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Transcriptome profiling of the *C. elegans* Rb ortholog reveals diverse developmental roles

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Abstract

LIN-35 is the single *C. elegans* ortholog of the mammalian pocket protein family members, pRb, p107, and p130. To gain insight into the roles of pocket proteins during development, a microarray analysis was performed with *lin-35* mutants. Stage-specific regulation patterns were revealed, indicating that LIN-35 plays diverse roles at distinct developmental stages. LIN-35 was found to repress the expression of many genes involved in cell proliferation in larvae, an activity that is carried out in conjunction with E2F. In addition, LIN-35 was found to regulate neuronal genes during embryogenesis and targets of the intestinal-specific GATA transcription factor, ELT-2, at multiple developmental stages. Additional findings suggest that LIN-35 functions in cell cycle regulation in embryos in a manner that is independent of E2F. A comparison of LIN-35-regulated genes with known fly and mammalian pocket protein targets revealed a high degree of overlap, indicating strong conservation of pocket protein functions in diverse phyla. Based on microarray results and our refinement of the *C. elegans* E2F consensus sequence, we were able to generate a comprehensive list of putative E2F-regulated genes in *C. elegans*. These results implicate a large number of genes previously unconnected to cell cycle control as having potential roles in this process.

Keywords: *lin-35*; Retinoblastoma; pRb; p107; p130; Pocket proteins; *C. elegans*; Development; Microarray

Introduction

The mammalian retinoblastoma protein (pRb) was amongst the first tumor suppressor proteins to be identified (Dryja et al., 1986; Friend et al., 1986; Lee et al., 1987), and loss or inactivation of pRb is thought to play a causative role in the majority of human cancers (Bindra and Glazer, 2006; Knudsen and Knudsen, 2006; Sherr, 2004; Sherr and McCormick, 2002; Yamasaki, 2003). Many studies on pRb, p107, and p130 have demonstrated their importance in the regulation of cell cycle progression, largely by binding E2F transcription factor family members using a cleft or "pocket" domain. This leads to the repression of E2F target genes and the consequent suppression of S-phase entry by inhibiting E2Fs promoting target gene expression (E2F1–3) or by augmenting E2Fs acting as transcriptional repressors (E2F4–6) (Dannenberg et al., 2000; Du and Dyson, 1999; Reed, 1997; Sage et al., 2000).

Although the interaction of pocket proteins with E2F family members is the most thoroughly characterized function of pRb-family members, they are far from the only transcription factors known to bind pocket proteins (Lipinski and Jacks, 1999; Morris and Dyson, 2001). For example, pRb has been reported to interact with C/EBPε to promote the terminal differentiation of granulocytes and adipocytes (Chen et al., 1996; Gery et al., 2004), with GATA-1 to induce erythrocyte differentiation (Rekhtman et al., 2003), and with MyoD, to promote myocyte differentiation (Gu et al., 1993). pRb also antagonizes the cell-differentiation inhibitor Id2, allowing for the terminal differentiation of several cell types (Iavarone et al., 1994; Lasorella et al., 2000). In addition, both pRb and p107 have been shown to act through the basic helix–loop–helix transcription factor, NeuroD1, which is critical for early development of the neural system in *Xenopus* (Batsche et al., 2005).

Notably, the majority of reports to date describe activities that have been observed in tissue culture systems and significantly less is known about pocket protein functions in the context of intact developing organisms. This lack of supporting in vivo data

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is in part due to the lethality of many pRb family knockouts in mice and flies (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992; Dimova et al., 2003; Du and Dyson, 1999; Wikenheiser-Brokamp, 2006). *C. elegans* has only a single pocket protein family member, *lin-35*, which is not essential for viability. In addition, *C. elegans* has only one DP homolog and three E2F homologs, further simplifying the analysis of the role of pocket proteins in this organism. Studies have demonstrated that *lin-35* also functions in G1–S-phase regulation during development (Boxem and van den Heuvel, 2001; Fay, 2005; Fay et al., 2002) and plays a number of diverse roles outside of cell cycle control. For example, *lin-35* has been shown to repress vulval developmental fates (Ceol and Horvitz, 2001; Lu and Horvitz, 1998) and to play roles in pharynx and vulva morphogenesis (Bender et al., 2007; Fay et al., 2003, 2004), asymmetric cell division (Cui et al., 2004), somatic gonad cell fate (Bender et al., 2004), larval growth (Cardoso et al., 2005; Chesney et al., 2006; Cui et al., 2004), germline gene repression (Wang et al., 2005) and even RNA interference (Lehner et al., 2006; Wang et al., 2005).

