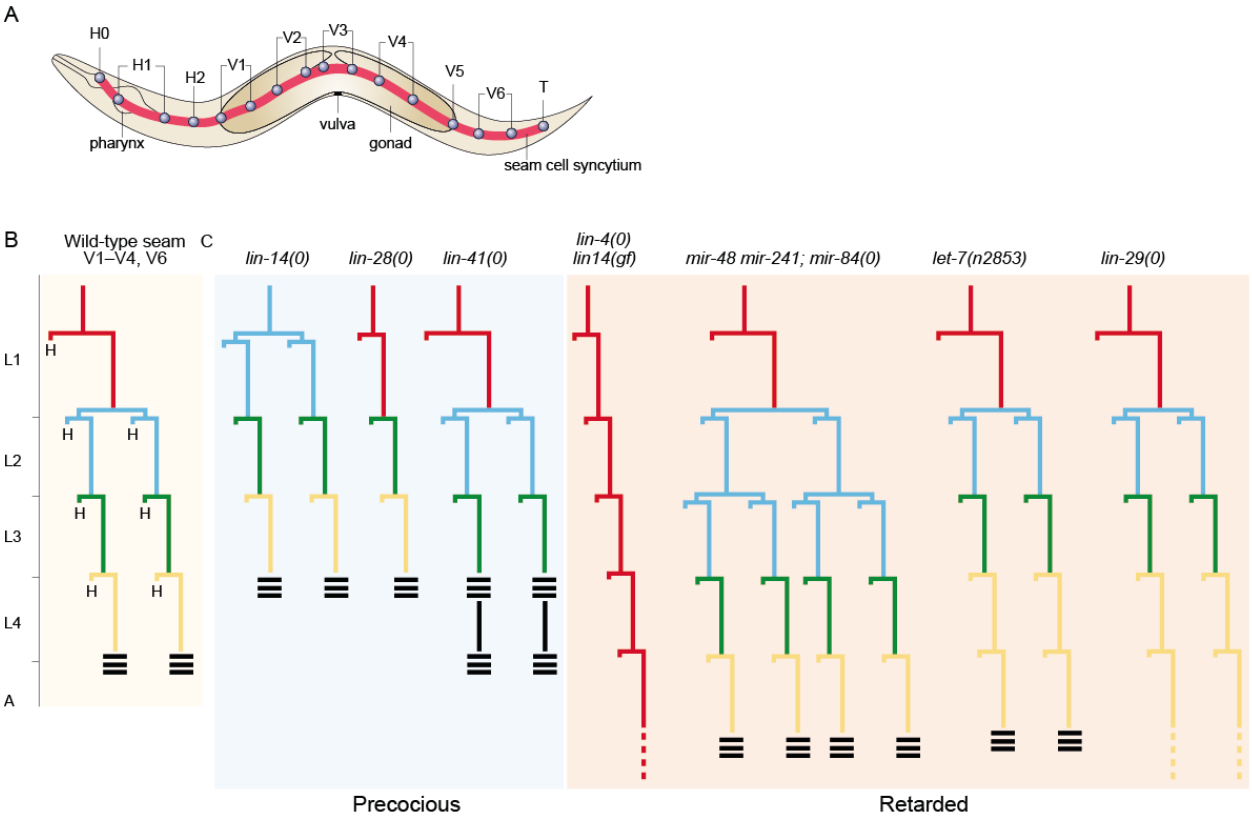

Figure 2 *C. elegans* seam cell development

Seam cells are a specified subset of hypodermal cells (H0-2, V1-6 and T) that line the lateral sides of the worm. All cells except H0 divide during larval development, giving rise to multiple daughter cells that join the hypodermal syncytium, as well as a total number of 16 adult seam cells on each body side. The seam cells fuse at the L4-to-adult transition and secrete adult specific cuticular ridges called alae. **A,** Schematic of an adult hermaphrodite, lateral view. Seam cell nuclei (blue) are indicated with their respective parental blast cells. **B,** Postembryonic cell lineage of the V1-4 and V6 cells in wild-type animals. The division event at the beginning of each larval stage produces a daughter that differentiates and fuses with the hypodermis (H) as well as a daughter that retains proliferative potential. During L2 stage, a symmetrical division precedes the asymetrical event, increasing the total number of seam cells. **C,** indicated heterochronic mutants that either skip (Precocious) or reiterate (Retarded) stage-specific division patterns. Notably, *let-7(n2853)* is not a null mutation, but a temperature-sensitive hypomorph that shows seam cell differentiation failure at 15°C but a lethal bursting phenotype at the L4-to-adult transition at 25°C. Seam cell fusion followed by secretion of alae is represented by black horizontal triple bars (modified from Rougvie 2001).

the environment and serves as a stable but very flexible exoskeleton. At the time of hatching, the hypodermis mainly consists of the large syncytial hyp7 cell and two major types of specialized epithelial cells (Sulston et al. 1983, Podbilewicz and White 1994). One group of these specialized cells are the seam cells, lateral hypodermal blast cells that maintain their proliferative potential after the completion of embryogenesis (Figure 2). Postembryonic lineaging revealed that the V1-V4 and V6 seam cells repeat a stem-cell-like division pattern during each larval stage (Sulston and Horvitz 1977). During this process one daughter cell fuses to the hyp7 syncytium while the other daughter maintains its proliferative potential

for the next larval stage. This asymmetric division increases the number of hypodermal cells during development and allows growth of the animal from approximately 0.3mm in L1 to the adult size of 1mm. Moreover, symmetric division of a subset of seam cells during the L2 stage raises their total number from 10 to 16 on each side. After the last asymmetric division in early L4 stage seam cell development finishes with the differentiation of the seam cells around the time of the L4 molt.

This differentiation is marked by the fusion of each seam cell line to a lateral syncytium. As a result of the differentiation, the hypodermis starts to produce a cuticle with adult-specific collagen compounds, and the seam cells secrete alae, adult specific cuticular ridges along the lateral sides of the worm (Singh and Sulston 1978). Developmental control of hypodermis development can be divided into two major categories: mechanisms that time the larval specific division events, and mechanisms that ensure differentiation and establishment of the final adult state at the right time. The first isolated mutant for seam cell development was *lin-4(e912)*, in which the L1 specific division pattern of the V1-V4 subset of the seam cells was reiterated, resulting in a failure to produce an adult specific cuticle (Horvitz and Sulston 1980, Chalfie et al. 1981). Soon after this discovery, mutations in the genes *lin-14* and *lin-28* were identified that had the opposite effect on hypodermis development. Both displayed precocious alae formation due to skipping of L1 or L2 fates, respectively, indicating that the two factors were required for execution of stage specific division events (Ambros and Horvitz 1984, Ambros and Horvitz 1987). Careful analysis of the identified mutants revealed the requirement of *lin-4* for their repression in order to permit progression to the next step in seam cell division (Ambros 1989). A second, exceptional class of *lin-14* mutations that phenocopied the retarded *lin-4(e912)* mutation was concluded to be immune to *lin-4* mediated repression. These interactions of the early heterochronic genes were deciphered exclusively through analysis of phenotypes combined with the laws of genetics, which resulted in a model according to which genes responsible for a given event needed to be repressed after their time of action by a master regulator. Surprisingly, when the molecular identity of the mutant genes was revealed, it became clear that *the lin-4* locus did not, as expected, produce a protein. Instead it was found to produce a small RNA molecule that regulated its targets at the mRNA level through partial complementarity to their 3' untranslated region (3'UTR) (Lee et al. 1993, Wightman et al. 1993, Moss et al. 1997). The exceptional *lin-14* mutants that showed the same division pattern as *lin-4* mutants turned out to be immune to *lin-4* regulation because they carried deletions for these complementary sequences. This finding suggested a totally novel mechanism of gene regulaton, however, the molecular principle remained obscure for almost a decade. In the meantime, scientists continued to identify additional heterochronic mutations *of C. elegans*, among them *lin-29*, which was integrated into the heterochronic pathway as the most

downstream factor, controlling mature cuticle formation (Papp et al. 1991, Liu et al. 1995). In worms mutant for *lin-29* seam cells fail to differentiate at the end of L4, and the worms continue to produce a new larval cuticle with each supernumerary molt. *lin-29* codes for a transcription factor that regulates the activation of the adult-specific collagen *col-19* and represses the larval specific *col-17* (Rougvie and Ambros 1995).

The surprise came in the year 2000, when a genetic screen for heterochronic mutants discovered a second small RNA regulating the L4-to-adult transition. Animals mutant for *let-7* (lethal 7) execute early seam cell divisions properly but fail to initiate the differentiation event at the larval to adult transition. Instead, the seam cells go through an additional round of cell divisions and continue to express a larval specific cuticle without alae (Reinhart et al. 2000). Analogous to the relationship between *lin-4* and *lin-14*, a mutation in a second novel heterochronic gene was identified that was able to suppress the retarded *let-7* phenotype. Worms mutant for both *let-7* and *lin-41* (lineage variant 41) show wild-type seam cell differentiation at the end of L4 stage, while a *lin-41* mutation alone results in precocious differentiation and alae formation (Slack et al. 2000). This phenotype is lost in *lin-29* mutants, which led Slack and coworkers to conclude that *lin-29* was repressed by *lin-41* and activated in a *let-7*-dependent manner. These features defined *let-7* as the second master-regulator of the heterochronic pathway, initiating the final switch from proliferation to differentiation rather than execution of earlier division events.

The microRNA *let-7*

Soon after the discovery of *let-7* as a second small RNA timing regulator, highly conserved *let-7* homologues were detected in other organisms ranging from simple molluscs to humans (Pasquinelli et al. 2000). Those homologues also showed temporal expression and were proposed to function in regulation of developmental timing, leading to their definition as small temporal RNAs (stRNAs). The picture expanded when numerous additional small RNAs with potential regulatory functions were detected in *C. elegans* and a variety of other animal species, which were termed microRNAs (miRNAs) (Lau et al. 2001, Lee and Ambros 2001, Lagos-Quintana et al. 2001) . This discovery opened a new chapter in gene regulation.

Biogenesis and function of microRNAs

miRNAs mediate posttranscriptional repression as small RNA molecules 22nt of length. Nonetheless, most miRNAs begin their life as long polyadenylated, 5' 7-methylguanosine capped primary transcripts derived from their own polymerase II dependent promoters (Lee et al. 2004, Cai et al. 2004). Characteristic for these primary transcripts or pri-miRNAs are long hairpin structures that harbor the mature miRNA sequence within their stem. These primary transcripts are processed in the nucleus by the microprocessor complex, which contains the endonuclease Drosha (Lee et al. 2003) and the cofactor Pasha/DGCR8 that mediates binding to the precursor molecules and facilitates cleavage (Denli et al. 2004, Yeom et al. 2006, Gregory et al. 2004, Landthaler et al. 2004). This first processing step generates the ~70nt long precursor microRNAs or pre-miRNAs, hairpin molecules of individual sequence with an imperfectly paired stem of about 30 nucleotides. These precursors are exported to the cytoplasm by Exportin-5 in vertebrates, or Embargoed and its homologue XPO-1 in *Drosophila melanogaster* and *C. elegans*, respectively (Yi et al. 2003, Bussing et al. 2010, Lund et al. 2004, Bohnsack et al. 2004), where a second endonucleolytic cleavage event mediated by the nuclease Dicer removes the loop of the hairpin (Grishok et al. 2001, Hutvagner et al. 2001, Ketting et al. 2001). This initially produces a miRNA duplex of 22nt, which is handed over to the miRNA induced silencing complex (miRISC) and incorporated into the Argonaute subunit (Grishok et al. 2001). Following incorporation the duplex is unwound and the miRNA passenger strand or miRNA* strand is discarded and degraded immediately, while the remaining mature miRNA facilitates binding of the complex to the 3'UTRs of target mRNAs (Meister and Tuschl 2004, Tomari and Zamore 2005). This targeting process depends on partial complementarity of the miRNA to the 3'UTR of the targets and leads to posttranscriptional repression of the target. Although miRNAs can in principle induce Argonaute-mediated endonucleolytic cleavage of targets in case of perfect complementarity, this process typically does not take place in animal cells, where binding occurs through imperfect base-pairing. Moreover, early studies in *C. elegans* found no effect of *lin-4* on *lin-14* and *lin-28* mRNA levels, which suggested a mechanism of translational repression (Olsen and Ambros 1999, Seggerson et al. 2002). Soon thereafter, several studies both in vivo and in vitro that used polysome-profiling to determine the translation status of miRNA targets could provide evidence for repression of translation at the initiation step (Pillai et al. 2005, Mathonnet et al. 2007, Ding and Grosshans 2009). Nonetheless, genome-wide studies that analyzed steady-state protein and mRNA levels of miRNA targets could show that repression was mostly caused by changes in mRNA levels (Baek et al. 2008, Hendrickson et al. 2009, Guo et al. 2010, Eichhorn et al. 2014). Today, it has become increasingly clear that the GW182 subunit of the miRISC is essential for both translational repression as well as degradation of miRNA targets, making it the effector protein for miRNA mediated repression. Proteins of the GW182 family (AIN-1 and AIN-2 in *C. elegans*,

TNRC6A-C in mammals) interact with Argonaute through their N-terminal GW repeats, while mid and C-terminal domains are required for silencing (Huntzinger and Izaurralde 2011)(Huntzinger and Izaurralde 2011). Indeed, artificial tethering of the silencing domain (SD) to mRNAs is sufficient to induce repression

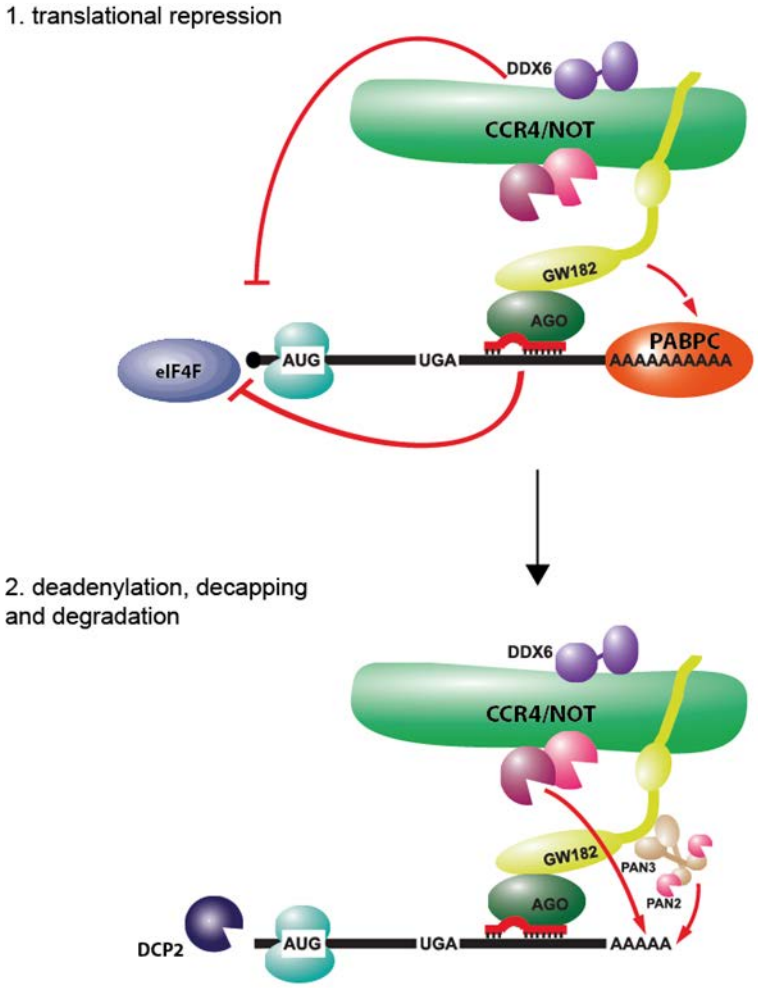

Figure 3 Posttranscriptional repression by miRNAs

When incorporated into Argonaute (AGO), the mature miRNA recruits the miRNA induced silencing complex (miRISC) to a target mRNA through partial sequence complementarity to miRNA binding sites typically located in the 3' untranslated region (UTR). Binding of RISC to the target immediately leads to repression of translation, most likely at the initiation step. This repression is mediated through direct interaction of AGO with the translational initiation complex, as well as binding of the RISC effector protein GW182 (AIN-2/AIN-2 in *C. elegans*) to the cytoplasmic poly(A) binding protein (PABPC). Moreover, GW182 can recruit the multiprotein complex CCR4/NOT and thereby the DEAD-Box ATPase DDX6, which contribute to repression of translation. For the majority of miRNA/target interactions, initial repression is immediately followed by deadenylation of the target due to recruitment of deadenylases such as PAN2/PAN3 and the CCR4/CAF1 subunits of the CCR4/NOT complex via the GW182 protein. This leads to decapping by DCP2 and associated factors and subsequent 5' to 3' degradation of the target (modified from Mathys et al. 2014).

and degradation, even in the absence of a miRNA loaded Argonaute (Lazzaretti et al. 2009, Zipprich et al. 2009, Chekulaeva et al. 2009). GW182 proteins were shown to interact with cytoplasmic poly(A)-binding protein (PABPC), thereby competing with the initiation factor eIF4G (Fabian et al. 2009, Zekri et al. 2009) which is required for mRNA circularization and reinitiation of translation. Molecular insight into the mechanism of mRNA degradation was obtained when GW182 proteins were shown to interact with the PAN2-PAN3 as well as the CCR4-NOT deandenylase complexes (Fabian et al. 2009, Braun et al. 2011, Chekulaeva et al. 2011). This interaction induces global deadenylation of miRNA targets, followed by decapping and ultimately mRNA decay through the 5'-3' exonuclease XRN-1 (Rehwinkel et al. 2005, Behm-Ansmant et al. 2006, Eulalio et al. 2009). Interestingly, the CCR4/NOT complex was also found to mediate

translational repression. Work recently published in Molecular Cell could now attribute this activity to recruitment of the DEAD-Box protein DDX6, which interacts with the complex through the CNOT1 subunit of CCR4/NOT. The study identifies CNOT1 as a platform for different functions of the complex, mediating repression of translation through recruitment and activation of DDX6 while inducing degradation through association with the deadenylase subunits CCR4 and CAF1 (Mathys et al. 2014, Chen et al. 2014). Moreover, recent studies have suggested a GW182 independent role for Argonaute in displacing the initiation complex subunit eIF4A from target mRNAs (Fukao et al. 2014, Fukaya and Tomari 2012, Fukaya et al. 2014).Taken together, these findings indicate that repression of translation and degradation are mediated through the same protein complex (Figure 3), and indeed, high resolution studies of mRNA kinetics have led to a sequential model of posttranscriptional gene repression, such that repression precedes deadenylation and degradation (Bazzini et al. 2012, Djuranovic et al. 2012, Fukaya and Tomari 2012, Bethune et al. 2012).

miRNA targets

Importantly, the miRNA pathway shares core components with a second cellular pathway involved in posttranscriptional regulation. Long double stranded precursor RNAs produced either by pathogens or from repetitive endogenous DNA loci can be processed into short duplexes of 22nt by Dicer (Bernstein et al. 2001, Ketting et al. 2001, Knight and Bass 2001). Incorporation of these short interfering RNAs (RNAi) into the RISC complex can target complementary mRNAs for endonucleolytic cleavage and degradation, and in some cases even transcriptional gene silencing (TGS) of the genomic locus of origin though heterochromatin formation (Hammond et al. 2000, Volpe et al. 2002, Mello and Conte 2004, Golden et al. 2008). To date, this phenomenon is widely used by researchers for transient knockdown of any gene of interest by RNA interference (RNAi) upon delivery of double-stranded RNA. A major difference between miRNAs and siRNAs in animals is the degree of complementarity to their targets. siRNAs are initially produced from their own targets, which results in perfect complementarity. miRNAs in contrast bind their targets through perfect Watson-Crick base pairing of their nucleotides 2-8 (miRNA “seed”) and variable, partial complementarity of the 3’ end (Carthew and Sontheimer 2009, Lai 2002). Therefore, identification of relevant miRNA targets has been a challenge ever since the discovery of miRNA mediated posttranscriptional regulation. The first miRNA targets were identified in *C. elegans* using classical genetic approaches by their ability to repress miRNA phenotypes (Reinhart et al. 2000, Ambros 1989). However,

this approach cannot distinguish between direct and indirect targets, and genetic screens are difficult to perform in higher organisms. MicroRNA targets can be predicted computationally based on the complementarity between miRNA seed and the 3'UTR of annotated genes. Today, the major target prediction tools calculate miRNA:target pairs based on base-pairing of the seed sequence and 3' end of the miRNA, free energy of this interaction, and conservation of the binding sites across species (Peterson et al. 2014). Based on these methods, up to 60% of mammalian protein-coding genes are predicted to subject to miRNA mediated regulation (Friedman et al. 2009). Depending on the miRNA of interest, these in silico approaches can result in hundreds of predicted targets, including numerous false positive hits. To complicate matters, individual 3'UTRs can contain multiple binding sites of a single miRNA, or even additional sites of multiple miRNAs (Bartel 2009). Thus, computational target prediction needs to be combined with biological validation to identify physiologically relevant interactions. A classical approach to verify miRNA mediated repression both in vitro and in vivo is the use of GFP or luciferase based target reporters. Here, the 3'UTR of the gene of interest is tested for its ability to mediate posttranscriptional repression to the reporter in the absence or presence of the candidate miRNA. Of note, while both in vivo and in vitro assays are insufficient to conclude functional relevance, analysis in vitro often depends on overexpression of the miRNA, which might alter miRNA:target interactions. Targets can also be predicted experimentally on a genome-wide level for their effect on global mRNA or protein abundance by RNA sequencing or SILAC proteomic approaches (Selbach et al. 2008, Baek et al. 2008). Again, such studies often use overexpression of the miRNA of interest and do not necessarily represent the in vivo situation. An alternative method to verify interactions is the biochemical detection of miRNA:target pairs by different crosslinking and immunoprecipitation (CLIP) techniques. For these experiments, target RNAs are chemically crosslinked to Argonaute by UV irradiation and coimmunoprecipitated from total lysates (Pasquinelli 2012, Hafner et al. 2010, Zisoulis et al. 2010). Bound RNAs can then be analyzed by high-throughput sequencing and screened for miRNA binding sites in silico (HITS-CLIP or CLIP Seq). Moreover, the recently developed technique of crosslinking, ligation, and sequencing of hybrids (CLASH) allows direct allocation of miRNA and respective target (Helwak et al. 2013). Actual effects on mRNA molecules can be concluded from their translation status, which can be determined by polysome profiling or global analysis of ribosome occupancy by ribosome profiling (Eulalio et al. 2008, Ingolia et al. 2009). Taken together, the methods described above provide a good set of tools to identify interesting miRNA target candidates (Jovanovic et al. 2010). Nonetheless, these interactions have to be tested for functional relevance. A major obstacle for validations in vivo is the fact that many miRNAs exist as members of families that can potentially repress the same targets. This functional redundancy might be one of the reasons for the lack

of obvious phenotypes in many miRNA knockouts. An elegant solution for this issue will be the targeted mutation of endogenous miRNA binding sites in a gene of interest using TALENS or the CRISPR/Cas9 system (Hsu et al. 2014, Kim et al. 2013). This will allow to determine both dimension and physiological impact of the targeting.

