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Transcriptional Control of Cell-Cycle Quiescence During *C. elegans* Development

Joseph E. Clayton[a], **Sander J.L. van den Heuvel**[b,1], and **R. Mako Saito**[a,1,*]

[a]*Department of Genetics, Dartmouth Medical School, Hanover NH 03755* [b]*Developmental Biology, Faculty of Science, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands*

Abstract

During the development of the *C. elegans* reproductive system, cells that give rise to the vulva, the vulval precursor cells (VPCs), remain quiescent for two larval stages before resuming cell division in the third larval stage. We have identified several transcriptional regulators that contribute to this temporary cell-cycle arrest. Mutation of *lin-1* or *lin-31*, two downstream targets of the Receptor Tyrosine kinase (RTK)/Ras/MAP kinase cascade that controls VPC cell fate, disrupts the temporary VPC quiescence. We found that the LIN-1/Ets and LIN-31/FoxB transcription factors promote expression of CKI-1, a member of the p27 family of cyclin-dependent kinase inhibitors (CKIs). LIN-1 and LIN-31 promote *cki-1*/Kip-1 transcription prior to their inhibition through RTK/Ras/MAPK activation. Another mutation identified in the screen defined the *mdt-13* TRAP240 Mediator subunit. Further analysis of the multisubunit Mediator complex revealed that a specific subset of its components act in VPC quiescence. These components substantially overlap with the CDK-8 module implicated in transcriptional repression. Taken together, strict control of cell-cycle quiescence during VPC development involves transcriptional induction of CKI-1 and transcriptional regulation through the Mediator complex. These transcriptional regulators represent potential molecular connections between development and the basic cell-cycle machinery.

Keywords

C. elegans; vulval development; cell-cycle quiescence; transcription; foxB; Ets; Mediator; CKI

INTRODUCTION

The development of multicellular organisms involves a precise choreography of numerous aspects of cell division, growth and differentiation. This intercellular coordination is often critically dependent on spatial and temporal control of cell divisions to ensure the co-existence of the appropriate cells. For example, heterochronic gene mutations disrupt the developmental timing of multiple cell lineages in *C. elegans* (Ambros and Horvitz, 1984). The heterochronic defect perturbs the developmental coordination between presumptive vulval and uterine lineages and results in a deformed adult structure that is unable to lay eggs (Euling and Ambros, 1996). The current study examines the mechanisms used by developmental programs to

[1]Previous address: Massachusetts General Hospital Cancer Center, Building 149, Charlestown, MA 02129
[*]Corresponding author. Fax: +1 603 650 1188 *E-mail address:* Richard.M.Saito@dartmouth.edu (R.M. Saito)

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regulate cell division in order to ensure that the appropriate cell populations are available for vulva organogenesis.

Multiple families of proto-oncogenes and tumor-suppressors are crucial for cell-cycle control during *C. elegans* development. The core cell-cycle machinery in *C. elegans* is highly conserved with higher metazoans, albeit with a simplified configuration as many gene families are represented by a single member. For example, cell-cycle entry is regulated by a single cyclin D/Cdk4 complex encoded by the *cyd-1* and *cdk-4* loci (Boxem and van den Heuvel, 2001; Park and Krause, 1999). The lack of functional redundancy in *C. elegans* allowed the demonstration that CYD-1/CDK-4 activity promotes G1/S progression by inactivating two partially redundant inhibitory pathways mediated by the sole Retinoblastoma family member, *lin-35*, and a p27 Cip/Kip cyclin-dependent kinase inhibitor, *cki-1* (Boxem and van den Heuvel, 2001). Importantly, the relationship between these pathways appears to be conserved between *C. elegans* and vertebrates (Park et al., 1999). By analogy with other systems, LIN-35 Rb likely inhibits S-phase by transcriptional repression of S-phase genes (Dyson, 1998) and potentially other mechanisms (Binne et al., 2007), while CKI-1 Cip/Kip directly binds and inhibits the cyclin E/Cdk2 complex (Ekholm and Reed, 2000). In fact, *in vivo* studies of *lin-35* function have identified multiple target genes, including negative regulation of cyclin E (Chi and Reinke, 2006; Grishok and Sharp, 2005; Kirienko and Fay, 2007). However, the mechanisms to integrate developmental signals and yield control of the cell-cycle machinery are not completely understood.

The highly reproducible cell cycles during *C. elegans* vulva development provide an ideal model system for identifying the mechanisms coordinating cell divisions in living animals ((Sulston and Horvitz, 1977) and Fig. 1A). During the first larval stage (L1), six vulval precursor cells (VPCs) are created which arrest immediately in an extended G_1 phase (Euling and Ambros, 1996). The VPCs remain quiescent until the mid-third larval stage (mid-L3) at which time they resume divisions and differentiate. Interestingly, the divisions that create the VPCs in L1 also produce neuroblast siblings that continue through several rounds of cell divisions without delay and ultimately develop as neurons. Thus, the VPCs are selectively targeted for a temporary cell-cycle arrest that is overcome specifically during the L3 stage. We utilized these characteristics of vulva production to study the mechanisms that regulate temporary cell-cycle quiescence during metazoan development.

Prior to the mid-L3 cell divisions, the quiescent VPCs are instructed to choose between the hypodermal (skin) and vulval fates through a series of well-characterized cell-cell interactions that include the RTK/Ras/MAPK, Notch and Wnt signaling cascades (Sternberg, 2005). Induction of VPC pattern formation is initiated during late L2/early L3 by LIN-3, a signal produced by the uterine anchor cell (Hill and Sternberg, 1992). Reception of this EGF-related signal initiates a conserved RTK/Ras/MAPK signaling cascade that includes the *let-23* EGF-R (Aroian et al., 1990), *let-60* Ras (Han and Sternberg, 1990), *lin-45* Raf kinase (Han et al., 1993), and *mpk-1* MAP kinase (Lackner and Kim, 1998; Wu and Han, 1994) gene products. Among the targets of the signaling cascade are the transcription factors LIN-1 (Beitel et al., 1995) and LIN-31 (Miller et al., 1993). LIN-3 strongly induces the VPC closest to the anchor cell to execute the 1° vulval cell fate. In contrast, the flanking VPCs adopt the 2° vulval cell fate likely as a result of *lin-12*-mediated lateral inhibition (Greenwald et al., 1983) and weak induction by LIN-3 (Simske and Kim, 1995), while the remaining VPCs take the 3° or hypodermal cell fate. Interestingly, in synMuv (synthetic multivulva) mutant animals, the surrounding hypodermis can induce adoption of vulval cell fates through ectopic expression of LIN-3 (Cui et al., 2006; Fay and Yochem, 2007). Genetic screens for mutations that enhance or suppress the mutant phenotypes of the RTK/Ras/MAPK pathway identified several transcriptional regulators, including components of the Mediator complex (Singh and Han, 1995). Detailed genetic analyses suggest that the Mediator may regulate expression of the genes

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targeted by the RTK/Ras/MAPK signaling cascade (Howard and Sundaram, 2002). As the Mediator may act more generally as a regulator of signal-activated transcription, such as TGF-β-dependent transcriptional activation (Kato et al., 2002), further studies are necessary to reveal the molecular basis of the cooperation between intercellular signaling and transcriptional regulation by Mediator complexes.