In this study, a microarray analysis was performed using *lin-35* null mutants to determine the role of this protein at different developmental stages in the organism and to identify which proteins and pathways it may be acting through. A number of differentially regulated genes identified in this study were homologous to mammalian genes also regulated by pocket proteins, suggesting a strong conservation of function of pRb-family members between nematodes and mammals. We have also refined the nematode E2F consensus binding site and have generated a compelling list of putative E2F targets in this organism. This, along with the discovery of several novel motifs that could represent binding sites for additional LIN-35 regulatory partners, may substantially expand the network of pRb-family-regulated genes and help to elucidate the routes by which they function.

Materials and methods

Strains and maintenance

All *C. elegans* strains were maintained according to established protocols (Stiernagle, 2006). All experiments were carried out at 20 °C. Strains used in these studies included the following: N2 (wild type) and of *lin-35(n745)* (Lu and Horvitz, 1998), which had been additionally backcrossed by our laboratory 5×.

Staging of organisms

Wild-type and mutant worms were staged by bleaching gravid adults to collect eggs, which were hatched onto NGM plates in the absence of food. After hatching, worms were transferred to NGM plates with bacteria (OP50). RNA harvested for embryonic stage worms was obtained from a total preparation of embryos. As a result of extracting embryos from young adult stage worms, predominantly early stage embryos were obtained. The differences in embryos between strains were similar to the differences between replicates in the same strain. For RNA from L1 stage larvae, collection occurred ~1 h prior to the apoptosis of T.ppp, which occurred ~10 or 11 h after hatching in N2 and *lin-35* mutants, respectively (Sulston and Horvitz, 1977). For L4 stage larvae, a distinct Christmas tree vulval morphology was used as a biomarker.

RNA extraction and microarrays

Embryos or larvae were washed off of NGM plates using M9. RNA was extracted using phenol followed by phenol:chloroform:isoamyl alcohol. RNA was precipitated overnight at 4 °C with 4 M lithium acetate and ethanol. RNA was DNase treated, purified on RNEasy minicolumns (QIAGEN) and verified to be free of genomic DNA contamination by quantitative reverse transcription-mediated real time PCR (qRT-PCR). cDNA synthesis, cRNA synthesis, labeling, fragmentation, GeneChip hybridization and scanning were performed according to specifications from Affymetrix. Triplicate chips were run for each strain at each stage. RNA and cDNA qualities were verified at each step using capillary electrophoresis (Bioanalyzer; Agilent Technologies). Gene expression values were determined using Robust Multiarray Analysis software (Irizarry et al., 2003). Relevant expression differences were identified using a modified Wilcoxon rank test (for details, see Results).

qRT-PCR analysis

cDNA synthesis reactions were performed with SuperScriptII (Invitrogen), and reactions contained 5 mg RNA were purified as above. Approximately 20 ng of cDNA was used for each qRT-PCR reaction. qRT-PCR reactions were performed in an iCycler iQ (Bio-Rad), using an SYBR Green fluorophore. Control reactions were performed using actin-specific primers, and threshold cycles for all reactions were normalized using a D/DCt method.

Regulatory motif analysis

To identify candidate regulatory sequences in the 5′ non-coding regions of putative *C. elegans* homologs of p107/p130-target genes, 1 kilobase of sequence data upstream of the start codon of each gene was retrieved from *C. elegans* genomic information using Regulatory Sequence Analysis Tools software (http://embnet.ccg.unam.mx/rsa-tools/). Regions of DNA with low sequence complexity were masked using RepeatMasker (http://repeatmasker.org), and then searched for overrepresented motifs using the program MEME (Multiple Em for Motif Elicitation) (http://meme.sdsc.edu/meme/intro.html). This analysis was performed locally, using background models of the fourth order. Motif sequence logos were created using an online program (http://weblogo.berkeley.edu/logo.cgi).