The *let-7* network in *C. elegans*

As described above, *let-7* acts as a regulator of differentiation in *C. elegans*. Analysis by Northern Blot has shown increasing expression of the mature miRNA from the early L3 stage on, reaching a plateau at the mid L4 stage (Reinhart et al. 2000, Esquela-Kerscher et al. 2005). This expression pattern coincides with the regulatory functions of *let-7* during the L4 stage. *let-7* mutants display a defect in seam cell differentiation at the L4 to adult transition. Instead of fusing with their neighbors to a lateral seam cell syncytium, some seam cells undergo an additional round of division, which results in increased seam cell numbers at the young adult stage and production of a larval specific cuticle without alae. These phenotypes are shared between *let-7* null mutants and the temperature sensitive *let-7(n2853)* allele. Moreover, *let-7* function is essential for viability, since both null mutants as well as the temperature-sensitive strain at the restrictive temperature of 25°C die by bursting through the newly formed vulva at the L4 to adult transition (Reinhart et al. 2000). Interestingly, the *let-7(n2853)*ts strain can be maintained at 15°C, where it shows abnormalities in seam cell differentiation and an egg-laying defect, but only infrequent bursting in about 40% of the animals (Reinhart et al. 2000). While *let-7(mn112)* null mutants that fail to produce any mature miRNA carry a longer deletion disrupting the stem-loop formation, it was discovered that the *let-7(n2853)* allele harbors a single G to A transition in the seed sequence of the mature miRNA. Expression of *let-7* is reduced, but not absent in these animals.

The first target of *let-7* to be identified was *lin-41*, which encodes a member of the conserved TRIM/NHL protein family of ubiquitin ligases. The family name derives from the tripartide motif (TRIM) of RING finger, B-box and coiled-coil domain, and the NHL domain , which was first identified in *C. elegans* NCL-1, human HT2A2 and *C. elegans* LIN-41 (Slack et al. 2000, Slack and Ruvkun 1998). Soon after its appearance on stage as a target of *let-7* in *C. elegans*, it was found to be repressed by the *let-7* homologues of zebrafish, mouse, chicken, human and fly, revealing a conservation not only of *let-7* and *lin-41*, but also of the relationship between miRNA and target (Kloosterman et al. 2004, Kanamoto et al. 2006, Lin et al. 2007, O'Farrell et al. 2008). Early studies in *C. elegans* identified LIN-41 as a repressor of the transcription factor

LIN-29, which can be detected at the protein level only from L4 stage on, even though the corresponding RNA is transcribed earlier (Rougvie and Ambros 1995, Bettinger et al. 1996). Therefore, LIN-41 was proposed to act as a translational repressor of *lin-29*, yet the mechanism remained elusive (Slack et al. 2000). More recently, two studies could demonstrate translational repression of a luciferase reporter mediated by the 3'UTR of identified LIN-41 targets in mouse and human (Chang et al. 2012, Loedige et al. 2013). This repression was attributed to the coiled-coil and filamin domains of the protein, while RNA binding crucially depended on the NHL repeats (Loedige et al. 2013). On the other hand, the TRIM domain of LIN-41 is found in a large group of mammalian proteins, multiple of which possess E3 ubiquitin ligase activity (Meroni and Diez-Roux 2005). Indeed, a study in mouse identified the Argonaute protein Ago2 as a target for mLin41 mediated ubiquitylation in stem cells , which was followed by degradation of Ago2 (Rybak et al. 2009). This suggested the interesting possibility of a counter-regulation between a miRNA target and the miRNA machinery. However, the physiological relevance of the Ago ubiquitylation remains elusive, and Ago destabilization could not be reproduced in recent studies (Chang et al. 2012, Loedige et al. 2013). Furthermore, the Drosophila LIN-41 homologue dappled/wech lacks the RING domain responsible for ubiquitylation (O'Farrell et al. 2008). In contrast to the functions of LIN-41, the requirements for the binding of *let-7* to the 3'UTR of *lin-41* have been extensively studied. Six *let-7* binding sites were predicted in silico based on partial sequence complementarity, and careful experimental validation identified 2 conserved *let-7* complementary sites (LCSs) as the major effectors of repression (Vella et al. 2004). LIN-41 was shown to inhibit hypodermis differentiation through repression of the terminal heterochronic factor LIN-29 on a posttranscriptional level. Expression of LIN-29 protein at the late L4 stage depends on *let-7*-mediated repression of *lin-41* (Slack et al. 2000). Despite this clear linear explanation of the *let-7* phenotypes, additional genes were identified as potential *let-7* targets by the presence of *let-7* binding sites in their 3'UTR. RNAi mediated knockdown of the transcription factors *daf-12, pha-4, lss-4, die-1* and *hbl-1* (Abrahante et al. 2003, Lin et al. 2003, Grosshans et al. 2005) was shown to rescue the lethal phenotype of *let-7* null mutants, pointing indeed to a *let-7* mediated regulation of these genes. Moreover, tissue-specific repression of several of these genes by *let-7* could be shown. The connection between the early and the late timer of the heterochronic pathway was identified with the discovery of additional *let-7* family members, the so called "let-7 sisters" *mir-48*, *mir-84* and *mir-241* (Lau et al. 2001). miRNAs of the same family share the seed sequence with their siblings and are thought to regulate the same targets. In *C. elegans* however, the *let-7* sisters are expressed earlier than *let-7* and show reiteration of the L2 specific seam cell division if present as a triple mutant (Abbott et al. 2005, Esquela-Kerscher et al. 2005), placing them upstream in the heterochronic pathway (Figure 4). Indeed,

the sisters were shown to target the transcription factor HBL-1 during L3 stage, thereby blocking repetition of the L2 specific seam cell division pattern (Abbott et al. 2005). This finding completes our view of the heterochronic pathway as a carefully timed succession of three miRNA triggers that repress stage-specific factors after their time of action to allow progression to the next developmental step (Ambros 2011).

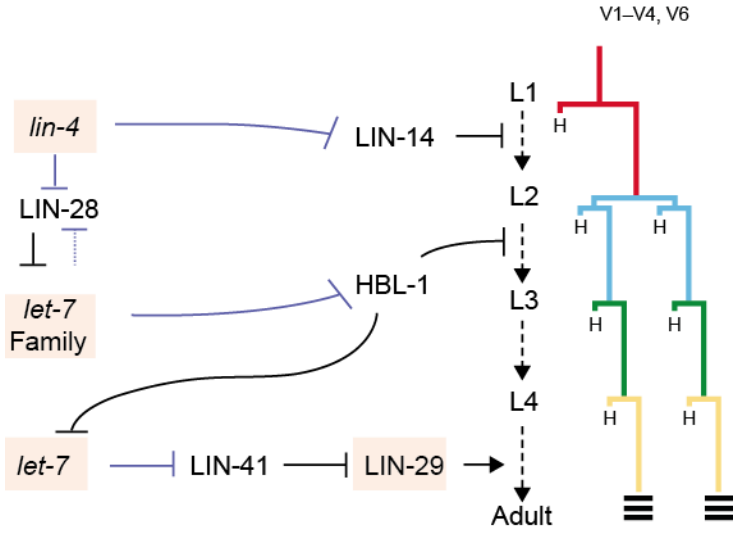

Figure 4 Simplified model of the heterochronic pathway

Major effectors of the heterochronic pathway and their effect on division of the V1-V4 and V6 subset of seam cells during larval development. LIN-14 is required in L1 stage but needs to be repressed by *lin-4* to permit progression to L2. The combination of symmetric plus asymmetric devision characteristic for L2 stage requires activity of HBL-1 and LIN-28, while progression to L3 depends on the *let-7* family members *mir-48*, *mir-84*, and *mir-241*. Finally, differentiation of the seam cells at the L4 to adult transition is mediated by posttranscriptional repression of *lin-41* by *let-7*, which allows activation of the transcription factor LIN-29. Factors in red boxes show retarded phenotypes if mutated (modified from Ambros 2011 and Rougvie 2001).

let-7 activity is regulated at multiple levels

The heterochronic function of *let-7* crucially depends on its temporally controlled activity. *let-7* is expressed in the form of two alternative primary transcripts that are subject to trans-splicing to the SL1 splice leader, resulting in the most abundant pri-*let-7* form (Bracht et al. 2004). Fusion of GFP to *the let-7* promoter reveals strong promoter activity in the seam cells as well as cells of the pharynx and intestine, muscles, and neurons from the L4 stage onwards (Johnson et al. 2003). This expression depends on the presence of a temporal regulatory element (TRE) as well as a second *let-7* transcription element (LTE) in the *let-7* promoter (Johnson et al. 2003, Kai et al. 2013).Surprisingly and in contrast to the expression pattern of the mature miRNA, low levels of pri- *let-7* can be detected by Northern Blot from the L1 stage

onwards (Kai et al. 2013). Maturation of *let-7* at these stages is repressed through binding of the heterochronic factor LIN-28, which recruits the poly(U) polymerase PUP-2 to target *let-7* for degradation (Lehrbach et al. 2009, Van Wynsberghe et al. 2011, Vadla et al. 2012). Moreover, full transcriptional activity of the promoter is repressed by the heterochronic gene HBL-1 until the end of L3 stage (Roush and Slack 2009). Hence, the heterochronic pathway itself controls the onset of activity of its late master-regulator *let-7*. Subsequent accumulation of mature *let-7* allows targeting of the *C. elegans* miRNA specific Argonaute ALG-1 to a conserved complementary sequence in the *let-7* precursor, which facilitates pri-*let-7* processing (Zisoulis et al. 2012). This creates a positive feedback loop that ensures robust *let-7* expression at the correct time point. In addition to transcription and processing, *let-7* function is controlled at the level of mature miRNA by active turnover. Both the exonuclease XRN-2 as well as the decapping enzyme DCS-1 in complex with the XRN-2 homolog XRN-1 have been shown to degrade mature *let-7* as well as other miRNAs in *C. elegans* (Chatterjee and Grosshans 2009, Bosse et al. 2013). Taken together, the many layers that control its expression imply that correct timing of *let-7* activity is of utmost importance for *C. elegans*. Indeed, artificial expression of *let-7* during earlier larval stages induces precocious seam cell fusion and alae formation, as well as egg-laying defects (Hayes and Ruvkun 2006).

The antiproliferative function of *let-7* is highly conserved in other organisms

let-7 and the heterochronic pathway of *C. elegans* are a beautiful example of posttranscriptional regulation of tissue development. Therefore, since its discovery *let-7* has gained enormous attention beyond the worm community. The absolute sequence conservation of mature *let-7* throughout most of the animal kingdom suggested an important function for the miRNA in all higher eukaryotes (Pasquinelli et al. 2000). Moreover, conservation of *let-7* targets such as *lin-41* (Slack et al. 2000, Kanamoto et al. 2006), *lin-28* (Moss and Tang 2003) and Ras (Johnson et al. 2005) as well as its temporal expression immediately pointed to a common role in the control of development. Indeed, *let-7* was soon found to be involved in a variety of developmental contexts in multiple species, such as mouse epithelial progenitor cell differentiation (Ibarra et al. 2007) or differentiation of neuronal stem cells (Rybak et al. 2008).Moreover, the effect of LIN-28 on *let-7* maturation turned out to be a second highly conserved aspect of the *let-7* pathway (Heo et al. 2008, Heo et al. 2009, Piskounova et al. 2011, Heo et al. 2012). It has become increasingly clear that *let-7* and its target *lin-28* play antagonistic roles in the control of stemness, LIN-28 acting as a pluripotency factor and *let-7* acting as its repressor. A study by Melton and

coworkers identified the *let-7* family miRNAs as the crucial factors to allow differentiation of mouse embryonic stem cells (ESCs) in the absence of canonical miRNA maturation in a Dgcr8$^{-/-}$ background (Melton et al. 2010, Shyh-Chang and Daley 2013, Wang et al. 2007). Moreover, they found *let-7* to antagonize the ESC cell cycle regulating (ESCC) miRNAs, an opposing class of stem cell specific miRNAs which are required for cell cycle progression and stem cell proliferation, and which were shown to support dedifferentiation (Judson et al. 2009, Wang et al. 2008). The authors could demonstrate repression of the pluripotency factors Lin28, N-myc and Sall4 by *let-7* using luciferase reporters, thereby offering an explanation for the antiproliferative function of *let-7*. Further insight on the downstream mechanism was gained when Trim71, the homologue of the *let-7* target LIN-41 in mouse, was found to repress Cdkn1a in concert with Ago2 and the ESSC miRNAs miR-290 and miR-302 (Chang et al. 2012). The cyclin-dependent kinase inhibitor Cdkn1a acts as a negative regulator of the transition from G1 to S phase of the cell cycle, thereby slowing ESC proliferation (Wang et al. 2008). Moreover, identification of the transcription factors and cell cycle regulators RBL1 and RBL2 as TRIM71 targets on the mRNA level by Loedige and colleagues provided a second line of evidence for the role of TRIM71/LIN-41 as a cell cycle promoting factor in mouse ES cells (Loedige et al. 2013). Recently, it was shown that knockdown of *let-7* can increase the efficiency of human induced pluripotent stem cell (iPSC) reprogramming, and that overcoming this "*let-7* barrier" largely depends on upregulation of LIN-41, unveiling LIN-41 a key downstream factor of *let-7* in ES cell differentiation (Worringer et al. 2014).

In line with its role as an inhibitor of proliferation and stemness, reduced *let-7* expression was found in lung cancer samples and associated with shorter survival of the patients (Takamizawa et al. 2004). Up to date, reduced *let-7* expression has been reported in numerous types of cancer (Bussing et al. 2008). Notably, the role of LIN28 in neuroblastoma formation through repression of *let-7* cements the importance of *let-7* as a tumor suppressor (Molenaar et al. 2012). A molecular explanation for this function of *let-7* was given when a number of potent oncogenes were discovered as *let-7* targets. Johnson and coworkers were able to demonstrate repression of two human RAS isoforms as well as the *C. elegans* homolog *let-60* by *let-7* through conserved binding sites in the 3'UTR of these genes (Johnson et al. 2005). Soon thereafter, MYC and HMGA2 were identified as a *let-7* targets in human Burkitt lymphoma and lung cancer (Sampson et al. 2007, Lee and Dutta 2007). Moreover, a study in a lung cancer cell line could detect a direct effect of *let-7* on cell cycle progression, which was accompanied by expression changes of a large number of cell cycle factors, among them CDK-6 and CDC25A (Johnson et al. 2007).

In summary, the microRNA *let-7* as well as its targets have been established as important regulators of development and differentiation in countless eukaryotic species. The work presented here characterizes the *let-7* regulatory network in the model organism *Caenorhabditis elegans*. Moreover, the high conservation of the pathway allows conclusions for other organisms, making this work a resource for *let-7* related research all the way up to humans.

Results

Project 1: A genome wide RNAi screen for characterization of the *let-7* network

Motivation and contributions

Numerous studies over the last decade have highlighted the importance of miRNA regulation both in development and disease (Ha and Kim 2014, Mendell and Olson 2012). Nonetheless, while function and effects of miRNAs have been extensively studied, little was known about the mechanisms that ensure the right dose of miRNA activity when I started my PhD. Therefore I decided to perform a genome-wide RNAi screen for suppressors of the lethal *let-7* bursting phenotype in *C. elegans*. A main goal was to identify potential negative regulators of *let-7* function, which might even emerge as general suppressors of miRNA activity. The existence of such negative regulators was highlighted by the study of a postdoctoral fellow in our lab who demonstrated in vitro that the exonuclease XRN-2 degrades mature *let-7* molecules, proven by in vitro rescue of *let-7* bursting upon knockdown of *xrn-2* (Chatterjee and Grosshans 2009). Nonetheless, the screen was also designed in a way that would allow to uncover novel players of the *let-7* pathway acting downstream of, or parallel to, *let-7*.

I performed the initial screening of the genome-wide RNAi library, validation of suppressors and analysis of the candidates in the context of the reporter system for hypodermis differentiation, as well as generation of the in vivo *let-7* target reporter and analysis of the suppressors in this system together with Matyas Ecsedi, a fellow PhD student. I then analyzed the suppressors for their effect on repression of endogenous *let-7* targets by qPCR and determined *let-7* levels upon suppressor knockdown by northern blot. Furthermore, I performed and analyzed the microarray experiments for gene expression in *let-7* null mutants. In-depth analysis of the suppressors CDK-1 and CDC-25.2 was performed by Hrishikesh Bartake.

Developmental Biology ■ (■■■■) ■■■–■■■

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

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

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ARTICLE INFO

Article history:
Received 5 January 2015
Received in revised form
13 February 2015
Accepted 17 February 2015

Keywords:
let-7
microRNA
C. elegans
Heterochronic
Developmental timing
LIN-29
CDK-1
NCC-1
CDC-25.2
Condensin
Condensin II complex
Hypodermis
Vulva
col-19
miRNA regulation
miRNA target
Cell cycle
Differentiation

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.

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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http://dx.doi.org/10.1016/j.ydbio.2015.02.013
0012-1606/

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

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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.

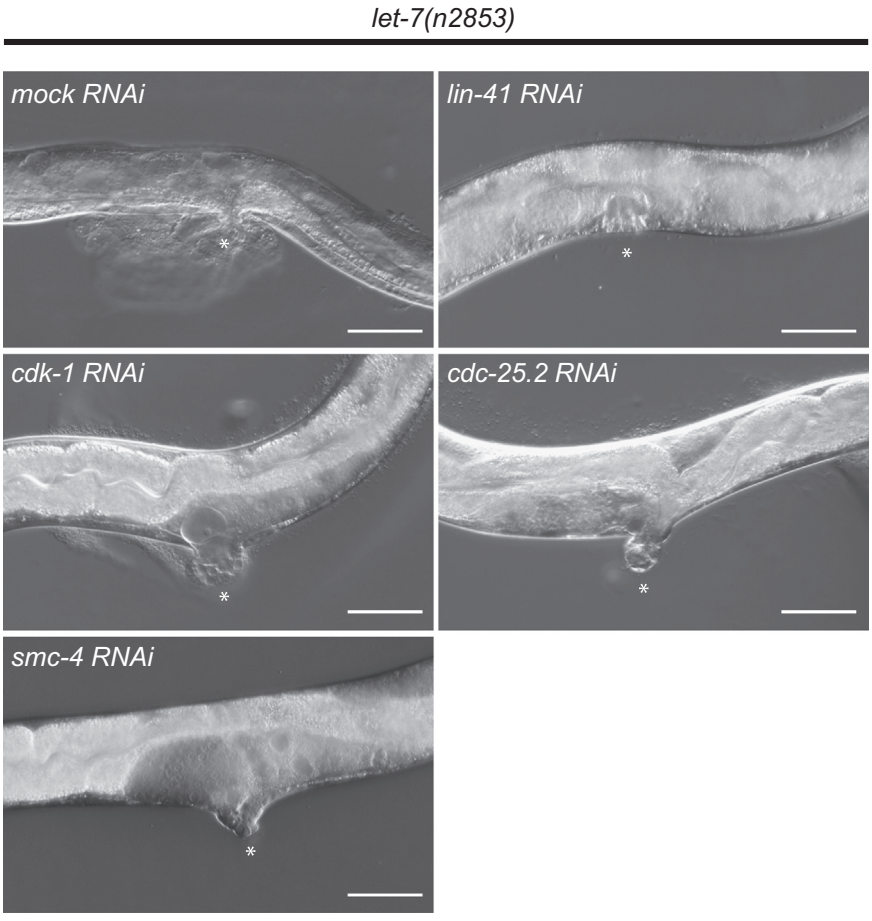

Fig. 1. A genome-wide RNAi screen for suppressors of *let-7(n2853)* bursting. Knock-down of the indicated suppressors by RNAi rescues bursting of *let-7(n2853)* worms. Vulvae are marked with asterisks. Scale bar indicates 50 μm.