In this study, we sought genes required for the VPC cell-cycle quiescent period during vulva development to identify the mechanisms regulating developmental control of cell-cycle entry and exit. Our genetic screen took advantage of the ability to easily visualize the progeny of individual VPCs to allow the identification of mutant strains producing excessive VPCs. Seven mutant strains that produced extra VPCs were isolated in the course of several screens. These include mutations of three genes, *lin-1, lin-31* and *mdt-13*, that encode transcriptional regulators required for normal control of VPC cell-cycle arrest during vulval development. Our results indicate that *lin-1* and *lin-31* control VPC cell-cycle quiescence by promoting the cell-type specific expression of the cyclin-dependent kinase inhibitor, *cki-1*. Isolation of the *C. elegans* TRAP240 homolog, *mdt-13*, led us to examine the components of the Mediator complex. We find evidence for the selective requirement of individual Mediator complex subunits. Collectively, these data illustrate the important contribution of a transcriptional network to regulate cell-cycle quiescence during development.

MATERIALS AND METHODS

Strains

The *Caenorhabditis elegans* strains were grown as previously described (Brenner, 1974), except where noted. The following wild-type (N2)-derived strains were utilized in this study: FX1238: *cdk-8(tm1238)* I; MH17: *sur-2(ku9)* I; MT587: *lin-31(n301) bli-2(e768)* II; MT2131: *lin-31(n1053)* II; SV510: *lin-31(he136)* II; SV327: *cdc-14(he118)* II; DR103: *dpy-10(e128) unc-4(e120)* II; SV519: *mdt-13(he135) unc-4(e120)/mnC1* II; SP364: *mdt-13(mn19) unc-4 (e120)/mnC1* II; SP635: *mnDf66/mnC1* II; PS3931: *ref-1(ok288)* II; SD378: *mpk-1(ga117)/ dpy-17(e164) unc-79(e1068)* III; GS589: *lin-12(n950)* III; *him-5(e1490)* V; MT1035: *lin-12 (n137n460)* III; MT688: *lin-12(n137)/unc-32(e189)* III; *him-5(e1467)* V; JT5205: *lin-12 (n950)* III*; lag-2(sa37)* V; VT825: *dpy-20(e1282)* IV; *maIs113*[*dpy-20(+) + cki-1::GFP*]; MT1355*: lin-1(e1777)* IV*; dpy-7(e1324) sup-7(st5)* X; MT8256: *lin-1(n304)* IV/*nT1* [*unc-? (n754)let-?*](IV:V) ; MT7949: *lin-1(n1761gf)* IV; SV325: *lin-1(he117)* IV; SV332: *lin-1 (he119)* IV; SD551: *let-60(ga89ts)* IV; MT5748: *let-60(n2022)* IV/*nT1* [*unc-?(n754)let-?*] (IV:V) ; MT2124: *let-60(n1046gf)* IV; UP604*: sos-1(cs41ts)* V; MT1821: *lin-25(e1446)* V/ *nT1* [*unc-?(n754)let-?*](IV:V); JN147: *gap-2(tm748)* X; CB652: *dpy-22(e652)* X; MT6290: *lin-8(n2403)* II; *lin-36(n766)* III; MT8189: *lin-15(n765)* X; MT465: *dpy-5(e61)* I; *bli-2 (e768)* II; *unc-32(e189)* III; MT464: *unc-5(e53)* IV; *dpy-11(e224)* V; *lon-2(e678)* X and the Hawaiian isolate, CB4856

Elm phenotype screen

The genetic screen for cell-cycle regulatory genes was performed using *lin-12(gf)* mutations to visually mark each VPC as a ventral protrusion (pseudovulva) on the adult. Multiple *lin-12 (gf)* strains (Table 1) were subjected to the following general protocol: new mutations were induced using ethylmethanesulfonate (EMS) (Brenner, 1974) or TMP/UV (Yandell et al., 1994), 1-10 F1 hermaphrodites were transferred to new plates, F2 progeny were screened using a dissection stereoscope for the production of supernumerary (greater than 6) pseudovulvae. Further classification of defects was achieved by VPC analysis, described below.

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VPC analysis

Following the isolation of Elm strains, a secondary screen was used to identify *elm* mutations that specifically disrupted control of cell-cycle quiescence. Eggs were obtained from gravid hermaphrodites using the standard hypochlorite method. Eggs were hatched in the absence of food to produce an arrested L1 population. Synchronously developing larvae were produced by feeding the starved L1 animals. Following 24 hours of development at 20° C, the L2/L3 molt animals were immobilized with 1 mM levamisole and examined using Nomarski optics for presence of daughter cells of recently divided VPCs. Although the timing of data collection undervalues the actual number of extra VPC divisions since extra divisions have been observed after the L2-to-L3 transition, the molt is a late event within the normal VPC quiescent period that provides a standardized developmental stage for direct comparisons. Cell-fate defects that disrupt cell fusion were analyzed using the AJM-1::GFP marker (Mohler et al., 1998) to visualize membrane boundaries which remain intact on VPCs. Animals displaying greater than six VPCs by the AJM-1 assay but not observed to undergo extra cell divisions by the Normarski assay were judged to be defective in cell-fate determination.

Molecular characterization of *elm* mutations

Newly isolated Elm strains were outcrossed at least two generations with N2 prior to characterization. The *elm* mutations were genetically mapped to a chromosome using the MT464 and MT465 triple-mutant strains. Because of the chromosomal locations and associated Muv phenotype of *lin-1(he119), lin-1(he117)* and *lin-31(he136)*, these mutations were tested for complementation with their respective null alleles, *lin-1(e1777)* and *lin-31(n301). mdt-13 (he135)* was localized to LGII between *dpy-10* and *unc-4* using three-factor mapping followed by CB4856-based SNP mapping (Wicks et al., 2001). The *mdt-13(he135)* mutant phenotype was rescued by introduction of a mix of overlapping cosmids T01B7, K08F8 and F07H5 or an approximately 13 kb PCR-generated genomic fragment containing the predicted gene K08F8.6. Sequence analysis identified the mutation within K08F8.6 of *mdt-13(he135)* animals. *ref(he137)* was localized to LGII and was examined for genetic complementation with *ref-1 (ok288)*. None of the *ref(he137)*/*ref-1(ok288) trans*-heterozygous animals (n=5 broods) displayed the Ref phenotype.

mdt-13 genetic analysis

For analyses of animals homozygous for the *mdt-13(he135), mdt-13(mn19)* or *mnDf66* mutations, strains harboring the mutant chromosomes balanced by *mIn1*[*dpy-10(e128) mIs14*] were allowed to self-fertilize and non-GFP expressing animals were analyzed. These strains were intercrossed to examine hemizygotes (*mdt-13(he135)*/*mnDf66* and *mdt-13 (mn19)*/*mnDf66*) and trans-heterozygotes (*mdt-13(he135)/mdt-13(mn19)*). Double-stranded RNA corresponding to exon 12 of *mdt-13* (between GAACTTGAA and ACACTTGGC) was injected into N2 hermaphrodite gonads to initiate RNAi. The *mdt-13* deficient animals were categorized as embryonic lethal (Emb) if the eggs did not hatch, larval lethal (Let) if development arrested during larval stages or sterile (Ste) if adulthood was achieved. Between 62 and 108 individuals were examined for each genotype.

Results

A genetic screen for defective cell-cycle quiescence in C. elegans

We used pseudovulvae as indicators of VPC production in a genetic screen for mutant animals with more than six VPCs. Wild-type *C. elegans* produce six VPCs during the L1 stage that remain quiescent until the L3 stage. Gain-of-function mutations (gf) of the Notch family receptor encoded by *lin-12* cause VPCs to develop into pseudovulvae, highly visible protrusions on the generally featureless contour of the adult worm body (compare Fig. 1B and

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1C). Although the *lin-12(gf)*-induced pseudovulval structures are ectopic clusters of cells, each pseudovulva results from an abnormal cell-fate decision of a single VPC and not a defect in cell-cycle regulation ((Greenwald et al., 1983) and Table 2). Therefore, developmental defects that allow the production of greater than six VPCs, for example through defects in cell-cycle quiescence that allow extra cell divisions during the L1-to-L3 period, result in an easily detected increase in the number of adult pseudovulvae (Hong et al., 1998; Saito et al., 2004). We refer to the production of greater than six pseudovulvae by a *lin-12(gf)* adult as the Elm (enhancer of *lin-12(gf)* multivulva) phenotype.

