Transcriptional control of cell-cycle quiescence during C. elegans development

Joseph E. Clayton a, Sander J.L. van den Heuvel b,1, R. Mako Saito a,⁎

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

Received for publication 22 June 2007; revised 25 October 2007; accepted 30 October 2007
Available online 12 November 2007

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 multi-subunit 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.

© 2007 Elsevier Inc. All rights reserved.

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 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 G1 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), whereas...
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 Yokochi, 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 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 vulval 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, mdr-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(e61) 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: mdr-13(he135) unc-4(e120) unc-11(e120) unc-11(e120) unc-11(e120) unc-11(e120) unc-11(e120) unc-11(e120) unc-11(e120) I; SP634: mdr-13(nm19) unc-4 (e120) mnC1 II; SP635: mnDf66/mnC1 II; PS3931: ref-1(ok288) II; SD738: mtk-1(1ga17)dpv-17(e164) unc-79(e1068) III; GS589: lin-12(nm950) III; him-5 (e1490) V; MT1035: lin-12(nm76n460) III; MT688: lin-12(nm137)/unc-3(2e189) III; him-5(e1467) V; JTS205: lin-12(nm950) III; lag-2(sa37) V; VTB82: dpy-20 (e1282) IV; maIs13(dpy-20+) -cki-1::GFP; MT1355: lin-1(e1777) IV; dpy-7 (e1324) sup-7(s5) X; MT2825: lin-1(e308) IV; nit-1(ref(he137)) IV; MT7949: lin-1(n176fgf) IV; SV325: lin-1(he117) IV; SV332: lin-1(he119) IV; SD51: let-60(ga89u) IV; MT5748: let-60(n202) IV; nit-1(ref(he137)) IV; MT1224: let-60(nm460g) IV; WP604: sos-1(c41s) VS; MT1821: lin-25 (e1466); SV107 IV; MT745: lin-1(he119) IV; MT296: cad-1(625) X; MT290: lin-1(nm950) II; lin-36(nm766) III; MT819: lin-1(nm765) X; MT465: dpy-5(e61) I; bli-2(e768) II; unc-3(2e189) III; MT464: unc-5(e53) IV; dpy-11(e224) V; lin-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 (pseudo-vulva) on the adult. Multiple lin-12(gf) strains (Table 1) were subjected to the following general protocol: new mutations were induced using ethyl-methanesulfonate (EMS) (Brenner, 1974) or TMP/UV (Yandell et al., 1994), 1–10 F1 hemaphrodites were transferred to new plates, F2 progeny were screened using a dissection scope to reveal the production of supernumerary (greater than 6) pseudovulvae. Further classification of defects was achieved by VPC analysis, described below.

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 h 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 Nomarski 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(eh119), lin-1(eh117) and lin-31(eh136), these mutations were tested for complementation with their respective null alleles, lin-1(e1777) and lin-31(nm301). mdt-13(eh135) 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(eh135) 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 and was examined for genetic complementation with ref-1(ok288). None of the ref(he137)/ref(1ok288) trans-heterozygous animals (n=5 broods) displayed the Ref phenotype.

Table 1

<table>
<thead>
<tr>
<th>Version</th>
<th>Genotype</th>
<th>Mutagen</th>
<th>n*</th>
<th>Isolated mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>lin-12(nm950)</td>
<td>EMS</td>
<td>2758</td>
<td>cdc-14(he118), lin-1(eh119)</td>
</tr>
<tr>
<td>2</td>
<td>lin-12(nm137)/460</td>
<td>EMS</td>
<td>2900</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>lin-12(nm137)/460</td>
<td>EMS</td>
<td>10,480</td>
<td>lin-1(eh117)</td>
</tr>
<tr>
<td>4</td>
<td>lin-12(nm137)/460</td>
<td>EMS</td>
<td>6550</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>lin-12(nm950); lag-2(sa37)</td>
<td>EMS</td>
<td>10,296</td>
<td>mdt-13(eh135), lin-31(eh136), ref(he137), he138</td>
</tr>
</tbody>
</table>

* Number of haploid genomes screened.
mdt-13 genetic analysis

For analyses of animals homozygous for the mdt-13(hc135), mdt-13(mn19) or mnDf66 mutations, strains harboring the mutant chromosomes balanced by mdr[tup-10(e128) mls14] were allowed to self-fertilize, and non-GFP-expressing animals were analyzed. These strains were intercrossed to examine hemizygotes (mdt-13(hc135)/mnDf66 and mdt-13(mn19)/mnDf66) and trans-heterozygotes (mdt-13(hc135)/mdt-13(mn19)). Double-stranded RNA corresponding to exon 12 of mdt-13 (between GAACATTGAA and ACACCTTGGC) 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 (Ld) if development arrested during larval stages or sterile (St) 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 Figs. 1B and C). 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), 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(n137/n460) (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 pre-
phenotype. An extraordinarily robust of two targets of Ras signaling, abnormal VPC-fate adoption by presumptive hypodermal P

mutant adult. Notice the posterior appearance of the extra pseudovulvae due to

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. 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. Astersisks indicate pseudovulvae. Animals are oriented as anterior, left and dorsal, up.

viously 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, 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).