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.

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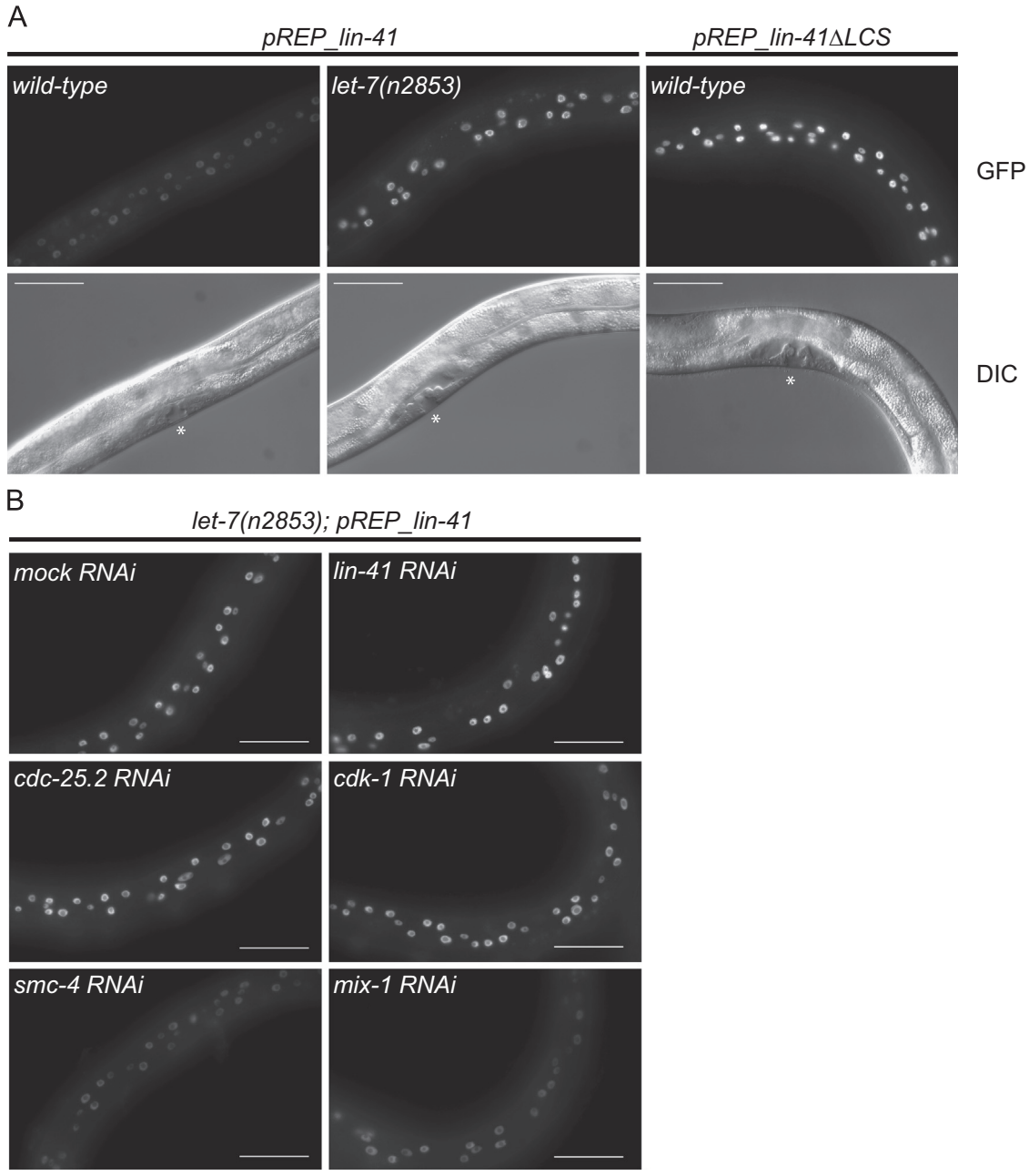

Fig. 2. *let-7* suppressor RNAi restores repression of a *let-7* target reporter. (A) Repression of a *let-7* target reporter (*Pwrt-2::gfp::lin-41 3′UTR*, "*pREP_lin-41*") in late L4 worms depends on *let-7* and is lost upon mutation of the *let-7* complementary sites (*pREP_lin-41ΔLCS*). Vulvae are 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

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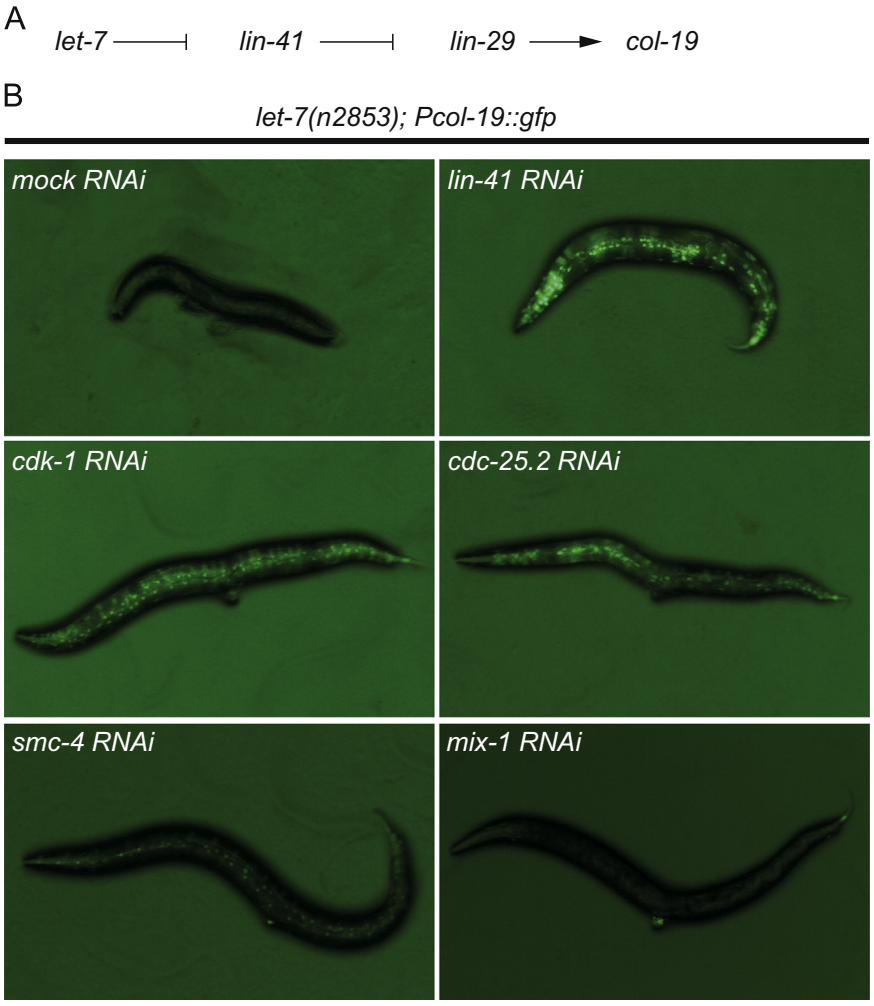

Fig. 3. RNAi of *let-7(n2853)* suppressors restores hypodermis differentiation. (A) Activation of the adult-specific *col-19* promoter is controlled by *let-7* through activation of the transcription factor LIN-29. (B) Expression of *col-19::gfp* in *let-7(n2853)* worms subjected to the indicated RNAi; 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 $= 5.1 \times 10^{-10}$, 'col-19 positives': p-Value $= 9.9 \times 10^{-6}$, 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

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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.

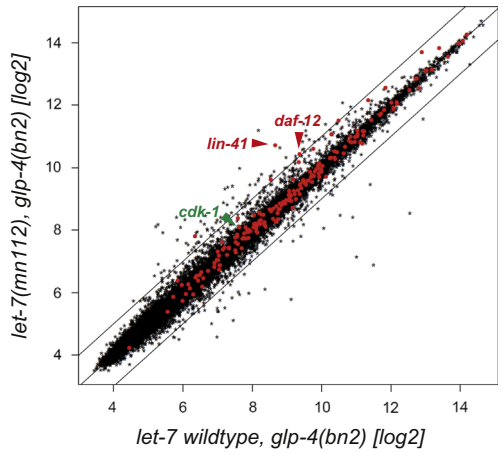

Fig. 4. Expression levels of novel *let-7* suppressors are not affected in *let-7* mutants. Microarray analysis of somatic gene expression in *let-7(mn112)* null mutant in germline-less *glp-4(bn2)* animals shows no changes in mRNA levels for genes identified as suppressors of the *let-7(n2853)* bursting 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

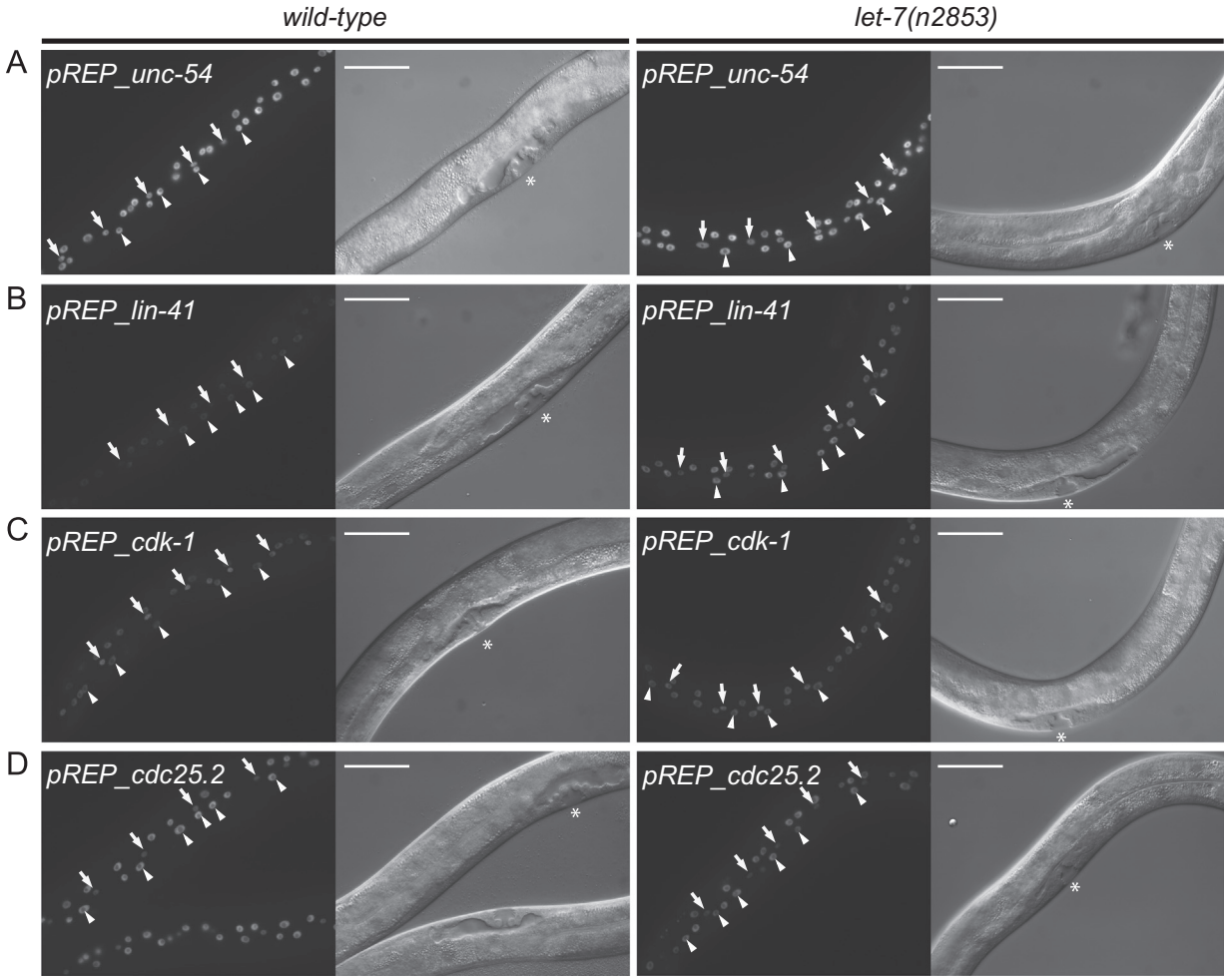

Fig. 5. The 3′UTRs of *cdk-1* and *cdc-25.2* do not confer *let-7*-dependent regulation. (A) A hypodermis specific target reporter (*wrt-2* promoter) containing *gfp* fused to the unregulated *unc-54* 3′UTR (*pREP_unc-54*) is expressed both in wild-type and *let-7(n2853)* background at the late L4 stage. (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.

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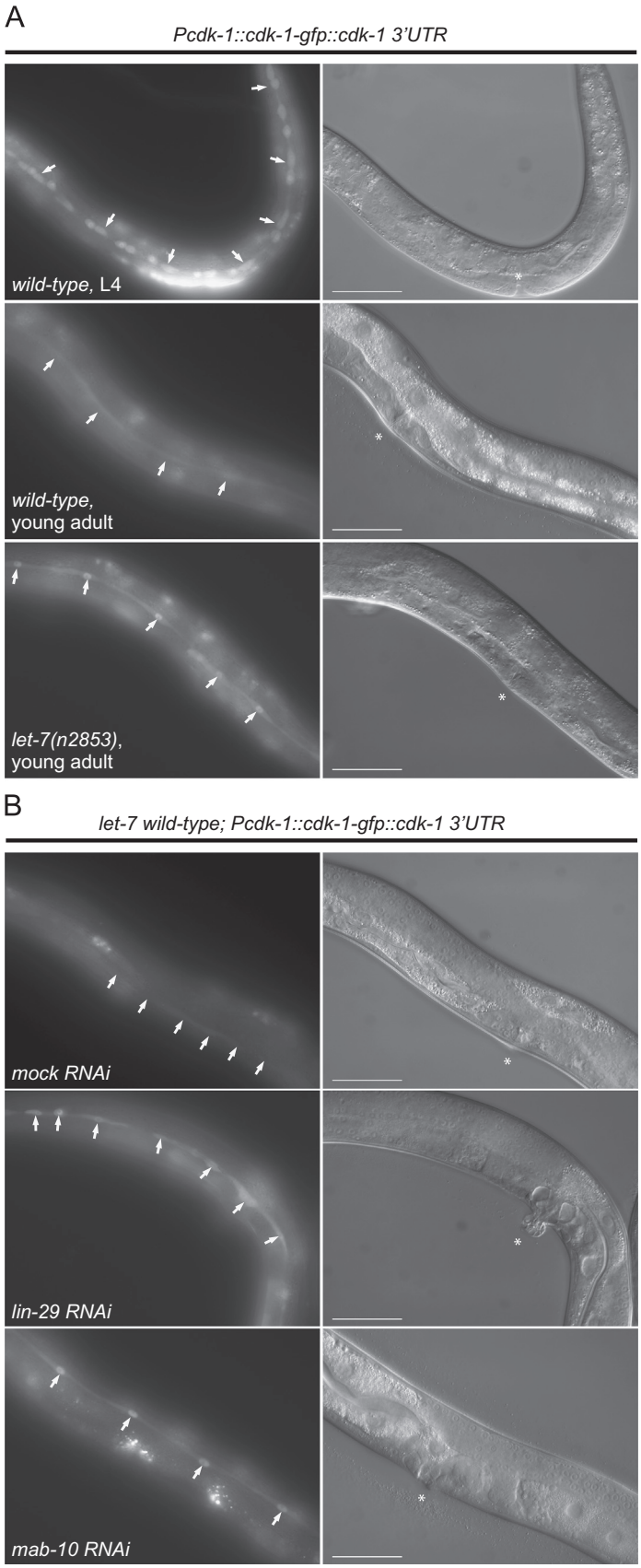

Fig. 6. Repression of *cdk-1::gfp* depends on LIN-29 and MAB-10 (A) Expression of *cdk-1::gfp* from the *cdk-1* promoter can be observed in seam cells (arrows) until the L4 stage. GFP levels decrease during L4 stage in wild-type background. *let-7(n2853)* mutant animals continue to express *cdk-1::gfp* 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.

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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

We thank Stéphane Thiry and Dimos Gaidatzis for performing the microarray hybridization experiment and help with its analysis, respectively, Dimos Gaidatzis and Sarah Carl for help with the ALG-1 target analysis, Craig Mello for the *cdk-1::gfp* strain, and Iskra Katic and Takashi Miki for a critical reading of the manuscript. Some strains were provided by the CGC, which is funded by NIH, United States Office of Research Infrastructure Programs (P40 OD010440). The research leading to these results has received funding from the European Union Seventh Framework Programme, Belgium (FP7/2007-2013) under Grant agreement no 241985 (European Research Council 'miRTurn'), and from the Novartis Research Foundation through the FMI Foundation. M. E. was an SNSF/SAMS Swiss MD/PhD-program fellow supported by an Oncosuisse fellowship.

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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Please cite this article as: Rausch, M., et al., A genetic interactome of the *let-7* microRNA in *C. elegans*. Dev. Biol. (2015), http://dx.doi.org/10.1016/j.ydbio.2015.02.013

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*]

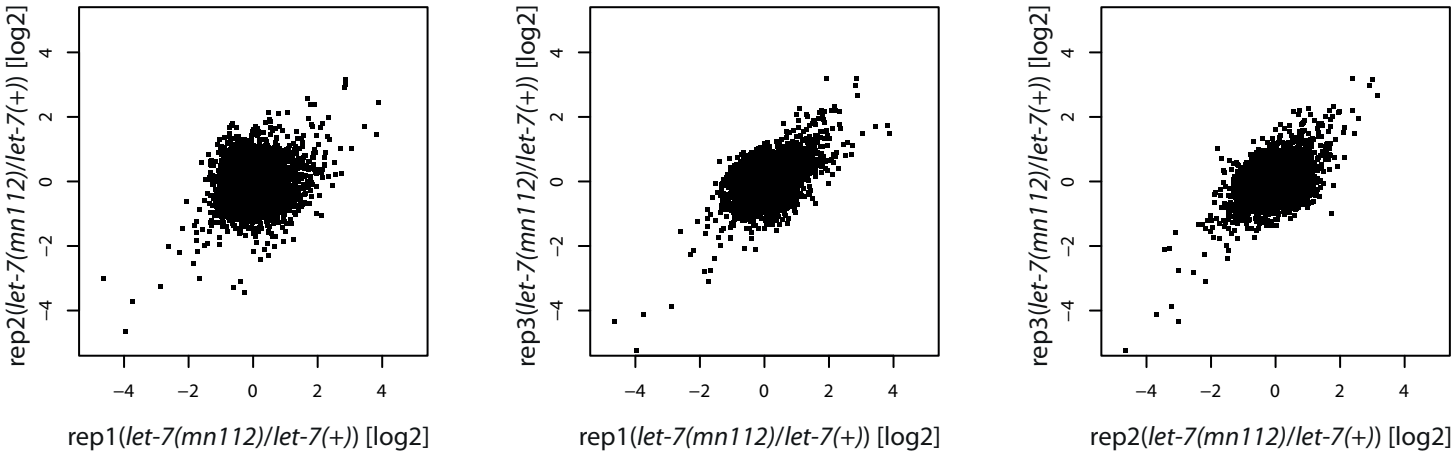


Fig. S1, Replicate correlation of gene expression profiling by microarray

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.

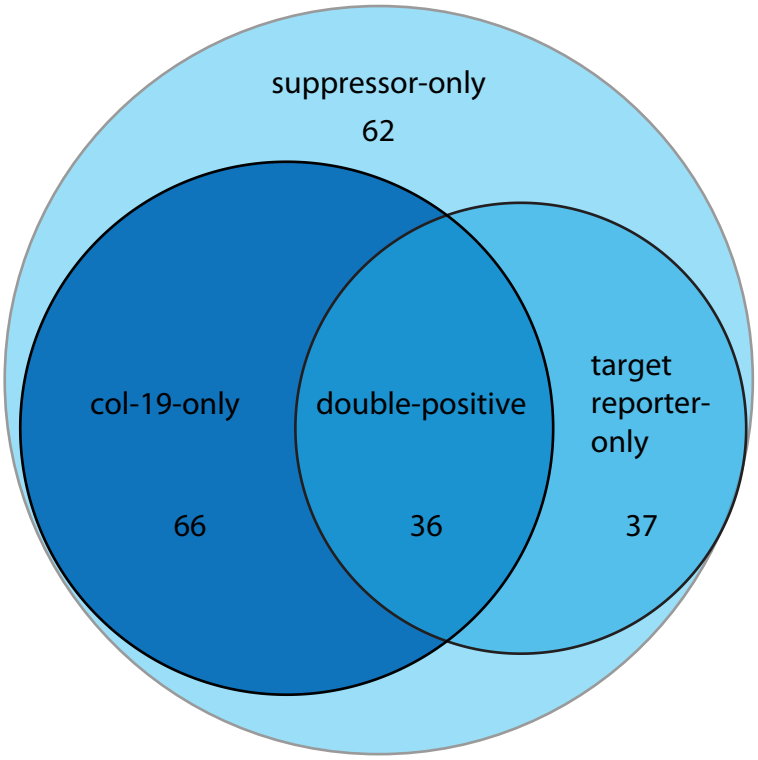


Fig. S2, The suppressors of *let-7(n2853)* can be grouped into four classes

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 S1: Suppressors of *let-7(n2853)* lethal bursting phenotype

Shown are all genes which upon knockdown by RNAi suppressed the *let-7(n2853)* bursting phenotype.
Suppression was defined as ≥20% worms alive after 48h incubation at 25 °C or 66h incubation at 20°C.