We performed five variations of the Elm screen, each using a *lin-12(gf)* mutation as the basis of visualizing VPCs (Table 1). Version 1 utilized a strong gain-of-function allele, *lin-12 (n950)*. Although the screen successfully isolated *elm* mutations, including *cdc-14(he118)* (Saito et al., 2004), the *lin-12(n950)* produced abnormally small broods that severely reduced the observable sample size of the mutant strains during screening (Fig. 1D). Additionally, although they were generally self-fertile, *lin-12(n950)* mutant hermaphrodites were cross-sterile because of an inability to mate. To perform genetic analyses, cross-fertility could be inefficiently restored to the *lin-12(n950)* animals using RNAi-based methods to decrease the *lin-12(gf)* activity (Saito et al., 2004). Versions 2 through 4 (Table 1) bypassed the mating and fertility defects by using the cold-sensitive gain-of-function allele, *lin-12(n137n460)* (Greenwald et al., 1983), however, the weaker Muv phenotype was not optimal for Elm phenotype screening. The most effective solution to restore mating and fertility to the *lin-12 (n950)*-containing strain was the addition of the *lag-2(sa37)* mutation (Tax et al., 1994). The *lin-12(n950); lag-2(sa37)* double mutant animals used in version 5 of the Elm screen produced a larger brood and restored cross fertility (Fig. 1D). Importantly, the *lag-2(sa37)* mutation did not significantly decrease production of the pseudovulvae as the *lin-12(n950); lag-2(sa37)* double mutant animals displayed similar pseudovulvae number compared to the *lin-12 (n950)* single mutant strain. Because of the combination of these characteristics, the *lin-12 (n950); lag-2(sa37)* double mutant animals became our primary strain for screening and characterization of new mutations that disrupt VPC developmental quiescence.

We isolated seven Elm mutant strains that represent six loci. Of these, five loci exhibited an extra VPC division defect, while one showed a cell-fate defect (see below and Fig. 1E). We determined the molecular identity of four loci that contribute to VPC cell-cycle quiescence. In addition to the previously described role of the phosphatase encoded by *cdc-14* (Saito et al., 2004), we report here the identification of three transcriptional regulators identified in the screen.

One of the elm mutations disrupts Pn.p cell fate

Of the seven isolated Elm strains, only animals harboring the *he137* mutation showed extra pseudovulvae that resulted from a cell-fate transformation (Fig. 2A). Transformation from a presumptive hypodermal cell into a VPC-like identity was previously observed upon loss of *ref-1* or *ref-2* (regulator of fusion), two genes that promote the hypodermal fate within a subset of Pn.p cells (Alper and Kenyon, 2001;Alper and Kenyon, 2002). In animals deficient for either *ref-1* or *ref-2*, posterior Pn.p cells (P9.p - P11.p) forego the syncytial hypodermal fate and instead adopt VPC-like characteristics, including maintenance of an autonomous cell boundary that can be observed using the AJM-1::GFP marker (Francis and Waterston, 1991;Mohler et al., 1998). Since the *he137* mutation genetically mapped to Chromosome II which contains the *ref-1* locus, *he137* was tested for genetic complementation of *ref-1(ok288)* (Materials and Methods). As measured by the Ref phenotype, functional complementation between *he137* and *ref-1(ok288)* strongly suggested that the *he137* mutation does not disrupt *ref-1*. Since we are interested in Elm mutations that disrupt cell-cycle regulation, we focused our analyses on the remaining *elm* genes.

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LIN-1, an Ets-family transcription factor, contributes to VPC quiescence

Two *lin-1* alleles, *he117* and *he119*, were identified that permitted an extremely low frequency of extra VPC divisions (Fig. 1E and Table 2). The defect is observed as one extra VPC division approximately every thirtieth animal. The *lin-1*-encoded *Ets*-family transcription factor has been described to inhibit the 1° vulval cell fate (Beitel et al., 1995). Accordingly, *lin-1 (he117)* and *lin-1(he119)* mutant adults displayed the Muv phenotype (Fig. 2B). We determined that strains harboring the null alleles *lin-1(e1777)* and *lin-1(n304)* also exhibited the weak defect in VPC cell-cycle quiescence (Table 2), indicating that the extra cell division defects are inherent to *lin-1* loss of function. The *lin-1(he117)* mutation introduced a premature stop at codon 99 (Glutamic acid) within the Ets domain encoding sequence while the *lin-1 (he119)* mutation has not yet been identified (Miley et al., 2004). Therefore, the isolation of *lin-1* mutations in the Elm screen for extra VPC divisions revealed a previously unrecognized role for *lin-1* in the developmental quiescence of VPCs.

LIN-31, Forkhead (foxB) transcription factor, is required for proper VPC quiescence

LIN-31, a member of the winged-helix/Forkhead box (Fox) family of transcriptional regulators (Miller et al., 1993), has been demonstrated to associate with LIN-1 during cell-fate determination by the VPCs (Tan et al., 1998). Consistent with the physical interaction with LIN-1, animals deficient for *lin-31* activity also display extra VPC divisions during the normal quiescent period as well as a Muv phenotype ((Miller et al., 1993) (Miller et al., 2000), Fig. 2C and Table 2). Similar to other strong loss-of-function alleles, *lin-31(he136)* disrupts the predicted DNA-binding domain by changing the Serine at amino acid position 60 to Phenylalanine. Based on the extra VPC phenotype, *lin-31(he136)* was indistinguishable from the null allele, *lin-31(n1053)* (Table 2). Since the progeny cells produced by the extra divisions retained the VPC identity as demonstrated by the ability to produce pseudovulvae in the *lin-12 (gf)* background, the ectopic cell divisions are the result of a defect in cell-cycle control and not heterochronic or other cell-fate abnormalities. Therefore, given the interaction between *lin-1* and *lin-31* during cell-fate determination, isolation of mutations within both loci in the Elm screen suggests a similar cooperation during VPC cell-cycle regulation.

lin-1 and lin-31 promote VPC quiescence by regulating cki-1 expression

Since *lin-1* and *lin-31* encode members of conserved transcription factor families, we examined genes that control cell-cycle entry and exit as potential targets of regulation. We observed that the normal pattern of expression of *cki-1*, which encodes a CIP/KIP-related cyclin-dependent kinase inhibitor (Hong et al., 1998), requires *lin-1* and *lin-31* activities (Fig. 3). We compared the expression of a GFP reporter driven by an 8 kb fragment of the *cki-1* promoter in wild-type, *lin-1(he119)* and *lin-31(n1053)* animals. The location and timing of GFP expression from this reporter coincided with *cki-1* activity during development (Hong et al., 1998). Within the ventral cord of all strains examined, the terminally differentiated neurons adjacent to the VPCs expressed the *cki-1::*GFP reporter. In contrast, unlike the robust expression observed in the wild-type animals during L2, the GFP expression by the VPCs of either *lin-1* or *lin-31* mutant animals was consistently reduced or not detected (Fig. 3). The decreased GFP expression indicated that *lin-1* and *lin-31* activities were required for efficient activation of the *cki-1* promoter in the newly formed VPCs at a time consistent with its role in cell-cycle quiescence. We did not observe appreciable changes to reporter expression in other tissues. These findings suggested that *lin-1* and *lin-31* control *cki-1* expression in a tissue-restricted manner and that the extra cell divisions observed in the *lin-1* and *lin-31* mutants are the result of decreased transcription of *cki-1* Cip/Kip.