To verify the relevance of motifs returned by the software, a PWM was generated for each motif by MEME. For the E2F consensus site, this matrix was applied to all 1949 responsive genes, and RSAT software was used to examine the relative representation of the motif in genes within the list of responsive genes as compared with the entire genome. This analysis was performed with two different types of cutoff ranges, using scores obtained by applying the PWM to the relevant genes. First, successive ranges of cutoff scores were used. Score ranges were obtained using cutoff scores between the highest score obtained and sequentially lower scores (yielding sequentially larger ranges with lower cutoffs). Second, interval score cutoffs were examined. These were obtained using PWM score intervals (with a range of two between selected points).

For the other reported motifs, sequences were derived and PWM were generated as above. Motifs were reported if they exhibited significant ($p<0.01$) overrepresentation in genes that were in the cluster (see below) within which the motif was identified compared to the remaining non-E2F genes, overrepresentation within the non-E2F-regulated genes as compared with E2F-regulated genes and overrepresentation within the non-E2F-responsive genes as compared with the entire genome. The cutoff scores for these calculations were set by the lowest score obtained by applying the PWM to the genes that the motif was identified within.

Clustering

Gene expression levels were normalized to gene-specific relative maxima across both strains and all stages (six points per gene). Genes were then divided into ten clusters using profiles that were specific for strains and stages. Clustering was performed using MultiExperiment Viewer software (http://www.tm4.org/mev.html), with a number of different clustering algorithms tested, including Hierarchical Clustering, K-Means/K-Medians Support, Self-Organizing Maps,

K-Means/K-Medians Clustering and Quantitative Threshold Clustering. K-Means/K-Medians clustering yielded groupings of genes that were the closest to the average expression line for each cluster, so this method was used to further divide the differentially expressed genes in each cluster into subclusters according to their fold-change profile. This division was based on relative expression level of the genes in the cluster.

Results

Time points and primary data analysis

To identify genes that are responsive to LIN-35 during development, RNA was harvested from synchronized *lin-35 (n745)* mutant and wild-type (N2) worms at embryonic, L1 and L4 stages. Our specific time points were chosen in order to provide information about LIN-35 activities at several distinct developmental stages and because previous studies have implicated biological roles for LIN-35 during these stages (also see Introduction). To ensure the precise developmental synchronization of animal populations for these studies, we used several stage-specific developmental markers as opposed to chronological timing, which can be variable between strains and individual replicates (see Materials and methods). RNA was purified from each stage and used to generate cRNA, which was fragmented and hybridized to *C. elegans* Affymetrix GeneChips®. Three independent biological replicates were performed for each stage and expression values for each gene were generated using Robust Multiarray Analysis software (Irizarry et al., 2003).

To determine relevant changes in gene expression, a modified Wilcoxon rank test was performed using a minimal quotient of >1.15, which has been shown to efficiently eliminate false positives and to yield results with high biological relevance (Braatsch et al., 2004; Harpster et al., 2006; Moskvin et al., 2005; Pappas et al., 2004; Zeller et al., 2005). In addition, a minimal mean and median fold-change limit for individual genes was set at 1.5, which was stringent enough to return an analytically manageable number of genes while still retaining sufficient sensitivity. Using these criteria, a false discovery rate of <1.5% was calculated, suggesting that these applied conditions were highly effective in identifying statistically significant, differentially regulated genes (data not shown).