GenePairs Name	Wormbase ID	Gene Name	Wormbase Concise Description	Wormbase conserved orthologous group	Found in Ding et al. 2008	Chromosome	second retest: [% burst]@ 25°C	second retest: [% burst] @ 20°C	Pcol-19::gfp - penetrance @25°C, t1	Pcol-19::gfp - penetrance @25°C, t2	Pcol-19::gfp - penetrance @20°C, t1	Pcol-19::gfp - penetrance @20°C, t2	Pwrt-2::gfp::lin-41 repression	Pwrt-2::gfp::daf-12 repression	Pwrt-2::gfp::unc-54 repression
Y54E10A_15	WBGene00000413	cdt-1	cdt-1 encodes the C. elegans ortholog of the replication-licensing factor Cdt1; CDT-1 activity is essential for DNA rep		+	I	20.3	36.9	++	+++	+	+++	+		+
C03D6.1	WBGene00000466	cel-1	cel-1 encodes a mRNA capping enzyme, with a N-terminal reg	mRNA capping enzyme	+	I	58.1	92.5	---	---	---	---	+	+	
B0511.10	WBGene00001228	eif-3.E	eif-3.E encodes the C. elegans ortholog of the translation initiation factor 3 subunit e (eIF3e/Int-6); by homology, EIF		+	I	6.9	33.3	---	---	---	---	+++	+++	+++
B0511.10	WBGene00001228	eif-3.E	eif-3.E encodes the C. elegans ortholog of the translation initiation factor 3 subunit e (eIF3e/Int-6); by homology, EIF		+	I	24.1	52.4	---	---	---	---	+++	+++	+++
C41D11.2	WBGene00001231	eif-3.H			+	I	70.3	92.3	---	---	---	---	+	++	+
C47B2.5	WBGene00001234	eif-6	eif-6 encodes the C. elegans ortholog of vertebrate anti-assoc	EUKARYOTIC TRANSLATION INITIATION FACTOR 6 (EIF	+	I	50.0	60.3	---	---	---	---	+		
T08B2.9	WBGene00001497	frs-1	frs-1 encodes a predicted phenylalanyl-t-RNA synthetase.	phenylalanyl-tRNA synthetase	-	I	44.1	51.6	---	---	---	---	+	+	++
ZK484.2	WBGene00001819	haf-9	haf-9 is orthologous to the human gene ATP-BINDING CASSET	transporter protein	-	I	92.6	77.1	---	---	---	++	---		
T03F1.9	WBGene00001832	hcp-4	The hcp-4 gene encodes a centromere protein (CENP)-C homolog,	holocentric protein (HCP)-4.	+	I	39.6	73.2	+	++	---	---	++		+
Y110A7A.1	WBGene00001833	hcp-6	hcp-6 encodes a HEAT motif-containing protein that displays similarity to the XCAP-D2/CNAP1/Cnd1/YCS4 family of proteins that a			I	33.3	46.6	---	++	---	+++	++	+++	+
F28B3.7	WBGene00001860	him-1	him-1 encodes a homolog of the conserved eukaryotic protein, SMC1, a member of the SMC (structural maintenance of chromosor			I	51.8	69.8	++	+++	---	+++	+		
Y48G1A_54.	WBGene00002079	xpo-2	imb-5 encodes an importin-beta-like protein orthologous to m	importin beta, nuclear transport factor	+	I	52.0	94.4	+	+	---	++	+	+	
Y48G1A_54.	WBGene00002079				+	I	37.7	84.0	---	---	+	+	---	++	
C01G8.7	WBGene00002717	let-526	let-526 encodes a homolog of a component of the SWI/SNF complex; the let-526(h185) mutation results in early lan		+	I	37.9	58.0	+	++	+	++	---		
C01G8.8	WBGene00002717				+	I	76.0	58.6	+	++	---	+	---		
F57B10.1	WBGene00002783	let-607			+	I	32.5		---	---	---	---	---	+	
C12C8.3	WBGene00003026	lin-41	lin-41 encodes a novel RBCC (Ring finger-B box-Coiled coil) protein that is a member of the NHL (NCL-1, HT2A, and L		+	I	13.2	16.7	+++	+++	+++	+++	---		
T19A6.2	WBGene00003596	ngp-1		AUTOANTIGEN NGP-1	+	I	47.6	51.0	---	---	---	---	---	+	
F56A3.3	WBGene00003792	npp-6	npp-6 encodes a protein with weak similarity to mouse nuclear	pore complex protein Nup160, and affects embryon	+	I	54.7	30.2	+	++	---	+	+++	+++	+
T19B4.2	WBGene00003793	npp-7			+	I	20.6	17.1	---	---	---	---	---	++	
Y71F9A_27	WBGene00003836	nxt-1			+	I	58.9	69.9	+	+	---	---	---		
Y106G6H.2	WBGene00003902	pab-1	pab-1 encodes a polyadenylate-binding protein (i.e., poly(A)-b	RNA recognition motif. (aka RRM, RBD, or RNP domai	+	I	11.5	100.0	---	---	---	+	---		
W02D9.1	WBGene00004181	pri-2	pri-2 encodes a homolog of the DNA polymerase alpha-prima	DNA primase 58 KD subunit	+	I	29.9	51.6	+	+	---	+	++	+	+
C03D6.8	WBGene00004437	rpl-24.2	rpl-24.2 encodes a large ribosomal subunit L24 protein paralo	ribosomal protein	+	I	31.0	59.5	---	---	---	---	---		
K12C11.2	WBGene00004888	smo-1	smo-1 encodes the C. elegans ortholog of SUMO, a small ubiquitin-like moiety that, when attached to protein substr		+	I	45.5	88.6	---	---	---	---/Burst	---		
F56A3.4	WBGene00004955	spd-5	spd-5 encodes a coiled-coil domain protein; spd-5 activity is required for centrosome maturation, symmetry breakin		+	I	52.4	47.6	+	++	---	++	---		
Y63D3A.5	WBGene00006565	tfg-1			+	I	21.2	30.8	---	---	---	---	+++	++	++
C36B1.3	WBGene00007971	rpb-3		DNA directed RNA polymerase II	+	I	0.0	21.0	---	---	---	---	++	+++	
Y53C10A.3	WBGene00008670	F11A3.2		guanine nucleotide exchange factor	-	I	84.8	78.9	---	---	---	---	+	++	+
F14B4.3	WBGene00008781	F14B4.3		DNA-directed RNA polymerase I	+	I	68.8	84.4	---	---	---	---	---		
F14B4.3	WBGene00008781	rpoa-2			+	I	57.8	69.2	---	---	---	---	---		
M04C9.6	WBGene00008878	ppfr-1			-	I	92.5	44.2	---	---	+++	+++	---		
F20G4.1	WBGene00008990	smgl-1			+	I	21.3	34.0	---	---	---	---	---		
F58H10.1	WBGene00010291	F58H10.1			-	I	82.1	42.2	++	+++	++	+++	---		
K04G2.1	WBGene00010560	iftb-1	iftb-1 encodes the C. elegans ortholog of translation initiation	translational initiation factor 2 beta subunit	-	I		37.5	---	---	---	---	+++	++	+++
K07A1.2	WBGene00010609	dut-1	dut-1 encodes a deoxyuridine triphosphate nucleotidohydrola	deoxyuridine 5'-triphosphate nucleotidohydrolase	-	I	74.5	96.6	---	---	---	++	+		
R06C7.5	WBGene00011064		The R06C7.5 gene encodes an ortholog of the human gene AD	adenylosuccinate lyase	-	I	60.4	61.9	2/3 burst, rest +	++	---	+	---		
T23D8.3	WBGene00011944	T23D8.3	T23D8.3 encodes an ortholog of yeast and human LTV1 that inhibits DHC-1 in vivo; in mass RNAi assays, T23D8.3 is r		+	I	45.7	62.5	---	---	---	---	---	+	
W04A4.6	WBGene00012234	W04A4.5			+	I	43.9		---	---	---	---	+	++	
W06H12.1	WBGene00012317	ztf-6			-	I	94.1	57.9	---	---	++	+++	---		
Y105E8C.d	WBGene00013676	ekl-4			-	I	92.9	17.9	+	+	+++	+++	---		
B0511.6	WBGene00015232	B0511.6	B0511.6 encodes a DEAD-box helicase; loss of B0511.6 activity	helicase	+	I	11.6	0.0	---	---	---	---	---		
C43E11.9	WBGene00016607	C43E11.9			-	I	39.5	76.7	---	+	---	+	---		
C53H9.2	WBGene00016907	C53H9.2	C53H9.2 encodes at least three protein isoforms orthologous to the GTP-binding proteins human GNL1 (HSR1; OMIM		+	I	32.0	32.2	---	---	---	---	+	+	
F55A12.8	WBGene00018866	F55A12.8			-	I	39.6	28.1	---	---	---	---	---		
F55F8.3	WBGene00018891	F55F8.3			-	I	10.7	23.8	---	---	---	---	---		
H27M09.2	WBGene00019246	rpb-5	H27M09.2 encodes the C.elegans ortholog of RPB5 which, in e	RNA polymerase	-	I	5.9	14.0	---	---	---	---	++	++	++
K06A5.4	WBGene00019432	knl-2			-	I	20.0	46.6	---	++	---	++	+++	++	
T09B4.9	WBGene00020383	T09B4.9			+	I	50.0	48.7	---	---	---	---	---	+	
W01B11.3	WBGene00020915	nol-5			+	I	100.0	0.0	---	---	---	---	---		
Y48G1A_54.	WBGene00021660	Y48G1A.4			+	I	61.7	77.7	---	---	---	---	---	+	
Y54E10A.10	WBGene00021830				-	I	70.7	49.6	---	---	---	---	---		
Y54E10BR.5	WBGene00021844				-	I	54.7	43.8	---	---	---	---	---		
Y54E10B_15	WBGene00021845	rpb-7			+	I	79.5	56.5	---	+	---	+	++	+	
Y65B4B_10.	WBGene00022042	Y65B4BR.5			+	I	73.2	73.8	---	---	---	---	---		
Y110A7A.m	WBGene00022458	Y110A7A	Y110A7A.8 is orthologous to the human gene U4/U6 SNRNP-A	mRNA splicing protein	-	I	66.7	38.6	---	---	---	---	---	++	
ZC581.1	WBGene00022631	nekl-2	nekl-2 encodes a putative serine/threonine protein kinase ort	ser/thr-protein kinase	+	I	39.5	11.9	---	---	---	---	---		
ZC581.1	WBGene00022631	nekl-2	nekl-2 encodes a putative serine/threonine protein kinase orthologous to Aspergillus nidulans nimA and human NEK		+	I	39.2	47.7	---	--(Burst-	---	---	---		
M106.5	WBGene00000293	cap-2	The beta subunit of actin capping protein that regulates actin	F-actin capping protein beta subunit	na	II	41.0	87.2	---	---	---	---	---		
R06F6.1	WBGene00000411	cdl-1	cdl-1 encodes a homolog of human hairpin (stem-loop) binding proteins (HBP/SLBP) that bind to the hairpin (stem-lo		na	II	16.7	16.1	---	+	---	+++	---		
F33A8.3	WBGene00000472	cey-1	cey-1 encodes a protein with a cold-shock/Y-box domain that	'Cold-shock' DNA-binding domain	na	II	91.1	26.1	---	---	+++	+++	---		
F11G11.10	WBGene00000606	col-17	col-17 encodes a collagen which is expressed in all developme	collagen	na	II	95.3	24.0	---, burst	---	---	++	---		
R53.3	WBGene00001207	egl-43	egl-43 encodes a zinc finger protein that affects HSN cell migr	Zinc finger, C2H2 type (4 domains)	na	II	68.8	23.8	+	+	---	++	---		
M110.4	WBGene00002066	ifg-1	ifg-1 encodes, by alternative splicing, two orthologs of the tra	eukaryotic initiation factor	na	II	36.4	13.2	---	---	---	---	+++	++	+++
Y17G7A.2	WBGene00003015	lin-29	lin-29 encodes a zinc finger transcription factor of the C2H2 ty	transcription factor lin-29	na	II	88.0	54.5	---	---	---	---	---		
Y17G7B.5	WBGene00003154	mcm-2		MCM2/3/5 family	na	II	58.3	43.3	+	++	++	+++	---		
R06F6.10	WBGene00003367	mix-1	The mix-1 gene encodes a homolog of SMC2 involved in chron	mitotic chromosome and X-chromosome associated N	na	II	13.8	8.8	---	---	---	++	+++		
K12D12.2	WBGene00003789	npp-3	npp-3 encodes a nucleoporin that is a homolog of vertebrate	Nup205; npp-3 is required for normal nuclear pore cor	na	II	18.9	0.0	---	+	---	+	---	++	
K01C8.9	WBGene00003821	nst-1	nst-1 encodes a homolog of human GNL3 (OMIM:608011, nuc	GTP-binding protein	na	II	72.7	60.9	---	---	---	---	---	++	
F10C1.5	WBGene00004015	phb-2	phb-2 encodes one of two subunits of the mitochondrial proh	Prohibitin	na	II	27.0	44.3	---	---	---	---	+++	+++	++
W07E6.4	WBGene00004188	prp-21		splicing factor	na	II	48.8	12.5	+	+	---	++	---	+	

ZK1290.3	WBGene00004398	rol-8	rol-8 encodes a collagen that is enriched in dauer larvae; mut	Cutical collagen 6, col-6	na	II	93.1	30.0	---	---	---	++	---			
C09H10.2	WBGene00004454	rpl-41	rpl-41 encodes a large ribosomal subunit L41 protein; by hom	60S ribosomal protein	na	II		12.2					++	++		+++
C08B11.5	WBGene00004723	sap-49	sap-49 encodes the C. elegans ortholog of mammalian SAP49,	Poly(A) RNA binding protein	na	II	51.7	45.2	---	---	---	+	---			
B0491.2	WBGene00005016	sqt-1	sqt-1 encodes a cuticle collagen; during larval and adult devel	cuticle collagen SQT-1	na	II		63.6	---	---	---	++	---			
C01B12.1	WBGene00005017	sqt-2	sqt-2 encodes a collagen required for normal alae formation a	cuticular collagen	na	II	75.9	80.8	---	---			---			
F10G7.1	WBGene00006497	tag-151			na	II	23.8	84.4	---	+	---	---	---			
C56E6.1	WBGene00006522	abcx-1			na	II	43.9	48.5	---	---	---	---	---			
D2085.3	WBGene00008428	D2085.3	D2085.3 is orthologous to the human gene UNKNOWN (PROT	translation initiation factor (EIF)	na	II	21.8	48.2	---	---	---	---	++	+++		+
F44G4.1	WBGene00009711	F44G4.1			na	II	66.2	87.2	---	---	---	+	+			
R03D7.1	WBGene00010988	metr-1	R03D7.1 is orthologous to the human gene METHIONINE SYN	5-methyltetrahydrofolate-homocysteine methyltransl	na	II		67.0	---	burst	---	+	---			
W03H9.4	WBGene00012230	cacn-1	cacn-1 encodes an ortholog of Drosophila CACTIN and human	C19orf29 that is required for normal distal tip cell mig	na	II	40.9	60.0	---	---	---	---	++	+		
Y48B6A.1	WBGene00012978	Y48B6A.1	Y48B6A.1 encodes an ortholog of human BOP1 (OMIM:610596,	overexpressed in colon cancer) and S. cerevisiae ERI	na	II	31.3	36.4	---	---	---	+	---			
C01F1.3	WBGene00015298				na	II	54.1		---	---	---	+	++	++		++
F40H3.5	WBGene00015941	C18A3.3			na	II	57.8	30.8	---	---	---	---	---	++		
C18A3.3	WBGene00015941				na	II	63.0	55.7	---	---	---	---	++	++		+++
C44B7.3	WBGene00016625	aff-1	aff-1 encodes a cell-surface protein required in late L4 larvae for various cell fusions, of which at least one (AC-utse)		na	II	74.7	66.7	+	+	+	++	---			
F18A1.5	WBGene00017546	rpa-1	rpa-1 encodes the C. elegans ortholog of the replication prote	replication factor A	na	II	8.7	0.0	---	+	---	+	---	++		
F47F6.1	WBGene00018572	lin-42	lin-42 encodes three PAS domain-containing proteins orthologous to insect and vertebrate Period proteins that func		na	II	22.6	0.0	+++	+++	+++	+++	---			
F47F6.2	WBGene00018572				na	II		4.2	+++	+++	+++	+++	---			
F54A3_31.e	WBGene00018782	cct-3	cct-3 encodes a putative gamma subunit of the eukaryotic cytosolic ('T complex') chaperonin, orthologous to humar		na	II	23.9	45.7	---	---	---	---	++	+		+
C32D5.11	WBGene00018869	F55C12.1			na	II		66.7	---	---	+	+	---			
R153.1	WBGene00020114	pde-4	pde-4 encodes a cAMP phosphodiesterase orthologous to Dro	cyclic nucleotide phosphodiesterase	na	II	100.0	59.4	---	---	+	+++	---			
Y51H7B_5.b	WBGene00022042				na	II	53.4	81.0	---	---	---	---	---			
ZK430.1	WBGene00022739	toe-1			na	II	22.0	33.3	---	---	---	+	---	+		
T12C9.2	WBGene00022739				na	II	54.2	84.9	---	---	---	---	---			
ZK430.7	WBGene00022742	ZK430.7		ribosomal processing protein	na	II	69.8	27.5	---	---	---	---	+			
ZK1127.5	WBGene00022852	ZK1127.5			na	II	60.3	77.5	---	---	---	+	---			
F54H12.1	WBGene00000041	aco-2	aco-2 encodes an aconitase homolog that is required for emb	Aconitate hydratase	na	III	56.3	85.0	+	++	---	++	++			
F57B9.5	WBGene00000276	byn-1	byn-1 encodes a homolog of mammalian BYSTIN-LIKE (BYSL; OMIM:603871).		na	III	47.4	83.8	---	---	---	+	---			
T05G5.3	WBGene00000405	cdk-1	cdk-1 encodes a cyclin-dependent kinase, orthologous to and	serine/threonine kinase (CDC2/CDKX subfamily)	na	III	54.9	14.7	++	++	+	+++	---			
R08D7.3	WBGene00001227	eif-3.D	eif-3.D encodes the C. elegans ortholog of the translation initiation factor 3 subunit d (eIF3d/Moe1); by homology, E		na	III	24.1	52.4	---	---	---	---	+++	+++		++
W07B3.2	WBGene00001561	gei-4	gei-4 encodes a protein with a coiled-coil domain (predicted to mediate protein-protein interactions), an internal re		na	III	88.1	72.5	---	+	+	+++	---			
T20B12.8	WBGene00001974	hmg-4	hmg-4 encodes a protein with strong similarity to the highly conserved high mobility group protein SSRP1 (structure		na	III	23.7	15.9	---	---	---	---	+++	++		++
Y22D7AL.5	WBGene00002025	hsp-60	hsp-60 encodes a nuclear-encoded mitochondrion-specific chaperone that is a member of the GroE/Hsp10/60 super		na	III	89.6	79.0	---	---	---	---	---	++		
C02F5.1	WBGene00002231	knl-1	knl-1 encodes a novel acidic protein with a coiled-coil region at its C-terminus; KNL-1 is an essential kinetochore com		na	III	55.6	76.7	---	+	---	+++	+			+
C16A3.3	WBGene00002850	let-716			na	III	11.5	66.3	---	---	---	---	---			
C29E4.8	WBGene00002879	let-754	let-754 was identified in screens for ethyl methane sulfonate-	Adenylate kinase	na	III	70.0	90.9	---	---	---	---	---			
C07H6.7	WBGene00003024	lin-39	lin-39 encodes a homeodomain protein homologous to the De	C. elegans homeobox gene lin-39	na	III	68.1	68.9	+	++	+	+++	---			
R13A5.12	WBGene00003063	lpd-7	lpd-7 encodes a BRCT domain-containing protein that is orthologous to Saccharomyces cerevisiae Nop7p and the ve		na	III	32.1	30.2	---	---	---	---	++			+
ZK632.1	WBGene00003158	mcm-6	mcm-6 encodes a protein that has similarity to human DNA re	Mcm2/3	na	III	35.1	7.7	++	++	++	+++	---			
F59A2.1	WBGene00003795	npp-9		Nucleoporin	na	III		86.4	---	+	---	---	++	++		
K06H7.1	WBGene00004042	plk-1	plk-1 encodes a serine/threonine polo-like kinase homologous	Protein kinase	na	III	57.8	64.3	+	+	---	+++	---	+		
C14B9.4	WBGene00004042				na	III	40.0	65.8	+	++	---	++	+	+		
F58A4.4	WBGene00004180	pri-1	pri-1 encodes a homolog of the DNA polymerase alpha-prima	DNA primase 49Kd subunit	na	III		71.4	---	+	---	+++	++			+
F58A4.4	WBGene00004180	pri-1	pri-1 encodes a homolog of the DNA polymerase alpha-primase	subunit D that is required in embryos for the norma	na	III	37.1	59.0	---	++	---	++	+++	++		
K04G7.10	WBGene00004390	rnp-7		SN-RNP U1	na	III	11.1	47.5	---	---	---	---	+			
	WBGene00004391	rnr-1	rnr-1 encodes the large subunit of ribonucleotide reductase; b	Ribonucleoside-disphosphate reductase large chain	na	III	49.3	65.9	++	++	---	+++	---			
C54C6.1	WBGene00004451	rpl-37	rpl-37 encodes a large ribosomal subunit L37 protein.	60S ribosomal protein L37	na	III	20.5	36.4	---	---	---	---	++	++		++
Y47D3A.26	WBGene00004873	smc-3			na	III	38.7	55.6	+	+++	++	+++	++	+		+++
F35G12.8	WBGene00004874	smc-4	The smc-4 gene encodes a homolog of the SMC4 subunit of m	chromosome segregation protein	na	III	39.8	74.4	---	++	---	++	++			
ZK652.1	WBGene00004918	snr-5	snr-5 encodes an ortholog of human small nuclear ribonucleo	small nuclear ribonucleoprotein Sm F (snRNP core prc	na	III	23.1	30.4	---	+	+	+	---			
T27E9.1	WBGene00006439	ant-1.1	ant-1.1 encodes one of four C. elegans mitochondrial adenine	ADP/ATP carrier protein	na	III	4.3	17.1	---	---	---	---	---			
T27E9.1	WBGene00006439	ant-1.1	ant-1.1 encodes one of four C. elegans mitochondrial adenine	nucleotide transporters; by homology, ANT-1.1 is pre	na	III	1.1	22.3	---	---	---	---	---			
Y92C3B.2	WBGene00006697	uaf-1	uaf-1 encodes the large subunit of splicing factor U2AF (U2	Auxiliary Factor), orthologous to mammalian and Drosop	na	III	22.1	10.8	---	---	---	++	++	++		
R10E11.2	WBGene00006911	vha-2	vha-2 and vha-3 encode an ortholog of subunit c of the memb	Vacuolar ATP synthase subunit	na	III	79.2	80.2	---	---	---	---	+	+		
Y111B2C.m	WBGene00007030	epc-1		enhancer of polycomb-like	na	III	87.8	79.8	++	++ (many burst)	+++	+++	---			
Y111B2A.11	WBGene00007030	epc-1			na	III	18.9	13.2	---	---	---	---	---			
M01F1.7	WBGene00010813	pitp-1	pitp-1 encodes a phosphatidylinositol transfer protein, with a	RETINAL DEGENERATION B PROTEIN (PROBABLE CALC	na	III		70.6	---	---	---	---	++	+++		+++
T04A8.6	WBGene00011408	T04A8.6	T04A8.6 encodes an ortholog of S. cerevisiae NOP15 that may	ribonuleoprotein	na	III	63.0	71.8	---	---	---	---	---			
	WBGene00012059	T26G10.1		RNA helicase	na	III	22.0	27.6	---	---	---	---	---			
	WBGene00012936	Y47D3A.2	Y47D3A.29 encodes the catalytic subunit of DNA polymerase a	DNA polymerase alpha subunit	na	III	14.0	46.8	---	+	---	---	---	++		
Y49E10.19	WBGene00013038	ani-1	ani-1 encodes one of three C. elegans anillins; ANI-1 activity is	PH (pleckstrin homology) domain	na	III	69.7	30.0	+	+	---	++	+			
C16A3.4	WBGene00015809	C16A3.4			na	III	72.0	65.9	---	(but many have burs	---	---	---	++		
C16A3.6	WBGene00015811				na	III	54.2	82.6	---	---	---	---	---			
C23G10.8	WBGene00016015	C23G10.8		S. pombe adenylyl cyclase-associated protein (weak)	na	III	57.0	81.9	---	+	+	+	---			
C23G10.9	WBGene00016015				na	III	88.2	86.7	---	+	+	---	---			
C29E4.2	WBGene00016202	kle-2			na	III	44.2	54.7	+	+++	---	+++	+			
F09F7.3	WBGene00017300	F09F7.3			na	III	17.4	21.7	+	+	---	---	---			
F26F4.11	WBGene00017830	rpb-8			na	III	42.9	60.6	---	---	---	---	++			
F37C12.13	WBGene00018154	exos-9			na	III	32.4	62.7	---	---	---	---	---			
H06I04.3	WBGene00019168				na	III	36.6	17.0					---			
H06I04.3	WBGene00019168				na	III	38.6	31.3	---	---	---	---	---	+		
K12H4.3	WBGene00019678	K12H4.3			na	III	38.0	18.8	---	+	---	---	---	+		
K12H4.4	WBGene00019679	K12H4.4		Signal peptidase	na	III	80.3	48.2	---	---	---	---	---			
F57H12.1	WBGene00000183	arf-3	arf-3 encodes a member of the ADP-ribosylation factor relate	GTP-binding protein	na	IV	69.6	92.5	---	---	---	---	+	++		
Y41E3.2	WBGene00001066	dpy-4	dpy-4 encodes a cuticle collagen; dpy-4 activity is required for	CUTICLE COLLAGEN 1	na	IV	36.8	8.1	---	---	---	++				
F35H10.1	WBGene00001904	his-30	his-30 encodes an H2A histone; by homology, HIS-30 is predic	histone H2A	na	IV	60.7	73.3	---	---	---	---	+++	+++		+++
T11G6.1	WBGene00002001	hars-1	hrs-1 encodes predicted cytoplasmic and mitochondrial histid	histidyl-tRNA synthetase (cytoplasmic), histidyl-tRNA	na	IV	42.3	60.6	---	---	---	---	---			
F32E10.4	WBGene00002074	ima-3	ima-3 encodes one of three C. elegans importin alpha nuclear	importin alpha, nuclear transport factor	na	IV	69.4	93.5	---	---	---	---	---			