In several developmentally regulated cell cycles, including those of the VPCs, *cki-1* acts in parallel to a pathway that includes the sole *C. elegans* Retinoblastoma family member, *lin-35* (Boxem and van den Heuvel, 2001). If *lin-1* and *lin-31* act within the *cki-1*-mediated

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pathway by promoting *cki-1* expression, then simultaneous loss of *lin-35* activity in the *lin-31* or *lin-1* mutant animals should enhance the Elm phenotype. While *lin-35(RNAi)* did not enhance the rare extra divisions found in *lin-1(he119)*, the number of extra VPC divisions in *lin-31(he136)* mutant animals was significantly increased upon *lin-35(RNAi)* treatment (Table 2), consistent with a role for *lin-31* in promoting *cki-1* expression.

We examined the Elm phenotype of *cki-1(gk132)* heterozygotes for enhancement upon further loss of *lin-1* to obtain genetic evidence of *lin-1* as a regulator of *cki-1* expression. Similar to the haploinsufficiency reported for p27 in mouse and humans (Philipp-Staheli et al., 2001), *cki-1* heterozygous mutant animals display a cell-cycle quiescence defect ((Saito et al., 2004) and Table 3). As expected if *lin-1* promotes *cki-1* expression, the *cki-1(gk132)*/+; *lin-1 (he119)* animals displayed increased extra VPC divisions compared to *cki-1(gk132)*/+ animals (Table 3). Similar analysis of *lin-31* was not performed because of the genetic linkage between *lin-31* and *cki-1*. The enhanced Elm phenotype observed in *lin-35(RNAi); lin-31(he136)* and *cki-1(gk132)*/+; *lin-1(he119)* animals support a model in which the cell-cycle quiescence of the VPCs is controlled by non-overlapping activities of *lin-35* and a pathway mediated by *lin-1, lin-31* and *cki-1*.

Lastly, evidence of *cki-1* as a downstream target of *lin-1* and *lin-31* regulation during VPC quiescence was obtained by analyzing genetic interactions between *lin-1, lin-31* and *cdc-14*. *cdc-14* encodes a phosphatase that positively regulates *cki-1* activity, most likely through control of CKI-1 stability or localization (Saito et al., 2004). The extra cell divisions in the *cdc-14* mutant animals were suppressed by increased *cki-1* expression (Saito et al., 2004). If *lin-1, lin-31* and *cdc-14* encourage cell-cycle quiescence by promoting *cki-1* expression and activity, respectively, *lin-1* gain of function might reduce the defects caused by *cdc-14* loss of function. Indeed, the *lin-1(n1761gf)* mutation partially, but significantly ($p \leq 0.01$), suppressed the extra cell division defect of *cdc-14(he118)* null mutants (Table 3). Conversely, loss of *lin-1* or *lin-31* activities together with *cdc-14* mutation should result in greater penetrance of the extra division defect. We observed an enhanced extra cell-cycle phenotype in the *lin-31 cdc-14* double mutant animals (Table 3). Together with the previous results the genetic interactions with *cdc-14(he118)* indicate that *lin-1* and *lin-31* promote *cki-1* activity during VPC quiescence.

lin-1 and lin-31 may affect VPC quiescence independent of the let-60 Ras pathway

lin-1 and *lin-31* encode well-characterized targets of a RTK/Ras/MAPK signaling cascade that directs cell-fate specification of the quiescent VPCs beginning late in the L2 stage (reviewed in (Sternberg, 2005)). If the contribution of *lin-1* and *lin-31* in VPC quiescence reflect their roles in the RTK/Ras/MAPK cascade, then mutations of other genes acting within the pathway may also result in the Elm phenotype. We examined genes that control the VPC cell-fate decision for earlier requirements during the cell-cycle quiescent period. Mutations that activate or inactivate RTK/Ras/MAPK signaling upstream of LIN-1 and LIN-31 did not cause extra VPC divisions between the mid-L1 and mid-L3 stage (Table 2). Thus, the roles of *lin-1* and *lin-31* in establishing or maintaining VPC quiescence are likely independent of their previously described roles as targets of the RTK/Ras/MAPK cascade. However, *lin-25* and *mdt-23/ sur-2*, two genes with roles in VPC cell fate determination were found to also affect VPC quiescence (Table 2 and (Ferguson et al., 1987)). The products of these genes likely act in conjunction with the multi-subunit Mediator complex, a coregulator of RNA polymerase II transcription (see below).

TRAP240 is required for proper control of VPC quiescence

A mutation within the gene encoding the *C. elegans* TRAP240 homolog, *mdt-13*, was isolated in the Elm screen. The TRAP240 subunit is associated with the Cdk8 module (or Srb8-11 in

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yeast) of the Mediator complex that is implicated in negative regulation of transcription by RNA polymerase II (Carlson, 1997; Hengartner et al., 1998; Sun et al., 1998; Taatjes et al., 2002; van de Peppel et al., 2005). As would be expected for a generally used transcriptional regulator, homozygous *mdt-13(he135)* mutant animals displayed a complex phenotype indicating pleotropic functions. *mdt-13(he135)* animals derived from heterozygous mothers were small and sterile, superficially appearing developmentally arrested as late larvae. However, adult structures such as alae and vulvae were produced. When examined at the L2 molt, these homozygous *mdt-13(he135)* animals displayed a potent extra VPC phenotype (2.3 ± 0.9 average extra VPC divisions per worm, n=10 and Fig. 4A). In *mdt-13(he135); lin-12 (n950)* double mutant animals, the extra VPCs created during larval development contributed to a robust adult Elm phenotype (Fig. 4B). DNA sequence analysis showed that *mdt-13 (he135)* introduced a premature truncation at codon position 259 (Tryptophan-to-Opal mutation). Moreover, we examined a previously identified allele, *mdt-13(mn19)* (Yoda et al., 2005), and observed an equally penetrant extra cell division phenotype (data not shown). Together, these mutations revealed a crucial requirement for *mdt-13* function in the establishment and/or maintenance of VPC developmental quiescence.

Genetic analyses indicated that the *mdt-13(he135)* and *mdt-13(mn19)* mutations result in partial loss of function. The majority of *mdt-13(he135)* and *mdt-13(mn19)* mutant animals developed into small, sterile adults (87% and 89% Ste, respectively, Fig. 4C). However, more severe developmental defects resulted from *trans*-heterozygous combinations of either mutation with *mnDf66*, a chromosomal deletion that eliminates the *mdt-13* locus (Fig. 4C). The increase of embryonic and larval lethality of the hemizygous mutant animals indicated that the mutations result in a partial loss of function. Since the *mdt-13* mutant strains were maintained as heterozygotes with a wild-type allele, the homozygous progeny may use maternally contributed *mdt-13* activity to fulfill an embryonic requirement. Accordingly, inhibition of maternal as well as zygotic *mdt-13* function by RNAi resulted in completely penetrant embryonic lethality (Fig. 4C). These data indicate a general requirement for *mdt-13* during development. This is further supported by ubiquitous expression of transgenes containing the *mdt-13* promoter driving expression of the green fluorescent protein (GFP) ((Wang et al., 2004) and data not shown). Importantly, despite the essential function of *mdt-13* for viability, mutations reducing *mdt-13* function revealed a critical role in cell-cycle arrest of the VPCs.