Overview of stage-specific transcriptional responses

In this array study, a total of 1,949 genes showed altered expression in the *lin-35* mutant background as compared with wild type. There were 642 differentially regulated genes at embryonic stages, 945 at L1 and 1108 at the L4 stage; a full list of these genes can be located in Fig. S1. Importantly, a number of genes previously demonstrated to be regulated by pRb–E2F complexes in multiple species, including human, mouse and *Drosophila*, were amongst the targets identified (e.g., cyclin A, cyclin E, thymidylate synthase, BRCA, *mcm-2*, *mcm-3*, *mcm-4* and *mcm-5*). Moreover, consistent with the notion that LIN-35/pRB functions primarily as a transcriptional co-repressor, the large majority of identified responsive genes were upregulated in the *lin-35* null background; 81% in embryos, 70% at L1 and 64% at L4. To further validate the array findings, eleven genes with different developmental expression patterns and different biological functions were selected as targets for RT-PCR (Fig. 1). Although the microarray and RT-PCR data are not identical in all cases, they correlate well, strengthening the legitimacy of the array results.

Of the total 1,306 upregulated, responsive genes, only 123 (9%) were found to be differentially regulated at all three developmental stages (Fig. 2A). This relative lack of overlap is not simply due to a paucity of differentially regulated genes at any given stage, but more likely reflects distinct biological roles for LIN-35 at specific stages of development. In binary comparisons, L1 and L4 stages showed the greatest overlap with 33% (335/1029), despite the greater temporal separation of these stages as compared with L1 larvae and embryos. Furthermore, the overlapped genes showed a strong correlation in their

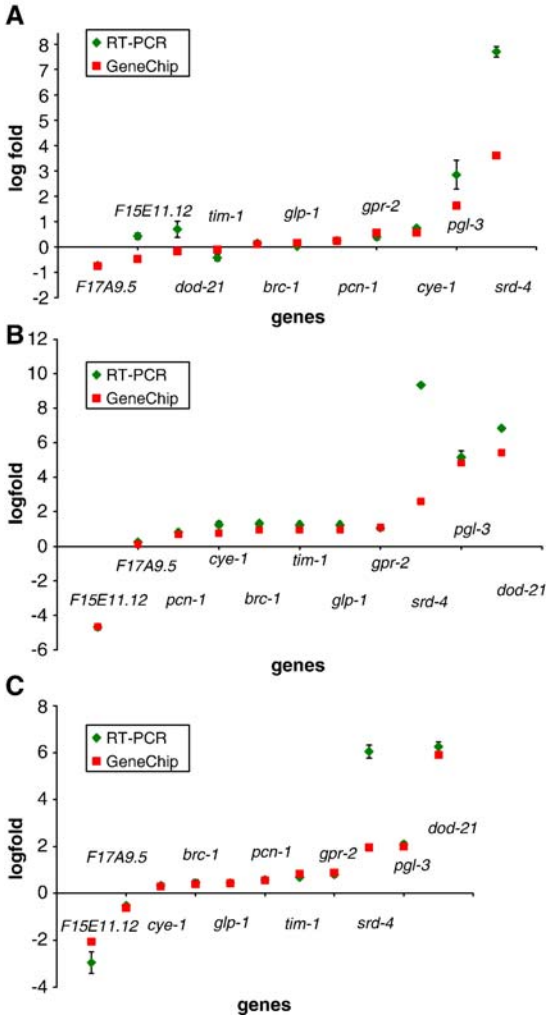


Fig. 1. Comparison of RT-PCR and GeneChip Data. RNA from (A) embryonic, (B) L1 and (C) L4 stage worms was used for RT-PCR. At least 6 reactions were performed for each gene. Error bars represent standard error (SE) for all replicates. Genes with well-characterized differential regulation showed appropriate expression levels in both GeneChip® and RT-PCR data (e.g., *cye-1*, *pcn-1*, *pgl-3*). Discrepancies in expression levels (e.g., *srd-4*) are likely due to a single primer set used for RT-PCR and averaging of multiple primer sets for microarray data.