M7.1	WBGene00002344	let-70	let-70 encodes a class I E2 ubiquitin conjugating enzyme; let-7	ubiquitin-conjugating enzyme E2-17 KD	na	IV	82.5	64.5	+	++	+	++	---			
F36H1.4	WBGene00002992	lin-3	lin-3 encodes a member of the EGF family of peptide growth f	lin-3 growth factor precursor	na	IV	23.8	23.1	+	+	+	+++	---			
C08F8.8	WBGene00003657	nhr-67	nhr-67 encodes a nuclear receptor that is orthologous to Dros	nuclear hormone receptor	na	IV	1.9	3.5	---	---	---	++	---			
Y41D4A_30	WBGene00003794	npp-8			na	IV	4.3	56.3	---	---	---	+	++	++		++
Y41D4A_30	WBGene00003794				na	IV	11.3	2.4	---	---	---	+	++	++		+++
Y41D4A_34	WBGene00003794				na	IV	2.3	2.4	---	---	---	---	+	++		+
Y41D4A_34	WBGene00003794				na	IV	17.3		---	---	---	---	+++	+++		++
T23F6.4	WBGene00004315	rbd-1	rbd-1 encodes an ortholog of S. cerevisiae Mrd1p; RBD-1 is re	RNA recognition motif. (aka RRM, RBD, or RNP domai	na	IV	74.0	85.7	---	---	---	+	++	+		++
Y62E10A.d	WBGene00004410	rla-2	rpa-2 encodes an acidic ribosomal subunit protein P2.		na	IV	69.0	48.2	---	---	---	---	+			++
C42D4.8	WBGene00004411	rpc-1		DNA-directed RNA polymerase II largest subunit	na	IV	31.0	75.6	---	---	---	---	---	++		
K11H12.12	WBGene00004427	rpl-15	rpl-15 encodes a large ribosomal subunit L15 protein.	60S ribosomal protein L15	na	IV	23.1	31.3	---	---	---	---	++	++		+++
K08E4.1	WBGene00005015	spt-5		translation initiation protein SPT5 like	na	IV	1.3	24.7	---	---	---	---	+++	+++		+
M03D4.1	WBGene00006974	zen-4	zen-4 encodes a kinesin-like protein that is a member of the k	kinesin-like protein	na	IV	30.2	49.1	+	+	---	+++	---			
C33D9.3	WBGene00007898	C33D9.3			na	IV	88.1	58.3	burst	burst	+(mostly burst)	+++(mostly burst)	---			
C47E12.4	WBGene00008149	pyp-1	pyp-1 encodes, by alternative splicing, three isoforms of an in	inorganic pyrophosphatase	na	IV	47.9	66.2	+	+	+	++	+			
C47E12.7	WBGene00008151	C47E12.7		Yeast YD78 like	na	IV	61.7	45.2	---	+	---	---	---	+		
F28D1.1	WBGene00009211	F28D1.1		Yeast hypothetical protein YER2 like	na	IV	64.4	73.7	---	---	---	---	---			
F40F11.2	WBGene00009587	F40F11.2			na	IV	15.2	69.8	++	++	---	---	++	++		+++
M18.5	WBGene00010890	ddb-1	M18.5 is orthologous to the human gene DAMAGE-SPECIFIC D	DNA repair protein like	na	IV	32.7	93.8	---	---	---	---/Burst	+	+		
T11G6.8	WBGene00011722			RNA recognition motif. (aka RRM, RBD, or RNP domai	na	IV	55.7	38.1	---	---	---	---	---	++		
Y45F10D.8	WBGene00012887	Y45F10D	Y45F10D.7 encodes a WD40 repeat-containing protein that is the	C. elegans ortholog of human WDR36, mutations i	na	IV	80.0	95.2	---	---	---	---	---			
C42C1.3	WBGene00016581	C42C1.3			na	IV	26.3	86.0	---	---	---	---	---			
H06H21.3	WBGene00019162	eif-1.A		translation initiation factor	na	IV	45.3	60.2	---	---	---	---	+	+		+
T22D1.10	WBGene00020687	ruvb-2			na	IV	69.4	82.8	---	+	---	+	++	+		
Y55F3A_75(	WBGene00021934	cct-8	cct-8 encodes a putative theta subunit of the eukaryotic cytosolic ('T complex') chaperonin, orthologous to human C		na	IV	2.9	35.4	---	---	---	---	---	+		
Y55F3A_75(	WBGene00021934				na	IV	0.0	45.5	---	---	---	---	---			
F29G9.3	WBGene00000159	aps-1	aps-1 encodes an adaptin: specifically, it encodes an ortholog	clathrin coat assembly protein	na	V	20.0	55.9	---	---	---	---	++			
K11C4.5	WBGene00000159				na	V	6.7	43.8	---	---	---	---	---			
F29G9.3	WBGene00000159	aps-1	aps-1 encodes an adaptin: specifically, it encodes an ortholog of the sigma1 subunit of adaptor protein complex 1 (A		na	V	16.7	57.5	---	---	---	---	+	+		
K07C5.1	WBGene00000200	arx-2	arx-2 encodes the C. elegans ortholog of the Arp2 subunit of t	actin	na	V	60.9	80.9	---	---/Burst	---	+++/or burst	---			
F16B4.8	WBGene00000387	cdc-25.2	cdc-25.2 encodes a putative homolog of Cdc25 phosphatase p	protein-tyrosine phosphatase	na	V	14.3	65.5	+	+	+	+++	---			
T06E6.2	WBGene00000868	cyb-3	cyb-3 encodes a member of the cyclin B family that is requirec	cyclin B like	na	V	49.2	31.4	+	++	---	+++	++			
F29G9.4	WBGene00001345	fos-1	fos-1 encodes two basic region-leucine zipper (bZip) transcript	BZIP transcription factor	na	V	47.5	52.7	+	++	---	+++	---			
C53A5.3	WBGene00001834	hda-1	The hda-1 gene encodes a histone deacetylase 1, similar to his	Yeast RPD3 protein like	na	V	42.9	80.9	---	---	---	---	---			
ZK742.1	WBGene00002078	xpo-1	xpo-1 encodes exportin-1, an importin-beta-like protein ortho	importin beta, nuclear transport factor	na	V	40.5	71.2	---	---	---	---	---			
Y73C8B.b	WBGene00002246	lag-2	lag-2 encodes a transmembrane protein of the Delta/Serrate/	intercellular signal protein	na	V	28.6	95.0	+	+	+	++	---			
C29A12.3	WBGene00002985	lig-1	lig-1 is orthologous to the human gene LIM HOMEOBOX PROT	DNA ligase I	na	V	49.0	52.2	+	++	---	+++	+			
F32D1.10	WBGene00003159	mcm-7		cell division control protein	na	V	21.4	53.2	++	+++	---	+++	---			
F38A6.1	WBGene00004013	pha-4	pha-4 encodes a FoxA transcription factor; during embryonic (	Fork head domain, eukaryotic transcription factors	na	V	4.1		+	+	---	+	---			
F18E2.3	WBGene00004738	scc-3	scc-3 encodes a cohesin complex subunit homologous to Saccharomyces cerevisiae Irr1p/Scc3p; SCC-3 is required du		na	V	16.7	39.6	+	+++	---	+++	---	+		
F23H12.4	WBGene00005018	sqt-3	sqt-3 encodes a cuticle collagen; during development, sqt-3 ac	collagen	na	V	95.0	40.3	---	Burst	---	+++	---			
D1054.15	WBGene00006481	plrg-1		beta transducin like protein	na	V	9.2		---	---	---	---	+	++		+++
Y80D3A.a	WBGene00006945	wars-1	wrs-1 encodes a predicted tryptophanyl-tRNA synthetase that	tryptophanyl-tRNA synthetase	na	V	48.7	72.2	---	---	---	---	---	+		
B0250.7	WBGene00007124	B0250.7			na	V	48.6	93.1	---	---	---	---	++	++		+++
C14C10.3	WBGene00007586	ril-2			na	V	32.0	77.1	---	---	---	---	+	+		
C15H11.9	WBGene00007617	rrbs-1		Human HA0609 protein like	na	V	27.8	10.5	---	---	---	---	---	+		
C27H6.2	WBGene00007784	ruvb-1	ruvb-1 encodes a AAA+ ATPase orthologous to the RUVBL1 fa	Yeast hypothetical 50.5 KD protein like	na	V	42.9	73.0	---	---	---	---	+	+		
F11A3.2	WBGene00008670				na	V	22.6	95.5	---	---	---	---	+	+		
C50F4.13	WBGene00008670				na	V	41.4	88.6	---	---	---	---	---	++		
F53B7.3	WBGene00009966	F53B7.3			na	V	55.2	21.2	---	---	---	---	---	++		
F53F4.11	WBGene00009993	F53F4.11			na	V	23.9	17.9	---	---	---	---	---			
F53F4.11	WBGene00009993				na	V	8.3	28.6	---	---	---	---	---			
F55C5.4	WBGene00010093	capg-2			na	V	12.5	26.5	---	++	---	+++	++	+		
F55C5.8	WBGene00010097	F55C5.8		signal recognition particle 68 KD protein	na	V	11.1	2.0	---	---	---	---	---			
K07C5.4	WBGene00010627	K07C5.4		yeast protein L8167.9-like	na	V	28.6	54.2	---	---	---	---	+			++
T06E6.1	WBGene00011538	T06E6.1			na	V	50.0	81.1	---	---	---	+	---	+		
C37H5.5	WBGene00016508	C37H5.5			na	V	54.3	85.9	---	+(many burst(	---	+(many burst)	---	+		
F09G2.4	WBGene00017313	cpsf-2		Cleavage and Polyadenylation Specificity Factor	na	V	56.7	16.2	---	---	---	+	++	++		
F25G6.2	WBGene00017797	symk-1			na	V	26.4	27.8	---	+	---	---	+	+		
F32D1.2	WBGene00017982	F32D1.2		ATP synthase epsilion chain	na	V	50.0	89.1	---	---	---	---	---			
T08B1.1	WBGene00020340	T08B1.1		sugar transporter	na	V		51.3	---/Burst	Burst	---	---	---			
T19A5.3	WBGene00020554				na	V	18.4		---	---	---	---	+	++		++
F08C6.1	WBGene00000083	adt-2		Thrombospondin	na	X	74.5		---	---	---	---	---			
C42D8.8	WBGene00000149	apl-1	apl-1 encodes two almost identical isoforms orthologous to human APP (OMIM:104760, mutated in familial Alzheim		na	X		9.1	---	---	---	---	+			
F18H3.5	WBGene00000406	cdk-4	cdk-4 encodes two isoforms of a cyclin-dependent serine/thre	serine/threonine kinase (CDC2/CDKX subfamily)	na	X	100.0	63.3	2/3 burst, rest	++ S	++(mostly burst)	+	+	---		
F11A1.3	WBGene00000908	daf-12	daf-12 encodes a member of the steroid hormone receptor superfamily that is homologous to human VITAMIN D RE		na	X	0.0	0.0	+++	+++	+++	+++	---			
C02C6.1	WBGene00001130	dyn-1	dyn-1 encodes the C. elegans ortholog of the dynamin GTPase	dynamin	na	X	10.8		---	---	---	---	+	++		
W01C8.2	WBGene00001182	egl-13	egl-13 encodes a SOX domain transcription factor; egl-13 is required for maintenance of the uterine pi	cell fate; mut	na	X	28.9	83.6	+	+++	+++	+++	---			
C33D3.1	WBGene00001250	elt-2	elt-2 encodes a GATA-type transcription factor most similar to	zinc finger protein (GATA type)	na	X	76.2	7.0	---	---	---	---	---			
F52D10.3	WBGene00001502	ftt-2		14-3-3 protein	na	X	82.9	42.9	+	+	++	+(also many burst)	---			
F13D11.2	WBGene00001824	hbl-1	hbl-1 encodes a C2H2-type zinc finger transcription factor rela	C2H2-type zinc finger	na	X	34.1	6.5	++	+++	+++	+++	---			
F46F2.2	WBGene00002203	kin-20		casein kinase I (epsilon/delta)	na	X	38.8	9.1	+	++	++	++	++	+		+
C17G1.6	WBGene00003553	nas-37	nas-37 encodes a Astacin-class metalloprotease required for f	zinc metalloprotease	na	X		62.7	---	---	---	---	---			
F11C1.6	WBGene00003623	nhr-25	nhr-25 encodes a nuclear hormone receptor orthologous to D	nuclear hormone receptor	na	X	43.1	13.2	+++	+++	+++	+++	+	+		
C49F5.1	WBGene00008205	sams-1	sams-1 encodes an S-adenosyl methionine synthetase; by hon	s-adenosylmethionine synthetase	na	X	39.2	85.4	---	---	---	---	---			

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	---	+	---	

Table S3: *let-7* suppressor genes that restore expression of the hypodermal differentiation marker *col-19::gfp* ('col-19 posi

Suppressors of *let-7(n2853)* bursting phenotype were screened for rescue of activation of the *Pcol-19::gfp* reporter in *let-7(n2853)* worms. Worms grown at 25°C were analyzed after 48h and 56h, worms grown at 20°C were scored after 56h and 72h. Shown are genes that resulted in weak (+), medium (++) or strong (+++) activation of GFP upon RNAi at one or multiple time points.