A subset of Mediator components is required for VPC cell-cycle quiescence

The observations that VPC cell-cycle quiescence required the Mediator-associated genes *mdt-13, mdt-23*/*sur-2* and *lin-25* prompted us to systematically examine predicted *C. elegans* components of the Mediator complex. The Mediator subunits are functionally and structurally conserved throughout eukaryotes and most components have readily identifiable *C. elegans* counterparts ((Bourbon et al., 2004) and Table 4). To determine if a Mediator subunit was necessary for VPC quiescence, we examined animals treated with RNAi to inhibit the expression of the subunit or, when available, we examined animals with disabling genetic mutations. Many Mediator subunits appear essential for normal development and viability as loss of function by either mutation or RNAi yielded a spectrum of defects that included embryonic and larval lethality (Let, Table 4). For these Mediator genes, VPC quiescence was examined in the “escapers”, the animals that survive and develop more extensively than their broodmates exposed to the lethal RNAi conditions. By examining mutant animals at the L2/L3 molt, we found that several Mediator components are required for establishing or maintaining VPC cell-cycle quiescence. Notably, in addition to the previously described *mdt-13, sur-2*/*mdt-23* and *lin-25* defects, extra VPC divisions were observed following RNAi-mediated inhibition of *cdk-8* (0.3 ± 0.5 average extra VPC divisions per worm, n=12), *mdt-1*/*sop-3* (0.1 ± 0.3 extra, n=30) and *mdt-12*/*sop-1* (0.1 ± 0.3 extra, n=32) activities, which encode homologs of Cdk8, TRAP220 and TRAP230, respectively. Interestingly, the Mediator

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components we identified based on their roles in VPC quiescence largely correspond to the Cdk8 module (Borggrefe et al., 2002; Carlson, 1997). This module is associated with a subset of Mediator complexes and has been implicated in transcriptional repression (see discussion).

DISCUSSION

The genes identified in our Elm mutant screen are required for the normal period of cell-cycle quiescence exhibited by the VPCs starting in the L1 stage of development. Two of the genes, *lin-1* and *lin-31*, encode transcription factors that control the cell-fate decision of the quiescent VPCs. However, the quiescence and cell-fate decisions are independent processes since several other genes controlling cell-fate selection by the VPCs did not display roles in regulating quiescence. Indeed, the Elm phenotype targeted by the screen depends on the ability of the cells to respond to the *lin-12(gf)* signal and therefore mutations that significantly alter the VPC identity could not be isolated from this screen. The screen also revealed a specific role in cell-cycle regulation for several components of the Mediator complex. Despite a general requirement for these transcriptional regulatory complexes, a specific function to regulate cell-cycle quiescence was revealed in our studies. To date, six of the seven strains identified by the Elm screen displayed defects in VPC cell-cycle regulation, indicating that the Elm screen provides a sensitive method for identification of genes that contribute to developmental control of cell division in *C. elegans*.

Contribution of *cki-1* transcriptional regulation by LIN-1 and LIN-31 to VPC quiescence

Upon identification of *lin-1* and *lin-31* in the Elm screen, we focused on *cki-1* as a candidate target gene and cell-cycle quiescence effector for several reasons. First, the mutant phenotypes display common characteristics, including enhancement by loss of *lin-35* activity ((Boxem and van den Heuvel, 2001) and Table 2). Second, the Fox family member, AFX, controls expression of p27 in Humans and mice (Medema et al., 2000). Third, over twenty consensus binding sites for Ets and Forkhead DNA-binding domains are contained within the *cki-1* promoter fragment that confers VPC expression concurrent with the initiation of cell-cycle quiescence ((Hong et al., 1998) and not shown). It is noteworthy that we observed a specific requirement within the VPCs for both *lin-1* and *lin-31* activities in expression of a *cki-1* promoter-driven GFP reporter. Intriguingly, the defect in *cki-1* promoter activation observed in the *lin-1* and *lin-31* deficient animals correlates with the penetrance of the cell-cycle quiescence defect of the mutant animals. Together, these findings support that *lin-1* and *lin-31* promote the expression of *cki-1* during the establishment or maintenance of cell-cycle quiescence.

Studies of cell-cycle regulation during *Drosophila* eye development proposed a “mop up” function for CKIs such as p27 during the establishment of cell-cycle quiescence (de Nooij et al., 1996). In their model of regulated cell-cycle quiescence, the role of the p27 family member encoded by the Dacapo gene is to bind and inactivate residual cyclin/CDK complexes that escaped inactivation during the previous cycle. This mop up function allows greater control of the activities of G_1 CDKs to achieve a strict enforcement of cell-cycle quiescence. Observations of *cki-1*-deficient development in *C. elegans* support the mop up model. In *cki-1* deficient animals, cells such as the VPCs do not show uncontrolled proliferation. On the contrary, on average each VPC undergoes only a single extra division and despite the resulting extra VPCs, development continued unperturbed (Boxem and van den Heuvel, 2001; Hong et al., 1998). For example, the progeny cells produced from a VPC that circumvents quiescence and undergoes an extra division during L2 will subsequently resume divisions in L3 at the normal time (Hong et al., 1998; Saito et al., 2004). Since these later divisions are performed by cells that are essentially “younger” than those in wild type, a mechanism utilizing a cell-intrinsic timer to regulate divisions is highly unlikely. The single extra cell cycle in the absence of *cki-1* activity is consistent with an unchecked activity of residual CDK complexes: uncontrolled

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proliferation was not observed since a constitutive proliferation signal was not provided. In contrast, the VPCs of animals deficient for both *cki-1* and *lin-35* activities can undergo two or more rounds of cell division during the normal quiescent period ((Boxem and van den Heuvel, 2001) and not shown). These observations are consistent with the mop up model in which residual cyclin activity in the *cki-1* mutants lead to but are degraded during the extra cell cycle, however, the increased expression of cyclin E allowed by loss of *lin-35* Rb function may promote further cell divisions.

VPC cell-cycle quiescence selectively requires components of the Mediator complex

Although Mediator is thought to generally regulate activated RNA polymerase II transcription (Kornberg, 2005), the effect on a specific gene depends on the cellular and developmental context (Yoda et al., 2005) and the composition of the complex (Taatjes et al., 2002). Biochemical studies have demonstrated that the large, multi-subunit Mediator complex exists in several forms that bridge site-specific DNA-binding proteins and the RNA polymerase II holoenzyme to regulate transcription. The homologs of CDK-8, MDT-12, MDT-13 and CIC-1 constitute a biochemically distinct Mediator subcomplex, referred to as the Cdk8 module, that interacts with the core components through an interaction with MDT-1 (Borggrefe et al., 2002; Carlson, 1997). Therefore, it is significant that we observed specific requirements for *cdk-8, mdt-12, mdt-13* and *mdt-1* during regulation of VPC cell cycles. Defects in cell-cycle entry were not observed following RNAi of *cic-1*; however, we were unable to determine the efficacy of *cic-1(RNAi)* treatment. Moreover, co-immunoprecipitation experiments demonstrated that SUR-2 associated with MDT-13-containing Mediator complex *in vivo* (Yoda et al., 2005). Our demonstration that these Mediator subunits are necessary for the control of cell-cycle quiescence is consistent with the biochemical evidence that they constitute a functional unit.

In our studies, loss of the Mediator component encoded by *mdt-13* caused a significant number of extra cell divisions. Unlike *lin-1* and *lin-31, mdt-13* does not appear to regulate the cell cycle through control of *cki-1* expression, since the *mdt-13(he135)* mutation did not appreciably reduce expression of the *cki-1::GFP* reporter in the VPCs (data not shown). In fact, Mediator complexes in yeast and mammalian systems that contain the Cdk8 module do not activate transcription of target genes, on the contrary, they likely inhibit activation (Carlson, 1997; Hengartner et al., 1998; Holstege et al., 1998; Sun et al., 1998; Taatjes et al., 2002; van de Peppel et al., 2005). Based on these observations we predict that the extra cell division phenotype displayed by *mdt-13(he135)* mutant animals results from ectopic expression of genes that promote cell-cycle progression. However, we did not observe alteration of *C. elegans cyd-1* cyclin D or *cye-1* cyclin E promoter activation in the VPCs of *mdt-13* mutant animals (data not shown). Wang *et al* concluded from decreased reporter gene expression following RNAi knockdown that *mdt-13* can activate transcription of certain target genes (Wang et al., 2004). Thus, it remains possible that the critical targets of the Cdk8 module-associated Mediator complex in VPC quiescence include cell-cycle inhibitors. In order to delineate the total contribution of Mediator to VPC cell-cycle quiescence, a comprehensive description of the regulatory targets and their dependence on Mediator components will be necessary.