Because 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.

Because we are interested in Elm mutations that disrupt cell-cycle regulation, we focused our analyses on the remaining

elm

genes.

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 30th 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 development of 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, 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 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(df)

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

Because

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 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. Although 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 3), 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 activity, respectively, to 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 ≤ 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

---

**Table 3**

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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. extra VPCs</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>cki-1(gk132)/+</td>
<td>0.3±0.5</td>
<td>22</td>
</tr>
<tr>
<td>cki-1(gk132)/++; lin-1(he119)</td>
<td>0.6±0.7</td>
<td>22</td>
</tr>
<tr>
<td>cdc-14(he118); lin-1(he119)</td>
<td>3.0±1.2</td>
<td>23</td>
</tr>
<tr>
<td>cdc-14(he118); lin-1(n1761gf)</td>
<td>2.9±0.9</td>
<td>30</td>
</tr>
<tr>
<td>lin-31(n301); cdc-14(he118)</td>
<td>1.9±1.2</td>
<td>17</td>
</tr>
<tr>
<td>lin-31(n301); cdc-14(he118); lin-1(n1761gf)</td>
<td>4.1±1.0</td>
<td>29</td>
</tr>
</tbody>
</table>

* b Number of L2 animals examined.

---

* a Presented as average number of VPCs displaying extra divisions/animal examined ±SD.

---

Fig. 3. Activation of the cki-1 promoter in arresting VPCs requires lin-1 and lin-31 activities. Nomarski (A and C) 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 ≥ 10).
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 co-regulator 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 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 pleiotropic 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 components we identified based on their roles in VPC quiescence largely correspond to the Cdk8 module (Borggreve et al., 2002; Carlson, 1997). This module is associated with a subset of Mediator complexes and has been implicated in transcriptional repression (see Discussion).

**Table 4**

<table>
<thead>
<tr>
<th><em>C. elegans</em> gene</th>
<th>Homolog</th>
<th>Let</th>
<th>Elm</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>mdt-1/sop-3</em></td>
<td>TRAP220/Med1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>mdt-7/let-49</em></td>
<td>Med7</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td><em>mdt-8</em></td>
<td>Med8</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td><em>mdt-10</em></td>
<td>Nu2/Med10</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td><em>mdt-11</em></td>
<td>Med11</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>mdt-12/sop-1/lpy-22</em></td>
<td>Trap230/Med12</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>mdt-13/let-19a</em></td>
<td>Trap240/Med13</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>mdt-14</em></td>
<td>Rgr1/Med14</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td><em>mdt-15</em></td>
<td>Arc105/Med15</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>mdt-18</em></td>
<td>Srb5/Med18</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td><em>mdt-19</em></td>
<td>Rox3/Med19</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td><em>mdt-22</em></td>
<td>Srb6/Med22</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td><em>mdt-23/sur-2b</em></td>
<td>Sur2/Med23</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td><em>mdt-27</em></td>
<td>Trap37/Med27</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>mdt-28</em></td>
<td>Med28</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>mdt-29</em></td>
<td>Med29</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>mdt-31</em></td>
<td>Soh1/Med31</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td><em>cdk-8a</em></td>
<td>Srb10/Cdk8</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td><em>cic-1</em></td>
<td>Srb11/Cyclin C</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Phenotypes were produced by RNAi treatment, unless otherwise noted.

a Phenotype examined in mutant animal and by RNAi treatment.
b Phenotype examined in mutant animal only.

**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 as 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 20 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 G1 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). Because 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 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 the 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 because 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 cyclin D or cyclin E promoter activation in the VPCs of mdt-13 mutant animals (data not shown). Wang et al. (2004) concluded from decreased reporter gene expression following RNAi knockdown that mdt-13 can activate transcription of certain target genes. 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. Because 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 elm genes to complete the description of the developmental network controlling cell-cycle quiescence will provide further insights toward our understanding of how the decision to divide during normal development is made.

Acknowledgments

We thank Victor Ambros, Patricia Ernst, Larry Myers and anonymous reviewers for careful reading and commenting on the manuscript. Some nematode strains used in this work were
provided by the National BioResource Project (NBRP) and the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR). This work was funded by the National Institutes of Health (SvDH), American Cancer Society (PF-98-114-01-DCC and IRG-82-003-21), the MGH fund for Medical Discovery and the Howard Hughes Medical Institute (76200-560801) to Dartmouth Medical School under the Biomedical Research Support Program for Medical Schools (RMS).

References