Predicted Gene	Wormbase ID	*col-19* activation 25 °C: 49h	*col-19* activation 25 °C: 58h	*col-19* activation 20 °C: 56h	*col-19* activation 20 °C: 72h	function
cell cycle/ chromosome maintenance & segregation						
plk-1	WBGene00004042	+	++	---	+++	polo-like kinase
cyb-3	WBGene00000868	+	++	---	+++	Cyclin B
cdc-25.2	WBGene00000387	+	+	+	+++	Cdc25 phosphatase homolog
cdk-1	WBGene00000405	++	++	+	+++	cyclin-dependent kinase
cdk-4	WBGene00000406	++	++	+	+	cyclin-dependent kinase
smc-4	WBGene00004874	---	++	---	++	condensin II complex
capg-2	WBGene00010093	---	++	---	+++	condensin II complex
mix-1	WBGene00003367	---	---	---	++	condensin II complex
hcp-6	WBGene00001833	---	++	---	+++	condensin II complex
kle-2	WBGene00016202	+	+++	---	+++	condensin II complex
him-1	WBGene00001860	++	+++	---	+++	structural maintenance of chromosome family
hcp-4	WBGene00001832	+	++	---	---	CENP-C homolog
scc-3	WBGene00004738	+	+++	---	+++	cohesin subunit
smc-3	WBGene00004873	+	+++	++	+++	cohesin subunit
spd-5	WBGene00004955	+	++	---	++	centrosome
knl-1	WBGene00002231	---	+	---	+++	kinetochore associated
knl-2	WBGene00019432	---	++	---	++	kinetochore associated
collagens						
col-17	WBGene00000606	burst	---	---	++	collagen
dpy-4	WBGene00001066	---	---	---	++	collagen
rol-8	WBGene00004398	---	---	---	++	collagen
sqt-1	WBGene00005016	---	---	---	++	collagen
sqt-3	WBGene00005018	---	burst	---	+++	collagen
cytoskeleton						
zen-4	WBGene00006974	+	+	---	+++	kinesin, mitosis
ani-1	WBGene00013038	+	+	---	++	actin binding protein
arx-2	WBGene00000200	---	burst	---	+++	Arp2/3 complex
gei-4	WBGene00001561	---	+	+	+++	cytoskeleton rearrangement, vulva dev.
DNA/replication						
cdt-1	WBGene00000413	++	+++	+	+++	repl. licensing
mcm-2	WBGene00003154	+	++	++	+++	repl. licensing
mcm-6	WBGene00003158	++	++	++	+++	repl. licensing
mcm-7	WBGene00003159	++	+++	---	+++	repl. licensing
rnr-1	WBGene00004391	++	++	---	+++	ribonucleotide reductase
lig-1	WBGene00002985	+	++	---	+++	ligase
pri-1	WBGene00004180	---	+	---	+++	primase
pri-2	WBGene00004181	+	+	---	+	primase

ruvb-2	WBGene00020687	---	+	---	+	recombination protein homolog
rpa-1	WBGene00017546	---	+	---	+	replication protein A homolog
Y47D3A.29	WBGene00012936	---	+	---	---	DNA polymerase alpha subunit
mRNA biogenesis						
rpb-7	WBGene00021845	---	+	---	+	RNA Pol II subunit
cpsf-2	WBGene00017313	---	---	---	+	cleavage and polyadenylation
symk-1	WBGene00017797	---	+	---	---	cleavage and polyadenylation
sap-49	WBGene00004723	---	---	---	+	spliceosome associated
snr-5	WBGene00004918	---	+	+	+	splicing
prp-21	WBGene00004188	+	+	---	++	splicing factor
uaf-1	WBGene00006697	---	---	---	++	splicing factor
specific transcription						
lin-42	WBGene00018572	+++	+++	+++	+++	
daf-12	WBGene00000908	+++	+++	+++	+++	nuclear hormone receptor
nhr-25	WBGene00003623	+++	+++	+++	+++	nuclear hormone receptor
hbl-1	WBGene00001824	++	+++	+++	+++	zinc finger TF
egl-13	WBGene00001182	+	+++	+++	+++	SOX domain TF
lin-39	WBGene00003024	+	++	+	+++	homeodomain TF
fos-1	WBGene00001345	+	++	---	+++	FOS TF
ztf-6	WBGene00012317	---	---	++	+++	zinc finger TF
egl-43	WBGene00001207	+	+	---	++	zinc finger TF
pha-4	WBGene00004013	+	+	---	+	FoxA transcription factor
nhr-67	WBGene00003657	---	---	---	++	nuclear hormone receptor
nuclear transport						
ftt-2	WBGene00001502	+	+	++	+	nuclear trafficking?
xpo-2	WBGene00002079	+	+	---	++	nuclear export receptor
npp-6	WBGene00003792	+	++	---	+	nuclear pore protein
npp-3	WBGene00003789	---	+	---	+	nuclear pore protein
nxt-1	WBGene00003836	+	+	---	---	export protein
npp-8	WBGene00003794	---	---	---	+	nuclear pore protein
npp-9	WBGene00003795	---	+	---	---	nuclear pore protein
nucleosome remodelling						
epc-1	WBGene00007030	++	++	+++	+++	NuA4 histone acetyltransferase complex
ekl-4	WBGene00013676	+	+	+++	+++	NuA4 histone acetyltransferase complex
let-526	WBGene00002717	+	++	+	++	SWI/SNF complex
pyp-1	WBGene00008149	+	+	+	++	pyrophosphatase, nucleosome remodelling?
ribosome biogenesis						
rpc-2	WBGene00017300	+	+	---	---	RNA pol III
C37H5.5	WBGene00016508	---	+	---	+	nucleolar protein
C43E11.9	WBGene00016607	---	+	---	+	ribosome biogenesis
tag-151	WBGene00006497	---	+	---	---	ribosome biogenesis
rbd-1	WBGene00004315	---	---	---	+	ribosome biogenesis
Y48B6A.1	WBGene00012978	---	---	---	+	ribosome biogenesis, chromosome segregation?
K12H4.3	WBGene00019678	---	+	---	---	ribosome biogenesis
C47E12.7	WBGene00008151	---	+	---	---	ribosomal RNA processing protein 1 homolog
signaling						
kin-20	WBGene00002203	+	++	++	++	protein kinase
lag-2	WBGene00002246	+	+	+	++	notch ligand
lin-3	WBGene00002992	+	+	+	+++	EGF ligand
other						
lin-41	WBGene00003026	+++	+++	+++	+++	RBCC/NHL domain protein

F58H10.1	WBGene00010291	++	+++	++	+++	
let-70	WBGene00002344	+	++	+	++	class I E2 ubiquitin conjugating enzyme
aff-1	WBGene00016625	+	+	+	++	cell fusion
adsl-1	WBGene00011064	+	++	---	+	ADenyloSuccinate Lyase homologue
aco-2	WBGene00000041	+	++	---	++	aconitase
ppfr-1	WBGene00008878	---	---	+++	+++	protein phosphatase subunit
cey-1	WBGene00000472	---	---	+++	+++	Y-box protein, DNA binding?
mig-38	WBGene00009587	++	++	---	---	DTC migration
pde-4	WBGene00020114	---	---	+	+++	cAMP phosphodiesterase
C33D9.3	WBGene00007898	burst	burst	+	+++	
cdl-1	WBGene00000411	---	+	---	+++	histone mRNA binding
haf-9	WBGene00001819	---	---	---	++	HAlF transporter (PGP related)
dut-1	WBGene00010609	---	---	---	++	deoxyUTPase
C23G10.8	WBGene00016015	---	+	+	+	
F55C12.1	WBGene00018869	---	---	+	+	
byn-1	WBGene00000276	---	---	---	+	
metr-1	WBGene00010988	---	burst	---	+	methionine synthase
ZK1127.5	WBGene00022852	---	---	---	+	RNA 3'-terminal phosphate cyclase?
toe-1	WBGene00022739	---	---	---	+	
pab-1	WBGene00003902	---	---	---	+	cytoplasmic PolyA binding protein
F44G4.1	WBGene00009711	---	---	---	+	
T06E6.1	WBGene00011538	---	---	---	+	
C01F1.3	WBGene00015298	---	---	---	+	
C16A3.4	WBGene00015809	---	+	---	---	

Remarks and open questions

We decided to perform this screen for novel *let-7* pathway members on a genome-wide scale because we reasoned that this would, in contrast to a candidate screen, allow to identify important genes that had not yet been suggested to be involved in the regulation of *let-7* or its downstream targets. While survival as opposed to bursting is a rather obvious phenotype, which allowed us to determine the initial list of suppressors with high confidence, it became more challenging to analyze these for specificity with microscopy-based in vivo assays. Since we were faced with a list of 200 candidates, we chose to analyze these in reporter assays in a semi-quantitative manner, ensuring a degree of reliance through independent analysis by two different people. However, we know that the lack of an additional RFP-coupled unregulated 3'UTR reporter as an internal normalization control may have resulted in false positive hits for the target reporter for *let-7* activity, where most suppressors scoring positive had modest effects. We tried to circumvent this by testing the positive candidates on a reporter with an unregulated 3'UTR (pREP_unc-54) to identify factors that affect GFP accumulation in the nucleus independently of *let-7*. However, the *wrt-2* promoter that was used to drive expression of the reporters was later found to be subject to a strong cyclic expression (Hendriks et al. 2014), and slight developmental delays caused by RNAi might result in differences in GFP levels. We can therefore not exclude that the list of genes modulating *let-7* function still contains false positive hits.

RNAi	Wormbase ID	average fold change *daf-12* [log2]	average fold change *lin-41* [log2]	SEM *daf-12*	SEM *lin-41*
knl-2	WBGene00019432	-0.98166	-0.45999	n.d.	n.d.
smc-4	WBGene00004874	-0.90392	-0.4119	0.022	0.083
plk-1	WBGene00004042	-0.72998	-0.23548	n.d.	n.d.
xpo-2	WBGene00002079	-0.65523	-0.44108	0.182	0.064
cyb-3	WBGene00000868	-0.15599	-0.5744	0.144	0.034
plk-1	WBGene00004042	-0.79784	-0.52685	0.016	0.073
aps-1	WBGene00000159	-0.05461	-0.50284	n.d.	n.d.

Table 1 Effects of suppressor RNAi on endogenous *let-7* target levels.

Shown are top hits for repression of *daf-12* (yellow) and *lin-41* (red). Note that two individual clones scored positive for *plk-1*, one of which affected both *lin-41* and *daf-12*. Experiments were performed in duplicates unless SEM indicated as "n.d.". SEM=standard error of the mean.

Nonetheless, the target reporter assay identified a number of interesting candidates. I decided to proceed by testing the effect of the positive candidates on expression levels of the *let-7* targets *lin-41* and *daf-12*,

which should ideally mirror the *let-7* dependent downregulation of the reporter. To my surprise, only few of the tested suppressors showed a significant effect on repression of endogenous *lin-41* or *daf-12* upon knockdown. Hence, it remains to be determined whether this signals a tissue-specific effect of the involved suppressors, or whether they constitute false positive hits. If the suppressors affect *let-7* activity and thereby *lin-41* levels only in the hypodermis or with moderate impact, the effects of RNAi might be masked by *lin-41* expression in the germline, which is strongly activated during the L4 stage (Tocchini et al. 2014). At any rate, those suppressors that affect endogenous *let-7* target levels still contain interesting candidates, including known heterochronic factors as well as novel candidates such as genes involved in cell cycle regulation (Table 1). Unfortunately, I could not detect effects on mature *let-7* levels for any of the candidates by northern blot. Therefore, it remains to be determined how these genes affect the ability of *let-7* to repress its targets.

Project 2: The role of *let-7* in *C. elegans* vulva development

Motivation and contributions

Our genome-wide RNAi screen for *let-7* suppressors identified a surprisingly large number of genes that were able to suppress the *let-7* bursting phenotype. This notion lead us to an important question: Since its discovery, the classical model for *let-7* function has been the hypodermis of *C. elegans*. However, while developmental phenotypes and molecular mediators are well characterized in this tissue, a satisfactory explanation for the lethal bursting phenotype has been missing. Therefore we decided to ask whether *let-7* was playing an additional role in the vulva that was independent from its function in the hypodermis.

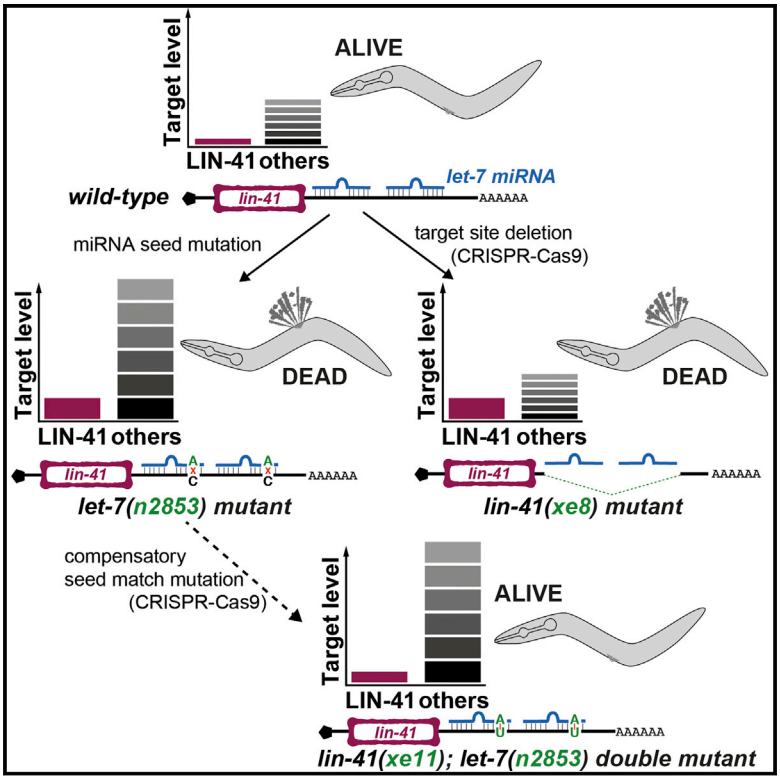
As a first step, I started to clone a construct for expression of full-length *pri-let-7*, as well as a number of ubiquitous and tissue-specific promoters that allowed to express *let-7* in different subsets of cells. These constructs were then used for tissue-specific rescue of the *let-7* null mutant by Matyas Ecsedi. When these initial experiments suggested a role for *let-7* in the vulva, he continued to analyze *let-7* function in this tissue in detail, both by studying vulva morphogenesis in *let-7* mutants as well as by developing a quantitative target reporter system to identify the relevant *let-7* target in this tissue. With this approach, he was able to propose *lin-41* as the crucial *let-7* target in the vulva. I subsequently confirmed this hypothesis both by phenotypic analysis as well as at the molecular level when I characterized the *lin-41* strains mutant for *let-7* binding that were generated through CRISPR/Cas9-mediated genome-editing by Matyas Ecsedi and Iskra Katic.

Article

Developmental Cell

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

Graphical Abstract



Highlights

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

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In Brief

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

Ecsedi et al., 2015, Developmental Cell *32*, 335–344
February 9, 2015
http://dx.doi.org/10.1016/j.devcel.2014.12.018

Developmental Cell
Article

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

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http://dx.doi.org/10.1016/j.devcel.2014.12.018

SUMMARY

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

INTRODUCTION

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

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

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

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

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

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

RESULTS

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

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

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

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

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

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

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

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

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

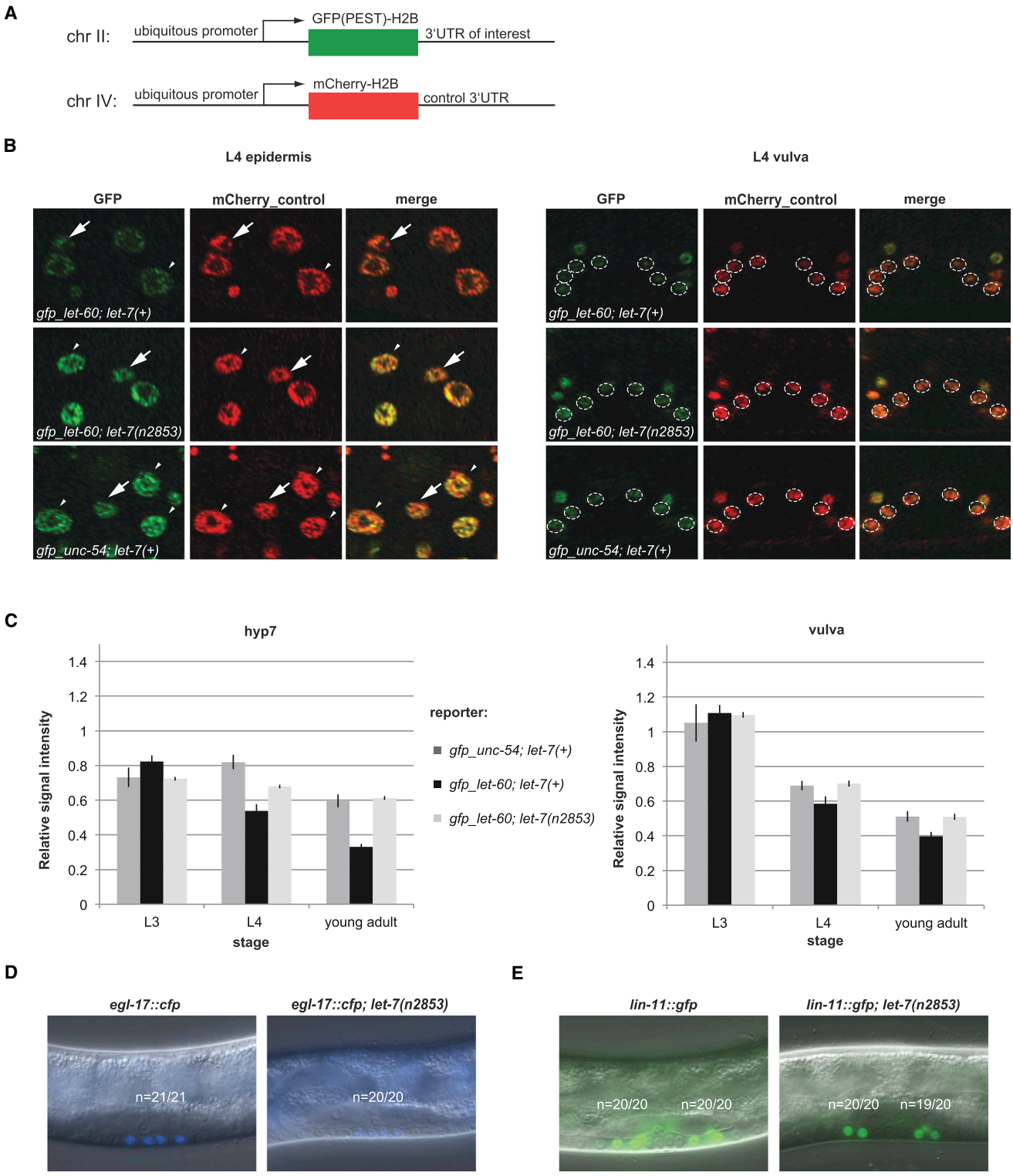


Figure 1. *let-7* and Its Regulation of *let-60* Are Dispensable for VPC Specification

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

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

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

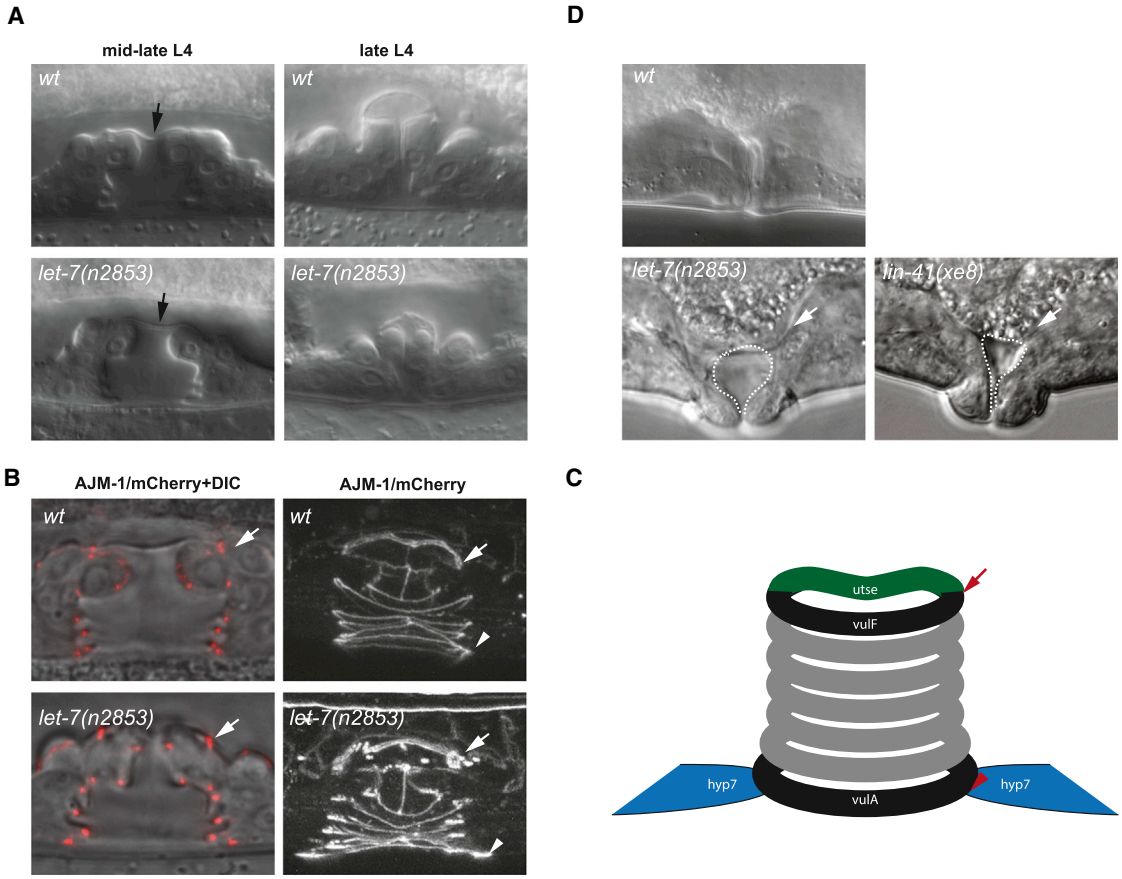


Figure 2. Loss of *let-7* Leads to Vulva Morphogenesis Defects at the Young-Adult Stage

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

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

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

See also Movie S1.