Concluding remarks

Our results demonstrate that the Elm screen presents a valid approach to identify *C. elegans* genes necessary for VPC quiescence during development. Since the normal function of the genes identified is to arrest cell division, their human homologs represent candidate tumor suppressors. We identified multiple mutations that disrupt normal cell-cycle quiescence of the VPCs. The identities of three genes isolated in the Elm screen, *lin-1, lin-31* and *mdt-13*, and their subsequent characterizations illustrate the important roles played by transcriptional regulation in the developmental control of cell division. Therefore, further identification of

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elm genes to complete the description of the developmental network controlling cell-cycle quiescence will provide further insights towards our understanding of how the decision to divide during normal development is made.

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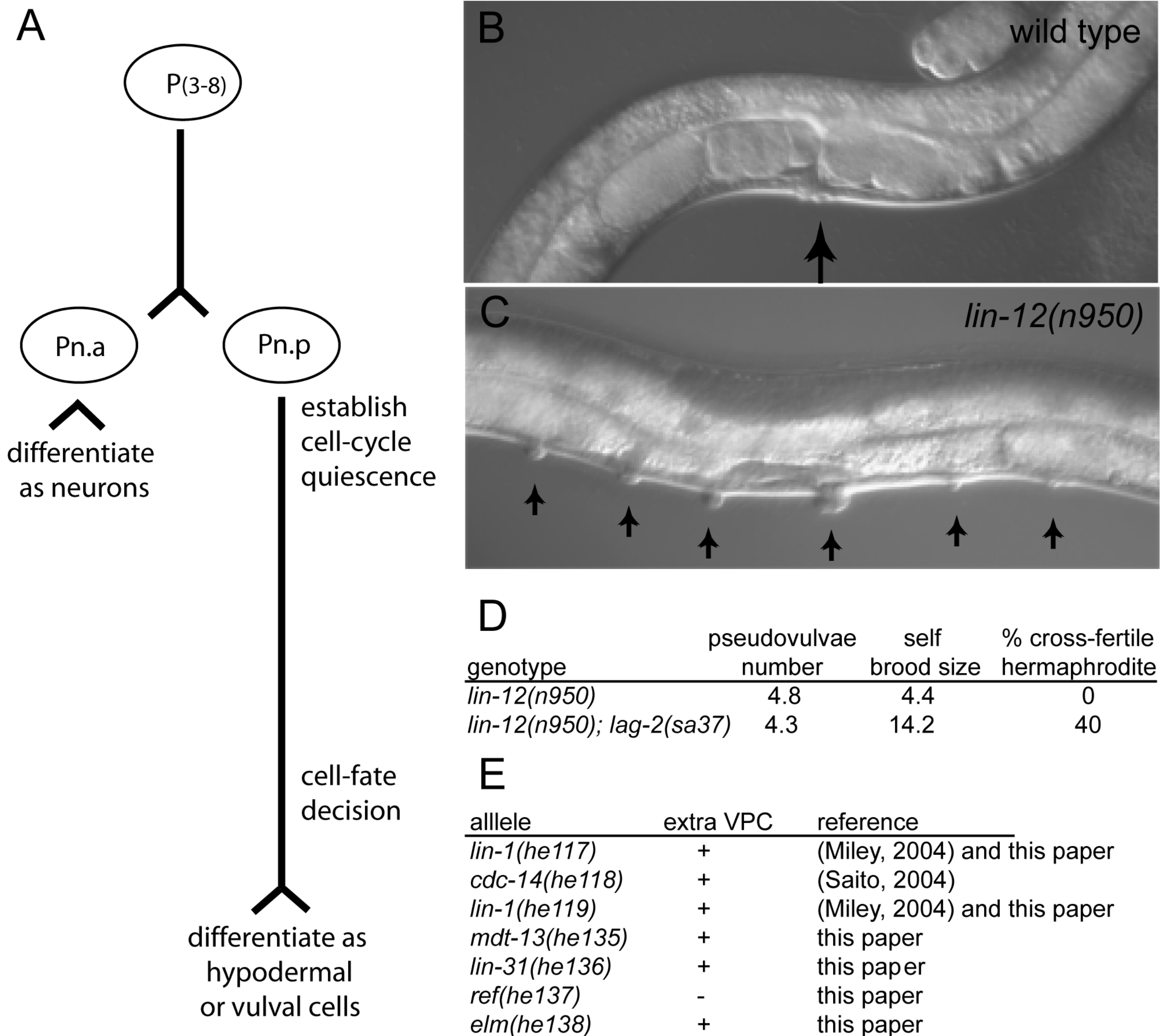

genotype	pseudovulvae number	self brood size	% cross-fertile hermaphrodite
lin-12(n950)	4.8	4.4	0
lin-12(n950); lag-2(sa37)	4.3	14.2	40

alllele	extra VPC	reference
lin-1(he117)	+	(Miley, 2004) and this paper
cdc-14(he118)	+	(Saito, 2004)
lin-1(he119)	+	(Miley, 2004) and this paper
mdt-13(he135)	+	this paper
lin-31(he136)	+	this paper
ref(he137)	-	this paper
elm(he138)	+	this paper

Fig. 1.

VPC development provides an attractive system for the study of temporal regulation of cell-cycle entry. (A) Schematic diagram of generalized post-embryonic P lineage. P cells divide mid-L1 to give rise to anterior (Pn.a) and posterior (Pn.p) progeny cells. The posterior progeny of P3 through P8 form the VPCs. Unlike the Pn.a siblings, the VPCs enter a quiescent period until mid-L3. During late L2/early L3 the quiescent VPCs receive a LIN-3-initiated signal that directs cell-fate selection. (B) Wild-type adult hermaphrodite with indicated vulva (arrow). (C) Adult *lin-12(gf)* hermaphrodite displaying inappropriately induced pseudovulvae (small arrows). Since the pseudovulvae originate from the VPCs (P3.p through P8.p), a maximum of six are observed. (D) *lin-12(gf)* strains harboring the *lag-2(sa37)* mutation produce larger broods, are cross-fertile and display generally improved health compared to *lin-12(gf)* single mutant animals. (E) The seven mutations isolated in the genetic screen for the Elm phenotype.