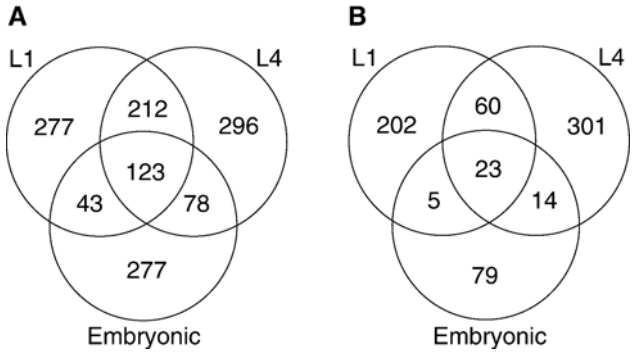


Fig. 2. *lin-35*-responsive genes at different stages. Venn diagrams representing overlap between upregulated (A) and downregulated (B) genes responding to the *lin-35* mutation at L1, L4 and embryonic stages.

patterns of differential expression (Fig. S2). In contrast, L1 and L4 stages showed only a 16% (166/1010) and 20% (201/1029) overlap with embryos respectively (Fig. 2A). Furthermore, the behavior of genes in the embryonic population did not strongly correlate with the expression patterns of these genes in larvae; a large number of genes that were upregulated in embryos were downregulated in larval stages (Fig. S2). These data suggest substantive differences in the nature of LIN-35 function at different developmental stages, particularly in larvae and embryos.

Considerably fewer genes were observed to be downregulated in *lin-35* mutants: 19% in embryos, 30% in L1 and 36% in L4. The increase in the number of genes specifically downregulated across developmental stages, from 79 in embryos to 301 in L4 stage worms, argues that some of this downregulation may be a result of non-specific causes. Furthermore, the degree of overlap observed in downregulated genes compared to upregulated genes (Fig. 2B) also suggests that much of the observed downregulation may be due to secondary effects.

With these analyses in hand, the responsive genes were divided into functional categories based on gene ontologies (GOs) and literature searches. Consistent with the general lack of overlap between certain developmental stages (Fig. 2), stage-specific profiles emerged. Both L1 and L4 stages showed a statistically significant enrichment for responsive genes characterized as having "extended cell cycle" functions, including genes involved in cell cycle control, DNA replication and metabolism, mitosis and meiosis (Fig. S3). Nearly all (93%) of the differentially regulated genes belonging to this extended cell cycle category were upregulated. The strong bias of enrichment and regulation of cell cycle-related genes indicates that LIN-35 plays an important role in controlling these processes during larval development.

In embryos, a significantly larger number of genes associated with neurogenesis and neurotransmission were represented as compared with larval stages (Fig. S3). This finding suggests a previously unknown role for LIN-35 in the nervous system during embryonic development. In stark contrast with larval stages, genes involved in cell cycle, DNA metabolism and cell maintenance were not statistically overrepresented at this stage (Fig. S3). These latter results indicate that LIN-35 may not play a major role in cell cycle regulation during embryogenesis.

Categorical analysis was further corroborated by evaluating stage-specific responses within the context of gene mountains (Kim et al., 2001). Mountain analysis first groups co-regulated genes, using a meta-array approach, and then attempts to ascribe functions to the mounts based on representative genes with established functions or known expression patterns. L1 and L4 stage mountain distributions showed expression patterns consistent with enrichment of meiosis and mitosis, and retinoblastoma complex members (Figs. S4B and C). Mountain analysis of embryos showed a dramatic overrepresentation of responsive genes in a mount associated with neuronal genes (Fig. S4A). These results corroborate those from those of the GO analysis and strengthen the hypothesis that LIN-35 may play an unrecognized role in the nervous system of *C. elegans* embryos.

Two mountains (7 and 11) described to possess germline genes were also overrepresented. Genes in both mountains were strongly enriched in E2F sites (Fig. S4), consistent with the involvement of LIN-35 in the direct regulation of these genes (also see below) and with previous reports demonstrating LIN-35 repression of germline-associated genes in somatic tissues (Wang et al., 2005).