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

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

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

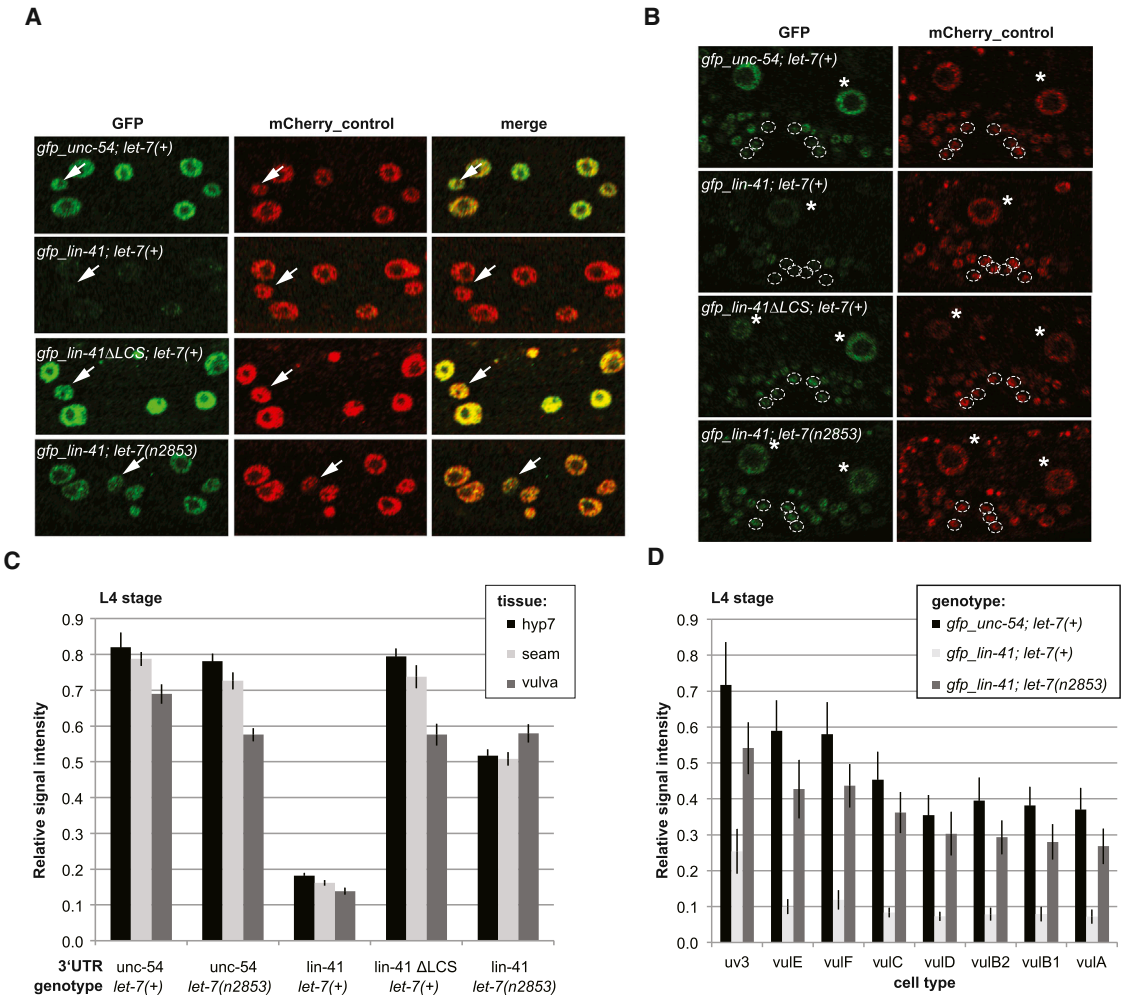


Figure 3. *let-7* Mediates Extensive Repression of *lin-41* in the Vulva

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

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

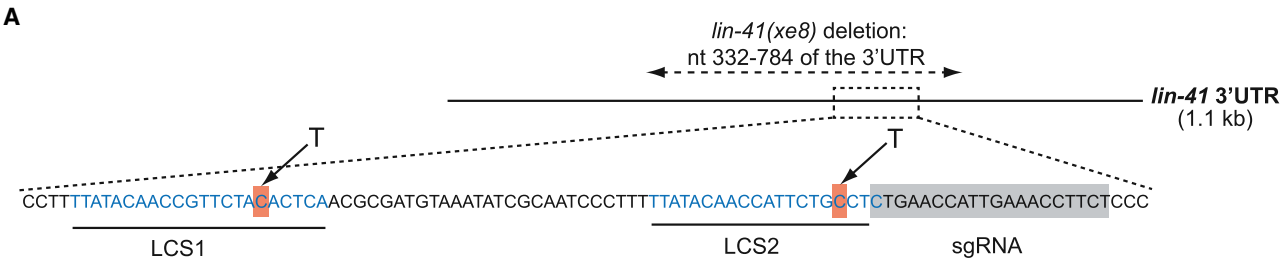
See also Figure S1.

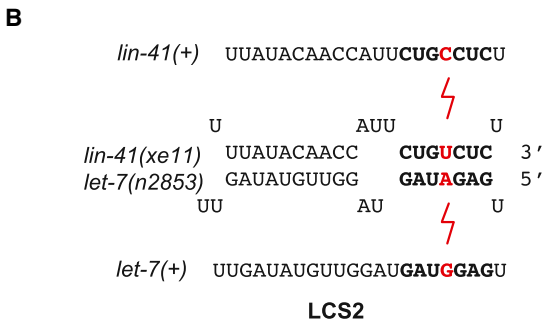
let-7 from the epidermis-specific *elt-3* promoter restored epidermal differentiation, but failed to suppress the bursting phenotype (Figure S1). Hence, *let-7* activity in other tissues, either in addition or alternatively to the epidermis, is needed to prevent vulva bursting.

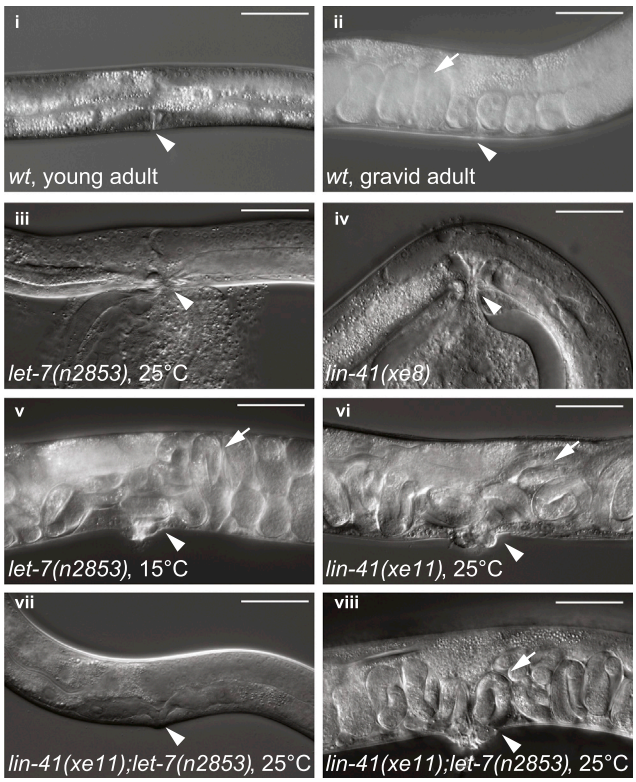
We were unable to find a promoter that drove *let-7* expression exclusively in the vulva (data not shown), either as a consequence of the shared developmental history of epidermis and vulva, or due to an epidermal enhancer element in the *pri-let-7* (Kai et al., 2013). This precluded direct demonstration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

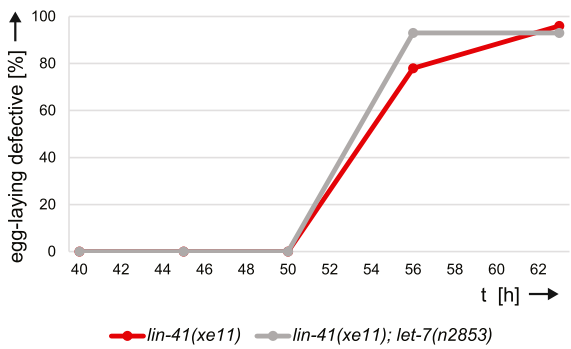
let-7 Is Highly Active against *lin-41* in the Vulva

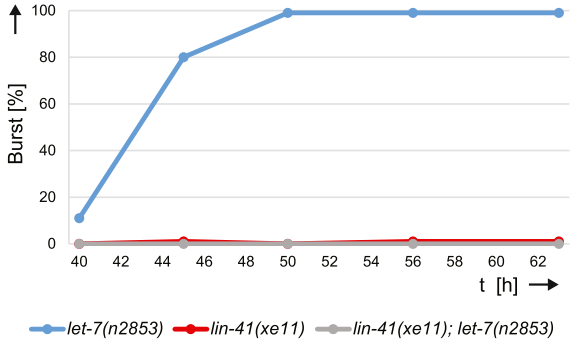
The above results suggested that *let-7* was functional in the vulva but argued against LET-60 as a relevant target. Hence, we sought to establish other targets. We focused on LIN-41 because of its important developmental functions and the fact that its regulation by *let-7* is highly conserved among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











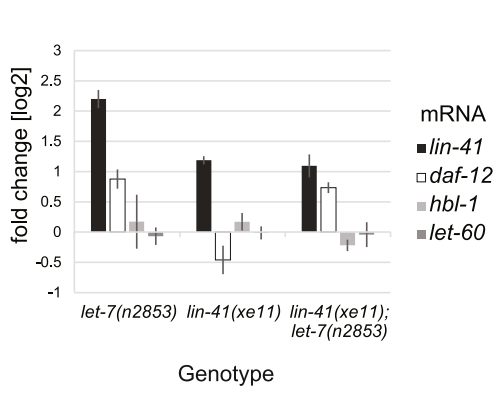


Figure 4. *lin-41* Is the Key *let-7* Target

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

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

(legend continued on next page)

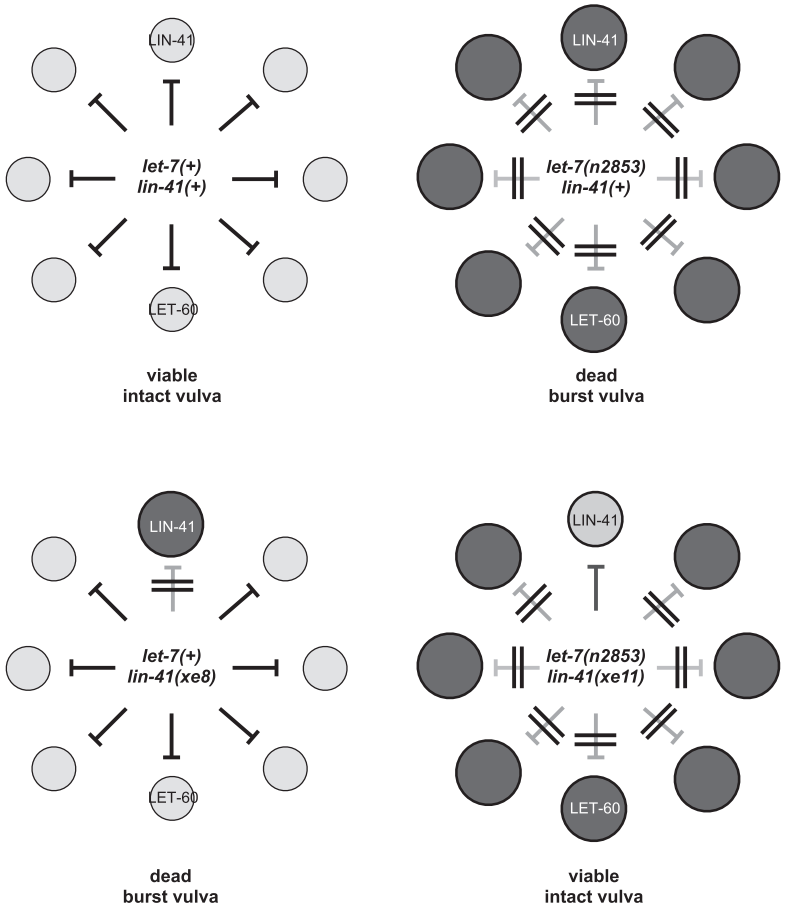


Figure 5. Schematic Depiction of the Effects of *let-7* and *lin-41* Alleles on *let-7* Target Expression and Phenotypes

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

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

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

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

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

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

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

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

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

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

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

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

DISCUSSION

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

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

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

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

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

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

EXPERIMENTAL PROCEDURES

Worm Handling and Strains

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

miRNA Target Reporters

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

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

AJM-1/mCherry Imaging and 3D Reconstruction

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

Time-Lapse Imaging

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

Tissue-Specific *let-7* Rescue

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

Targeted Genome Editing using Cas9-CRISPR

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

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

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

RNA Isolation and Quantitative Real-Time PCR

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

SUPPLEMENTAL INFORMATION

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

ACKNOWLEDGMENTS

We thank Iskra Katic for help with genome editing using CRISPR-Cas9 and Raphael Thierry for help with image analysis. We thank Alexander Hajnal for discussion, and Witold Filipowicz, Iskra Katic, and Alexander Hajnal for comments on manuscript drafts. Some strains were provided by the *Caenorhabditis* Genetics Center, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). The research leading to these results has received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement 241985 (European Research Council miRTurn), and from the Novartis Research Foundation through the Friedrich Miescher Institute Foundation. M.E. was a Swiss National Science Foundation/Swiss Academy of Medical Sciences M.D./Ph.D. program fellow supported by an Oncosuisse fellowship.

Received: July 9, 2014
Revised: October 31, 2014
Accepted: December 18, 2014
Published: February 9, 2015

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Developmental Cell

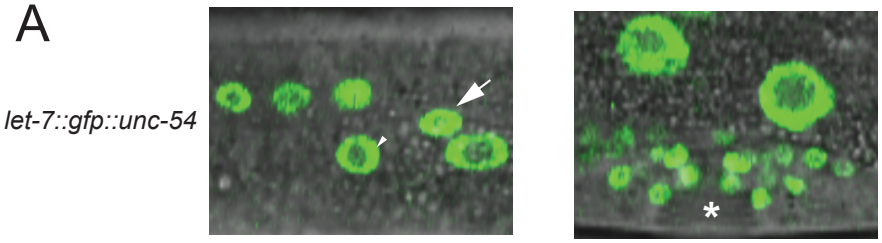
Supplemental Information

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

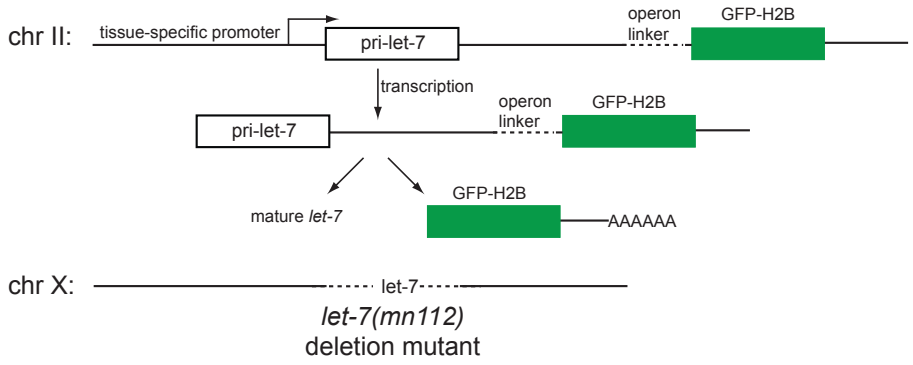
Matyas Ecsedi, Magdalene Rausch, and Helge Großhans

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

A



B



C

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

D

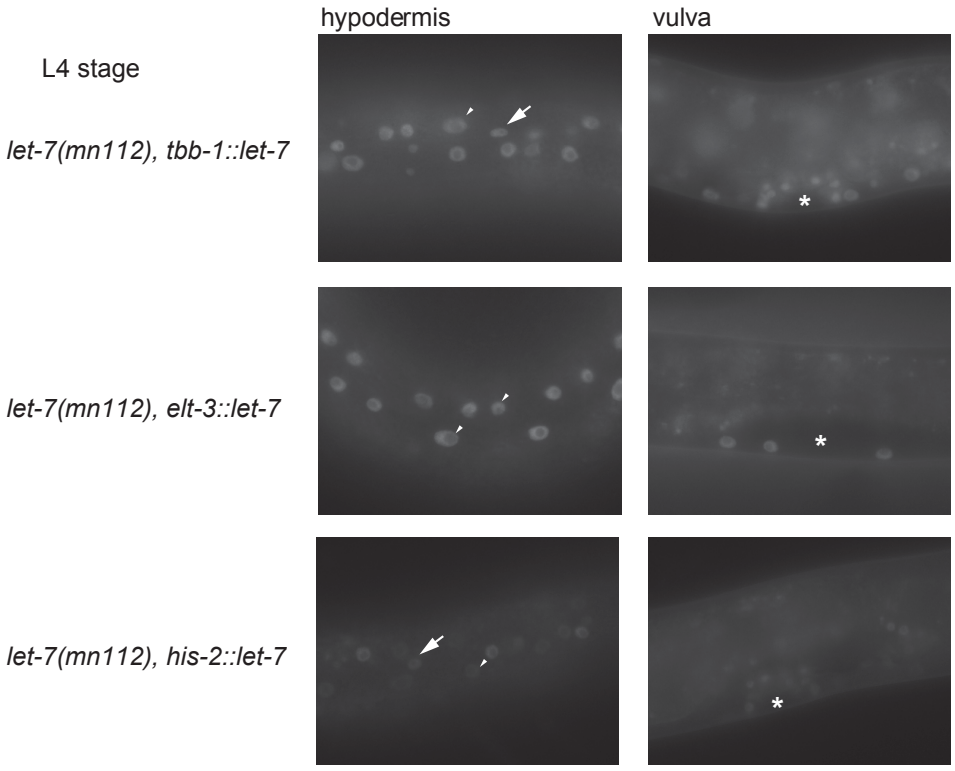


Fig. S1: *let-7* is active in the vulva. Related to Fig. 3.
A, *let-7* is transcribed in both hypodermis and vulva. Arrow: seam cell; arrowhead: hyp7, asterisk indicates vulva lumen.
B, Schematic of tissue-specific *let-7* expression. *Pri-let-7* is expressed in a tissue-specific manner in worms lacking 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	ggggactgctttttttgtacaaacttggAATCCGATAAGGACTGTG	GW primer to create promoter entry clone	
elt-3 GW fwd	ggggacaactttgtatagaaaagttgaaCGCTGATGGGGGTACGGTC	GW primer to create promoter entry clone	
elt-3 GW rev	ggggactgctttttttgtacaaacttgtGAAGTTTGAAATACCAGGTAGCCG	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	ggggactgctttttttgtacaaacttgcTACCCTTTTCTGAAAAAAGACGC	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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Remarks and open questions

With this study, we present the first systematic analysis of the *let-7* vulval bursting phenotype and the process of vulval morphogenesis in *let-7(n2853)* mutants. As a conclusion, we can exclude gross defects in vulval development and morphogenesis, especially during early stages when VPC specification and the division pattern of the vulval cells are induced. Unfortunately, we fail to identify the critical step, and the actual cause of bursting remains unexplained. This is at least in part due to the fact that the mechanisms that control the later stages of vulval morphogenesis are generally less well understood than the early steps. Moreover, while we can prove that *let-7* is required outside of the hypodermis, it remains to be determined if *let-7* function in the vulva is sufficient to rescue bursting. Even though we tested a number of candidate promoters, we had to accept the fact that exclusively vulva-specific promoters do apparently not exist in *C. elegans*. Hence, we cannot formally rule out that *let-7* functions in the uterus. One possible way to address this would be mosaic analysis of a transgene expressed from an extrachromosomal array.

The second major conclusion of this study is the fact that regulation of *lin-41* alone is sufficient to suppress *let-7* bursting in the background of the *let-7(n2853)* mutation. While this has somewhat been expected based on experiments from earlier publications, the work presented here can for the first time provide direct support of this hypothesis in the context of the endogenous genomic loci without using any transgenes. Our results suggests a major role for derepression of *lin-41* not only in respect to the hypodermal phenotypes, but also in the vulva (or, as mentioned, the uterus) of *let-7(n2853)* animals. It will be interesting to determine why the vulval cells, in contrast to the seam cells, do not present cell division defects. A potential explanation might be a tissue-specific function for LIN-41, maybe with different downstream effectors. To this end, the phenotypical differences between the unregulated *lin-41* mutants and *let-7(n2853)* and wild-type worms will have to be characterized in more detail, both in the context of the vulva as well as regarding seam cell differentiation. Furthermore, a combination of biochemical and genetic approaches may help to identify potential interacting-partners and targets of LIN-41 and could be the basis for further research.

Discussion

miRNAs: active switches or collective modulators?