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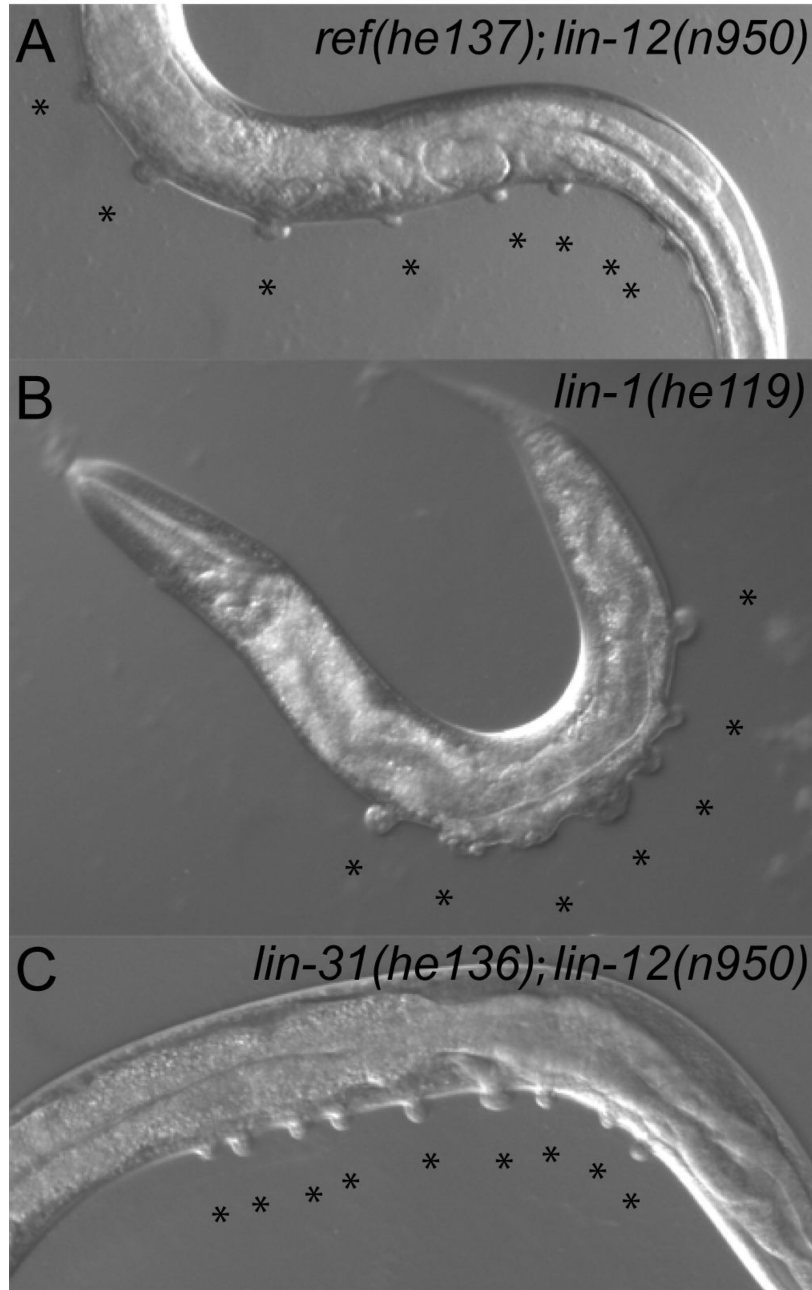

Fig. 2.

The Elm phenotype reveals extra VPCs that result from defects in either cell-fate or cell-cycle quiescence. (A) The Elm phenotype of the *ref(he137)* mutant adult. Notice the posterior appearance of the extra pseudovulvae due to abnormal VPC-fate adoption by presumptive hypodermal Pn.p cells. Mutations of two targets of Ras signaling, *lin-1* (B) and *lin-31* (C), result in the Elm phenotype. An extraordinarily robust *lin-31*; *lin-12* mutant adult is shown. Asterisks indicate pseudovulvae. Animals are oriented as anterior, left and dorsal, up.

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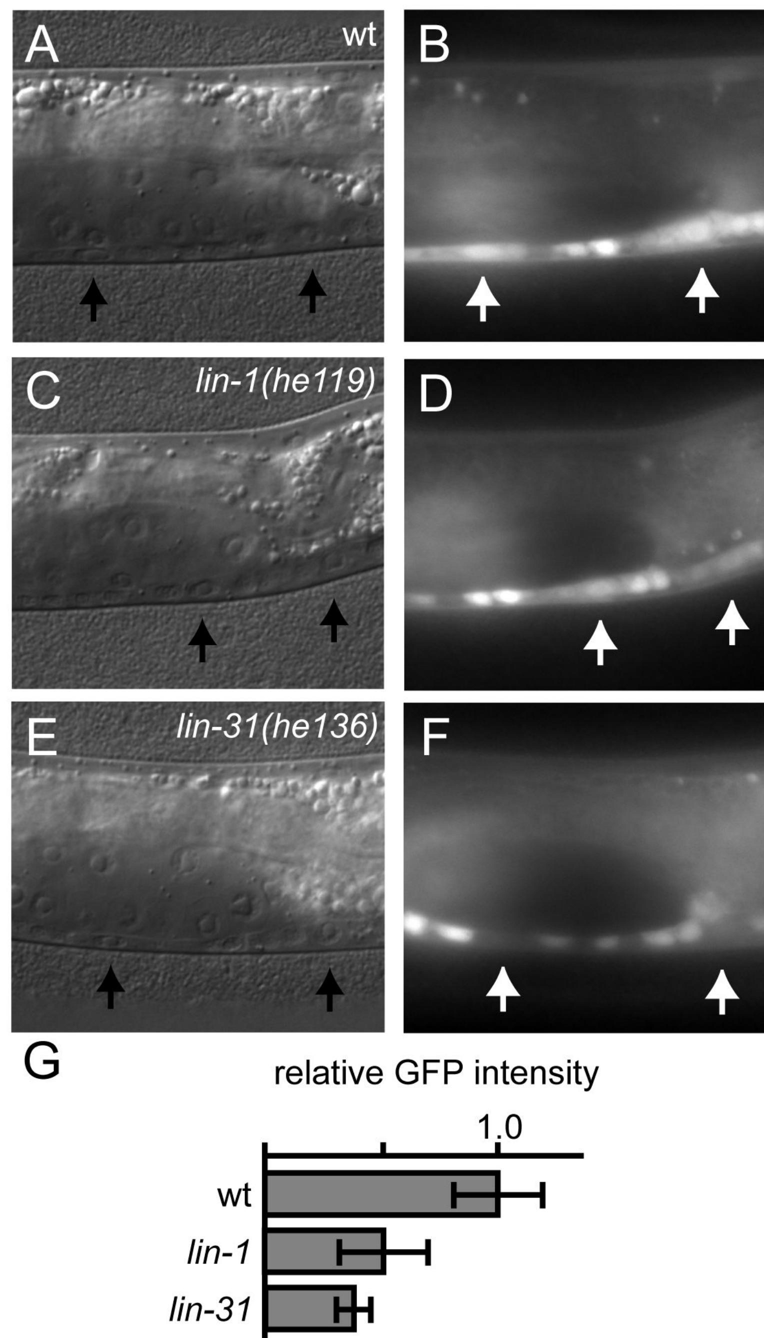

Fig. 3.

Activation of the *cki-1* promoter in arresting VPCs requires *lin-1* and *lin-31* activities. Nomarski (A, C and E) and epifluorescence (B, D and F) images of L2 animals expressing a *cki-1::gfp* reporter within the ventral cord (Hong et al., 1998). Compared to age-matched wild-type background (A and B), *lin-1(he119)* (C and D) and *lin-31(he136)* (E and F) mutant animals display weak or no detectable GFP expression above background. Animals are oriented as anterior, left and dorsal, up. The arrows indicate the VPCs, P6.p and P7.p. GFP expression is also observed within ventral cord neurons including the Pn.a descendants. (E) Quantitation of GFP signal within the VPC nuclei of the indicated genetic background ($n \geq 10$).

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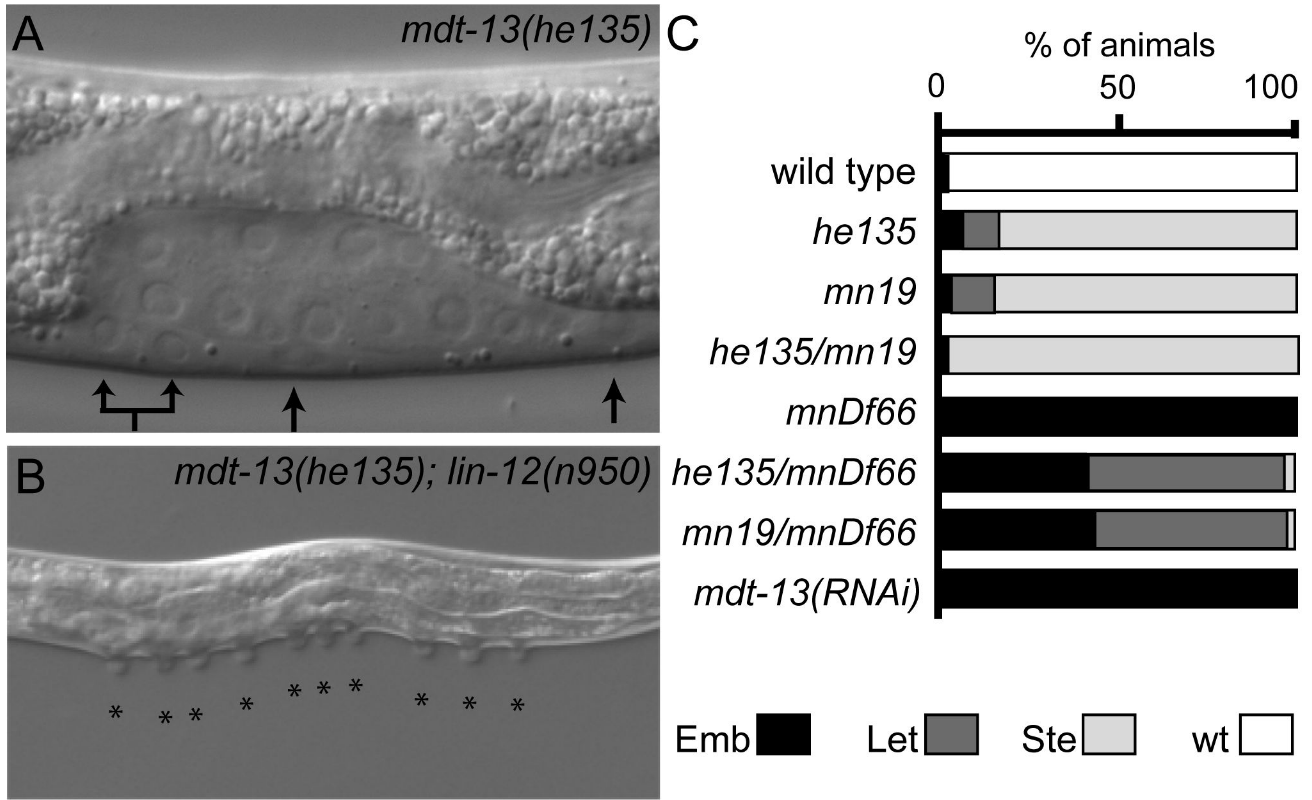

Fig. 4.

The *mdt-13* requirement in VPC cell-cycle quiescence is revealed by mutations that reduce activity. (A) Extra VPC divisions are observed during the normally quiescent L2 stage in the *mdt-13(he135)* mutant animals. Arrows indicate VPCs. In this example, the VPC, P5.p, has undergone an extra division (forked arrow). (B) The extra VPCs can be visualized as pseudovulvae (asterisks) on the *mdt-13(he135); lin-12(n950)* mutant adults. (C) Genetic analyses of the *mdt-13(he135)* and *mdt-13(mn19)* alleles indicate that the mutation partially disrupts *mdt-13* activity. Animals of the indicated genotypes were characterized by phenotype as normal (wt), embryonic lethal (Emb), larval lethal (Let) or sterile adult (Ste).

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Table 1

Summary of Elm phenotype screen

version	genotype	mutagen	*n*[1]	isolated mutations
1	*lin-12(n950)*	EMS	2,758	*cdc-14(he118), lin-1(he119)*
2	*lin-12(n137n460)*	EMS	2,900	
3	*lin-12(n137n460)*	TMP/UV	10,480	*lin-1(he117)*
4	*lin-12(n137n460); gaIs40*	EMS	6,550	
5	*lin-12(n950); lag-2(sa37)*	EMS	10,296	*mdt-13(he135), lin-31(he136), ref (he137), he138*

[1] number of haploid genomes screened

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Table 2

A subset of genes mediating the VPC cell-fate decision were also required for normal VPC quiescence

genotype[1]	**no. extra VPCs**[2]	***n***[3]
N2	0	many
lin-12(n950)	0	71
lip-1(RNAi)	0	25
sli-1(RNAi)	0	26
let-60(n2022)	0	19
let-60(n1046gf)	0	20
let-60(ga89), 15°C	0	23
let-60(ga89), 25°C	0	25
gap-2(tm748)	0	38
sos-1(cs41), 15°C	0	24
sos-1(cs41), 25°C	0	30
mpk-1(ga117)	0	34
lin-1(he117)	0.03 ± 0.2	35
lin-1(he119)	0.08 ± 0.3	59
lin-1(e1777)	0.06 ± 0.3	31
lin-1(n304)	0.03 ± 0.2	63
lin-31(he136)	0.4 ± 0.9	64
lin-31(n301)	0.3 ± 0.5	49
lin-31(n1053)	0.3 ± 0.6	64
lin-31(n301); lin-1(he119)	0.8 ± 1.2	25
lin-31(n301); lin-1(he117)	0.8 ± 0.9	89
lin-31(he136); lin-35(RNAi)	1.4 ± 1.0	14
lin-1(he119); lin-35(RNAi)	0	35
lin-35(RNAi)	0	30
lin-25(e1446)	0.8 ± 0.6	44
mdt-23/sur-2(ku9)	0.6 ± 0.3	35
sur-5(RNAi)	0	25
lin-8(n2403); lin-36(n766)	0	19
lin-15(n765)	0	27

[1] all strains were grown at 20° C, unless indicated.

[2] presented as average number of VPCs displaying extra divisions/animal examined ± SD.

[3] number of L2 animals examined.

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Table 3

Genetic interactions support *lin-1* and *lin-31* regulation of *cki-1* expression

genotype	**no. extra VPCs**[1]	***n***[2]
cki-1(gk132)/+	0.3 ± 0.5	22
cki-1(gk132)/+; lin-1(he119)	0.6 ± 0.7	22
cdc-14(he118)	3.0 ± 1.2	23
cdc-14(he118); lin-1(e1777)	2.9 ± 0.9	30
cdc-14(he118); lin-1(n1761gf)	1.9 ± 1.2[3]	17
lin-31(n301) cdc-14(he118)	4.1 ± 1.0	29

[1] presented as average number of VPCs displaying extra divisions/animal examined ± SD.

[2] number of L2 animals examined.

[3] $P \leq 0.01$, comparison between *cdc-14(he118)* and *cdc-14(he118); lin-1(n1761gf)*

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Table 4

A subset of Mediator genes control VPC quiescence

C. elegans gene	homolog	Let	Elm
mdt-1/sop-3	TRAP220/Med1	+	+
mdt-7/let-49	Med7	+	-
mdt-8	Med8	+	-
mdt-10	Nut2/Med10	+	-
mdt-11	Med11	-	-
mdt-12/sop-1/dpy-22	Trap230/Med12	+	+
mdt-13/let-19[1]	Trap240/Med13	+	+
mdt-14	Rgr1/Med14	+	-
mdt-15	Arc105/Med15	-	-
mdt-18	Srb5/Med18	+	-
mdt-19	Rox3/Med19	+	-
mdt-22	Srb6/Med22	+	-
mdt-23/sur-2[2]	Sur2/Med23	-	+
mdt-27	Trap37/Med27	-	-
mdt-28	Med28	-	-
mdt-29	Med29	-	-
mdt-31	Soh1/Med31	+	-
cdk-8[1]	Srb10/Cdk8	-	+
cic-1	Srb11/Cyclin C	-	-

Phenotypes were produced by RNAi treatment, unless otherwise noted:

[1] phenotype examined in mutant animal and by RNAi treatment.

[2] phenotype examined in mutant animal only.