The first known miRNAs, *lin-4* and *let-7*, were discovered for their obvious loss-of function phenotypes in genetic screens. Moreover, the absolute sequence conservation of *let-7* from nematodes all the way up to humans seemed to promise a crucial importance for miRNA mediated control of gene activity in development, similar to the effects observed for transcription factors. Today, almost fifteen years after the discovery of the biological process of posttranscriptional gene regulation by miRNAs, these initial hopes have been somewhat dampened. Apart from exceptional cases of genetic disorders that were linked to miRNA mutations, as found for the miR-183/miR-96/ miR-182 cluster in familiar hearing loss (Mencia et al. 2009), few examples of miRNAs essential in development have been found in humans. Moreover, the majority of miRNA knockouts in mice, despite the high conservation of these miRNAs across different species, do not show major defects. Even in *C. elegans*, where it was possible to analyze deletions for about 75% of the miRNAs encoded in the worm genome, the majority of them were found to be non-essential (Miska et al. 2007). A potential explanation for this is the predicted redundancy of miRNA families that share the same seed sequence and are thought to target the same mRNAs. However, when this was addressed in worms, few phenotypes were identified even if whole miRNA families were deleted (Alvarez-Saavedra and Horvitz 2010). Nonetheless, mammalian genomes encode hundreds of evolutionary conserved miRNAs, which argues for a profound role of these loci for survival of the species. To make matters more complicated, these miRNAs are computationally predicted to target conserved binding sites on hundreds of targets per miRNA, and mRNAs typically contain binding sites for several different miRNAs (Bartel 2009). The question of miRNA-target interactions remains a topic of intensive debate. Several large-scale studies suggest that the effects of miRNAs on protein levels are mostly modest (Baek et al. 2008, Hendrickson et al. 2009, Guo et al. 2010, Selbach et al. 2008). Even though coordinated effects of different miRNAs on a single target are experimentally very difficult to test and were not yet analyzed on a global level, these observations led to the conclusion that miRNAs might in most cases act as fine tuners rather than active switches of gene expression. Does this explain the lack of phenotypes for the majority of analyzed miRNA knockouts? If individual miRNAs act by moderate, but coordinated regulation of numerous targets, putting some targets under strong repression by an additive mechanism

in concert with other miRNAs, then phenotypes for individual miRNAs should not be detectable. In stark contrast to this hypothesis of miRNAs as broad fine-tuners of gene regulation is the existence of miRNAs such as *lin-4* and *let-7*, which show strong mutant phenotype and the significant repressive capacity towards their targets in *C. elegans*. Studying the heterochronic pathway, it became sufficiently clear that *lin-4* mediated its function solely through linear repression of *lin-14* and *lin-28*, even before it was understood that *lin-4* acted as a miRNA. My PhD work now proves that *lin-41* stands in a similar linear regulatory relationship with *let-7* (Ecsedi et al. 2015). *lin-41* has been proposed as the major effector of *let-7* phenotypes in the hypodermis, yet other targets of *let-7* have been found. In order to address this issue, we decided to artificially design a situation in which only *lin-41* would escape *let-7* mediated repression, while all other targets would remain unaffected. In order to create such a situation in vivo, we used CRISPR/Cas9 based genome engineering to modify the *let-7* complementary sites (LCSs) in the 3'UTR of *lin-41*. To our great satisfaction, we can indeed show that deletion of the LCSs can induce bursting, and, more importantly, that a molecular situation that changes the binding sites back to a Watson-Crick base-pairing between *let-7(n2853)* and *lin-41* can fully rescue the *let-7* bursting phenotype. This experiment is an elegant proof of concept for *lin-41* being the single *let-7* target responsible for the bursting phenotype in *C. elegans*. For now, miRNAs with such strong relations to key targets as in the case of *lin-4* and *let-7* seem to be the exception, rather than the rule. Nonetheless, even if miRNAs have subtle modulative effects in many cases, keeping targets within a physiological level, important examples for miRNAs actively inducing changes in gene expression over development do exist. Moreover, although results from *C. elegans* suggest otherwise, it cannot be excluded that miRNAs have crucial roles in higher organisms. The existence of multiple miRNA family members, e.g. in the case of humans, where *let-7* homologues are encoded in 13 genomic loci, might reflect the importance of these miRNAs and could provide a mechanism to protect the genome from detrimental effects in case of mutation of the miRNA. Unfortunately, this widespread distribution of miRNA family genes makes it extremely difficult to engineer fully functional miRNA deletions and prevents them from occurring spontaneously. Therefore, the genomes of higher organisms might harbor additional miRNAs with important functions in development.

let-7 targets in *C. elegans* – not quite as simple as "just" *lin-41*

My work has clearly highlighted the importance for *lin-41* as the key *let-7* target in development of *C. elegans*. Misregulation of *lin-41* alone can induce bursting, and repaired target binding of the point-

mutant *let-7(n2853)* allele only for *lin-41* in a situation where all other targets are derepressed can suppress the bursting phenotype. Nevertheless, multiple *let-7* targets were proposed and confirmed in different in vivo or in vitro systems, and several of them, such as *daf-12*, *hbl-1*, *lss-4*, *die-1* and *pha-4* have been found to rescue the bursting phenotype of *let-7* null mutants (Slack et al. 2000, Andachi 2008, Grosshans et al. 2005, Johnson et al. 2005, Hunter et al. 2013). This phenomenon still awaits a sufficient explanation. First of all, it remains to be determined if these factors are indeed physiological *let-7* targets. Using microarray analysis of *let-7* mutant animals, apart from *lin-41* I find only *daf-12* levels to change significantly upon loss of *let-7*. Indeed, high-resolution time-course studies performed in our lab indicate that only two genes initially respond to *let-7* upregulation during early L4 stage. These genes are the known *let-7* targets *lin-41* and *daf-12* (F. Aeschimann, unpublished data). However, if these suppressors are tissue-specific *let-7* targets, it might be impossible to pick up *let-7*-dependent mRNA changes in total worm lysates. Targets or not, we cannot exclude that these genes affect the processes that lead to vulval bursting, either downstream of or parallel to *lin-41/let-7*. Therefore it will be critical to identify the molecular and morphological determinants of this process. Notably, the transcription factors DAF-12 and PHA-4 co-regulate genes involved in the morphological changes upon dauer entry, which is a physiological response to stress caused by starvation (Ao et al. 2004). It might be possible that *lin-41* represents the crucial *let-7* target under normal conditions, regulated in a switch-like manner, while regulation of other targets might gain importance in stress conditions such as food withdrawal.

An alternative model predicts cross-regulation between the *let-7* targets. This idea is supported by the fact that the genes *daf-12*, *pha-4*, *die-1* and *hbl-1* that can suppress the lethality associated with the *let-7* null allele code for transcription factors, and *lss-4* acts as a chromatin remodeling factor (Grosshans et al. 2005). The *let-7* targets might activate expression of each other such that knockdown of one results in reduced expression of *lin-41*. However, this would have to account for a strong reduction in *lin-41* levels, since I find *lin-41* derepressed up to five-fold in *let-7(n2853)* mutants. Furthermore, I do not detect significant changes in *lin-41* levels upon RNAi of *daf-12* or *hbl-1*. Alternatively, the transcriptional regulators might dampen expression of the *let-7* precursor. Such a role has indeed been confirmed in the case of HBL-1 (Roush and Slack 2009).Nonetheless, this would not account for rescue in a *let-7* null background. A completely different mechanism would be the function of the other *let-7* targets as mRNA sponges that titrate *let-7* away from the physiological target *lin-41*. Indeed, I find *lin-41* and *daf-12* to increase repression of each other upon RNAi. This notion is further supported by my observation that loss of *lin-41* binding in *lin-41(xe11)* animals results in stronger repression of *daf-12*. However, rescue of *let-7* phenotypes in a context where knockdown of one *let-7* target would leave more *let-7* molecules “free” to

repress the other target again only makes sense in a situation where *let-7* is present as a hypomorph, but not in case of a *let-7* null. Therefore, this model does not provide a satisfactory explanation for the ability of the other targets to rescue the phenotype. It is therefore more likely that the other targets act parallel to *lin-41*, maybe even, as mentioned above, in a tissue-specific manner. Indeed, *hbl-1* RNAi can rescue only lethality, but not the hypodermis phenotype of *let-7* mutants (Vadla et al. 2012). Moreover, the fact that many of the *let-7* targets are expressed in the gut, it remains possible that homeostasis of this tissue has an effect on *let-7* bursting. Even *lin-41* itself seems to act in a tissue-specific manner. As a matter of fact, *lin-41* mutations do cause precocious alae formation in only about 50% of the animals (Slack et al. 2000), indicating that *let-7* might have additional targets in the hypodermis. This observation nicely correlates with the fact that in human induced pluripotent stem cell (iPS) generation, the beneficial effect of *let-7* repression can be explained largely, but not to 100% by *lin-41* upregulation (Worringer et al. 2014). Moreover, LIN-29, the major downstream target of LIN-41 in the hypodermis, is not required for male tale-tip development, a tissue in which LIN-41 controls activity of the transcription factors DMD-3/MAB-3 (Del Rio-Albrechtsen et al. 2006, Mason et al. 2008). We note that LIN-41 might also act in a different context in the vulva as compared to the hypodermis. First of all, we can show that VPC specification as well as early morphogenesis during L3 stage are not affected by *let-7* mutations. This correlates with the time of *let-7* expression and the onset of *let-7* phenotypes in the hypodermis only at the late L4 stage. However, we also do not observe any obvious morphogenesis effects during later stages of vulval development, and most importantly, no additional divisions of the vulval cells. This is surprising because it stands in contrast to the *let-7* hypodermis differentiation defect that is accompanied by a supernumeral division of the seam cells. Moreover, LIN-29 levels were found reduced in the seam cells of *let-7(n2853)* animals, but unchanged in the vulval cells at the late L4 stage, arguing that LIN-29 expression in the vulva might not be controlled by *let-7* (Reinhart et al. 2000). Third, LIN-29 mutants show a defect in fusion of the anchor cell (AC) with the uterine π cells (Newman et al. 2000). This phenotype does not occur in *let-7(n2853)* animals, were π cells and AC fuse to an utse cell of wild-type morphology. It has to be mentioned that this discrepancy could be caused by the remaining activity of the hypomorphic *let-7(n2853)* molecules, which might allow some basic LIN-29 activity. Or, *let-7* might indeed control vulval development through a tissue-specific factor downstream of LIN-41. If that is the case, then the other *let-7* targets might potentially affect this enigmatic factor. Taken together, a final explanation for the suppressive effect of the published *let-7* targets remains to be discovered.

miRNAs represent important regulators of the cell cycle

As opposed to knockout studies indicating that the majority of miRNAs are not of major importance during normal development of multicellular organisms in ideal environments, reports of miRNAs that have crucial functions if normal cell homeostasis is perturbed seem to accumulate exponentially. Indeed, it has become increasingly clear that miRNA loss-of-function can be detrimental for development if animals are subjected to stress (Leung and Sharp 2010). Moreover, countless examples for aberrant miRNA expression in pathological conditions have been found, which has resulted in a model according to which many miRNAs function to allow dynamic responses to pathological or physiological stress situations (Mendell and Olson 2012). The identification of deletions of the miR-15a/16-1 cluster in chronic lymphocytic leukemia (CLL) established cancer as the first disorder connected to miRNA misregulation (Calin et al. 2002). Likewise, since its initial discovery as a tumor suppressor in the lung, *let-7* has been linked to multiple types of cancers. Interestingly, the major characteristic of cancer, namely overproliferation of a specific cell type due to ectopic stimulation of the cell cycle, resembles the hypodermis phenotypes observed in *lin-4* and *let-7* mutants in *C. elegans*. Accordingly, interactions between the heterochronic pathway and cell cycle components have indeed been verified for the early timer consisting of *lin-4* and its target *lin-14*. LIN-14 directly or indirectly activates transcription of the cyclin-dependent kinase inhibitor *cki-1*, which results in a cell cycle pause prior to S phase in VPCs (Hong et al. 1998). My work presented here strongly indicates that such a miRNA-mediated regulation of cell cycle factors also occurs in the case of *C. elegans let-7*. The genes identified in the genome-wide suppressor screen comprise numerous factors required for cell cycle and mitosis, such as the genes *cyb-3* (Cyclin B) and *plk-1* (Polo-like kinase 1), as well as structural components required for mitosis, which suggests an important role for *let-7* in repressing the cell cycle of the seam cells. Moreover, in the case of the cell cycle promoting factor *cdk-1*, we can show direct control by *let-7* through the transcription factor LIN-29. Accordingly, a large number of cell cycle factors have been found to be downregulated upon *let-7* overexpression in human lung cancer cells, among them the G1 phase promoting kinase CDK6 as well as CDC25A, an activating phosphatase targeting CDKs (Johnson et al. 2007). My genome-wide RNAi screen has also identified a number of cell cycle factors that affect *let-7* activity. While the mechanism for this phenomenon remains to be identified, examples for a direct crosstalk with the cell cycle have been discovered for other miRNAs. The miR-16 family, which was found to be frequently mutated in human B cell chronic lymphocytic leukemias (B-CLL), is a prominent example for miRNA mediated regulation of cell cycle progression by

targeting factors like CDK6, CARD10, and CDC27 (Linsley et al. 2007, Liu et al. 2008, Calin et al. 2002, Klein et al. 2010, Bandi et al. 2009). Moreover, transcription of these miRNAs as well as rapid turnover are co-regulated with cell cycle progression, ensuring rapid repression of the inhibitory signal upon mitotic stimulation (Rissland et al. 2011). The fact that we have identified cell cycle factors to affect *let-7* function in *C. elegans* could point to a similar mechanism as in the case of miR-16. Accumulation of *let-7* can be detected from L3 stage onwards, a time when seam cells still have to undergo one more round of division. One could envision a situation in which cell cycle factors repress *let-7* activity in order to ensure completion of the last seam cell division, after which continuing accumulation of *let-7* represses the cell cycle and induces differentiation. Analysis by northern blot did not indicate substantial increase in mature *let-7* levels upon RNAi of the candidate factors. However, it remains possible that some suppressors act for example on RISC activity or support the transcription of *let-7* targets.

Notably, *let-7* is not the only miRNA for which cross-regulation with cell cycle components has been reported. A striking example is the interplay between p53 and miRNAs of the miR-34 family. The p53 transcription factor is a, maybe the, key mediator of a signaling pathway that induces checkpoint activation and cell cycle arrest upon DNA damage or other signs of cellular abnormalities, which, if not resolved during the cell cycle halt, induces apoptosis . In 2007, several groups found direct transcriptional activation of the mir-34 miRNAs by p53 to be crucial for cell cycle arrest and apoptosis (Bommer et al. 2007, Chang et al. 2007, He et al. 2007, Raver-Shapira et al. 2007, Tarasov et al. 2007). Conversely, the transcription factor Myc, which has emerged as a major proto-oncogene due to its strong capacity to induce cell growth and proliferation, was found to activate the prominent miR-17-92 cluster, a locus that has been identified to act oncogenic itself (Mendell 2008, He et al. 2005). Two of the miRNAs in this cluster, namely miR-17-5p and miR-20a, are capable of suppressing the cMyc target E2F1, a transcription factor that acts stimulating on cell cycle progression. However, E2F proteins have been proposed to induce apoptosis if too active (Ziebold et al. 2001). One model for the oncogenic capacity of the locus might therefore be that Myc activates transcription of E2F proteins, but in parallel counteracts apoptosis through the negative feedback loop established by the miR-17-92 cluster, resulting in full proliferative capacity of tumors (O'Donnell et al. 2005). An interesting notion in this context is the fact that regulation of *let-7* itself is tightly linked the cell cycle through transcriptional activation of the pluripotency factor LIN28 by Myc. LIN-28 was found to inhibit *let-7* processing at the level of Drosha in the nucleus, thereby blocking maturation. *let-7* in contrast can target and repress the *lin-28* mRNA through LCSs in the 3'UTR. This system generates a negative feedback loop that allows repression of *let-7* activity in proliferating ES cells and rapid downregulation of LIN-28 when differentiation is induced, while excessive activation of

Myc in cancer can shift this balance towards proliferation. A second prominent oncogene that was identified as a conserved *let-7* target is the small GTPase Ras, which acts as a major signal transmitter for proliferative stimulation (Esquela-Kerscher et al. 2008, Johnson et al. 2005). While we do not find a function for the *C. elegans* homologue *let-60* in vulval development, our data does not argue with its regulation by *let-7* in the hypodermis. This could indicate that ras is a tissue-specific *let-7* target in *C. elegans*. Alternatively, given the conservation of ras as a *let-7* target and its implications in cancer, this regulation could be important for the organism in situations that are, in the case of *C. elegans*, not encountered under lab conditions, or, in the case of cancer, represent a state of cellular stress.

These examples and my findings show that *let-7* and other miRNAs are indeed important players of the cell cycle, be it as active switches in development or as fine-tuners in the context of cell homeostasis. Notably, while many of the factors that actively regulate cell cycle progression have been found to interact with miRNAs, little is known about a potential contribution of structural factors to this system. With my genome-wide RNAi screen, I have discovered that many of the genes that act in the *let-7* pathway as well as several factors that affect *let-7* function comprise structural components required for chromosome integrity and cell division, such as the subunits of the condensin II complex. Therefore it might be worth to investigate the potential connections between *let-7* and these factors in more detail. A possible experiment to address this could be to study RNA expression in *let-7* mutants and wild-type worms both in presence and absence of such components. This experiment would have to be performed in a germline-less background of in a tissue-specific manner to exclude effects of meiosis. Prior to cost-intensive RNA sequencing experiments, the effect of individual suppressors on *let-7* activity could be analyzed quantitatively by using the microscope-based miRNA target reporter system developed by M. Ecsedi.

let-7 and *lin-41*- a special pair?

One the one hand, many miRNAs seem not to have strong effects on target levels and present inconspicuous phenotypes if deleted. In contrast, a subset of miRNAs do lead to significant degradation of target mRNAs, and their knockouts result in serious perturbations of development. Probably one of the most remarkable examples for this second class is *let-7*, mutations of which lead to severe developmental defects in *C. elegans*, and which is required for the differentiation of embryonic stem cells. Particularly the level of conservation not only in sequence, but also for its repressive function on *lin-41* from simple

multicellular organisms all the way up to humans is astonishing. Therefore, one wonders what makes this function of *let-7* so special. Or, to put it in a different way; what makes LIN-41 such an extraordinary target that its repression by *let-7* was maintained through evolution of the animal kingdom?

The answer to this question is obstructed by our little knowledge on the functions of LIN-41, and even more importantly, its mode of action. Research on *let-7* in vertebrates has largely focused on its interplay with a second major *let-7* target, namely LIN-28. But while this bistable switch explains how *let-7* activity is regulated, it never fully sufficed to explain how *let-7* was activating differentiation. Work from Worringer and colleagues has now nicely illustrated that a critical *let-7* target that orchestrates proliferation and blocks differentiation is TRIM71/LIN-41, which underlines its function as a major effector of the LIN-28/*let-7* "switch" (Worringer et al. 2014). Yet, LIN-41 has not fully stepped out of the shadows, because reports on its mode of action are inconsistent and many questions remain to be answered.

LIN-41 was originally proposed to repress *lin-29* by an unknown posttranscriptional mechanism, most likely at the mRNA level (Slack et al. 2000). However, the first molecular role of LIN-41 was linked to an E3 ubiquitin ligase function. Rybak and coworkers were able to show mLin41-dependent polyubiquitylation of the Argonaute protein Ago2 in mouse ES cells, leading to proteasomal degradation and an overall reduction of Ago2 (Rybak et al. 2009). The activity was associated with the RING and B-box domains but did not require the filamin or NHL domains. The observation that mLin41 colocalized with miRNA pathway members and caused degradation of Ago2 suggested a negative feedback loop to the miRNA machinery that targets mLin41 through *let-7*. On the other hand, the ESC cell cycle regulating (ESCC) miRNA families that oppose *let-7* function play an important role in ES cell proliferation, indicating that a global inhibition of miRNA function might not make sense in the context of ESCs. Indeed, a second study has identified a role for mLin41 in promoting the response to EGF (Chen et al. 2012). In this context, mLin-41 was found to bind SHCBP1, a mediator of FGF signaling that represses differentiation. Similar to the effect on Ago2, mLin41-dependent ubiquitylation of SHCBP1 was detected, however, Lin41 binding was shown to lead to stabilization, not degradation of SHCBP1. Notably, mLin41 was not shown to affect Ago2 levels in this study. In parallel, evidence for LIN-41 acting as a repressor of mRNA translation has accumulated. Two studies independently reported repression of a large number of mRNAs by mLin41 in mouse ES cells, a function that was mediated through the 3'UTR of the targets (Chang et al. 2012, Loedige et al. 2013). Here, filamin and NHL domains were crucial for repression, while the RING domain that is mediating E3 ubiquitin ligase activity was not required. Interestingly, both studies find cell cycle inhibitors among the Lin41 targets, which goes in line with the apparent connection between *let-7*/lin-41 and the cell cycle in *C.*

elegans. Moreover, a recent study identifies EGR1 as a major target of LIN41 at the mRNA level (Worringer et al. 2014). The transcription factor EGR1 is an important activator of genes expressed in differentiated fibroblasts that needs to be repressed during iPS cell induction (Fragola et al. 2013). Moreover, it contains a conserved LIN-29 domain and interacts with the cofactors NAB1 and NAB2, which are homologues of the LIN-29 cofactor MAB-10 in *C. elegans* (Harris and Horvitz 2011). This shows that not only the regulation of LIN-41 by *let-7* but also important downstream factors are conserved from worm to human. To make matters more complicated, further insight on LIN-41 came from a recent study in *C. elegans* that found LIN-41 to be required in the germ-line (Tocchini et al. 2014). Here, LIN-41 plays an important role in suppressing the premature onset of developmental plasticity characteristic for early embryonic development. While the authors can exclude a function for the RING domain, they also find that key RNA binding residues in the NHL domain are dispensable for the germ-line specific function of LIN-41, but not for somatic phenotypes. Notably, LIN-41 has been implicated in establishment of muscle attachment to the body wall of *Drosophila melanogaster* (Loer et al. 2008). In this context, function of LIN-41 depends on physical interaction with integrins and other proteins of the cytoskeleton, and not on translational repression. The hypothesis of a structural role for LIN-41 in formation of cell-cell contacts within the vulva or with the vulval musculature would be an attractive alternative to a function in gene-repression, especially since this would explain the different phenotypes of *let-7* animals in the vulva versus the hypodermis of *C. elegans*. Therefore, it will be exciting to determine the downstream factor of LIN-41 in the context of vulval bursting.

Taken together, LIN-41 seems to be involved in an astonishing variety of different processes that control proliferation and differentiation. Maybe this kaleidoscope of functions is what makes LIN-41 so special and the requirement for regulation by *let-7* so important. Future studies on individual LIN-41 functions and its interactions with yet undiscovered binding partners will help to expand our understanding of the *let-7* network, a regulatory system that is one-of-a-kind in its ability to affect proliferation and differentiation both in development and disease.

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