Comparison of pocket protein targets from different species

Genes previously demonstrated to be responsive to mammalian pRb (Finocchiaro et al., 2005) or to p107/p130 (Balciunaite et al., 2005) were used to identify best genome sequence matches from *C. elegans*. Genes were considered to be putative orthologs if a reciprocal search returned the original pocket protein target in *H. sapiens*. Based on this approach, >50% of the mammalian pocket protein responsive genes were found to possess likely *C. elegans* orthologs. Cross-referencing these genes with the list of LIN-35-responsive genes revealed that ~30% of the putative mammalian orthologs were also targets for regulation by LIN-35, a percentage that is highly statistically significant (Fig. S5B). Furthermore, similar levels of overlap were observed for both pRb and p107/p130 target genes with the identified LIN-35-responsive genes. This indicates that LIN-35 may carry out ancestral-type functions that are common to all three mammalian family members. This finding is also consistent with the placement of LIN-35 equally distant from all three mammalian pocket proteins and both *Drosophila* pocket proteins and as being located in an ancestral position to all five (Fig. S5A).

The majority of overlap between LIN-35 and the mammalian pocket proteins came from targets that were differentially regulated at either the L1 stage or at both the L1 and L4 stages. Polymerase subunits, replication factors, cyclins and histones were among the overlapping genes that are targets of the human and *C. elegans* pocket proteins (see Fig. S6 for a full list). As has previously been noted, many of these targets are known to act in cell cycle control and DNA replication, further demonstrating a conservation of function between LIN-35 and the mammalian pocket proteins.

In contrast, only nine of the 94 genes overlapping between LIN-35 and the mammalian pocket proteins were differentially regulated in embryos. This is consistent with previous data, such as the lack of conserved targets between embryonic and larval stages, further indicating distinct functions for LIN-35 in embryos that are unconnected to the cell cycle. Similar to embryos, LIN-35-responsive genes in the germline (Chi and Reinke, 2006), also failed to show high levels of overlap with the mammalian targets, indicating that LIN-35 plays a unique role in this tissue as well (Fig. S5). In both that study and in this one, responsive genes in embryos and the germline appeared to play a more prominent role in differentiation than in the extended cell cycle functions. We also note that the overlap of the mammalian pocket protein targets with genes responsive to the *Drosophila* pocket proteins, though statistically significant, was much weaker than that observed for LIN-35. This may reflect more divergent functions for the *Drosophila* proteins or may be due to differences in the experimental approaches used in these studies.

Search for evolutionarily-conserved Rb family binding partners

In order to find conserved Rb-family binding partners, the LIN-35-responsive orthologs of the mammalian p107/p130-responsive genes were searched for overrepresented motifs. Genes overlapping with p107/p130 targets were chosen instead of pRb-responsive genes because these data were derived from ChIP experiments rather than from a meta-analysis of multiple microarray experiments, thereby increasing the probability that the derived targets would be directly regulated by LIN-35. One kilobase of upstream sequence for each gene was retrieved from a *C. elegans* genomic database, regions of low sequence complexity were masked and an unbiased search for over-represented motifs was performed using MEME (see Materials and methods). This analysis returned a single motif (TTTSSCG-CGC) that was enriched in both the genes that overlapped with mammals and in the entire list of *lin-35*-responsive genes (Fig. 3A). This sequence bore a strong similarity to the consensus E2F binding site (TTTSGCGC) from mammals and also to a recently described consensus binding site for E2F in *C. elegans* (TTCSC-GCS) (Chi and Reinke, 2006). Given that this was the only motif enriched among this group of genes, E2F may be the only pocket protein binding partner in these organisms that is strongly evolutionarily conserved. While other conserved Rb-interacting proteins may exist and may play roles in limited temporal and/or spatial circumstances, their absence from this analysis suggests that they are not involved in organism-wide gene regulation.

E2F-regulated genes in C. elegans

A position–weight matrix (PWM) was derived for the putative *C. elegans* E2F consensus binding site (described above; see Materials and methods). A PWM determines a weighted probability of particular nucleotides occurring at individual motif positions based on the degree of conservation. This PWM was used to search the promoter regions of the 1949 LIN-35-responsive genes. Scores were generated for each gene containing a putative E2F motif, with higher scores indicating a better match. Several genes known to function in DNA replication and mitosis (including *mcm-3* and *mcm-4*, *pcn-1* and *pri-2*) obtained high scores using this matrix.

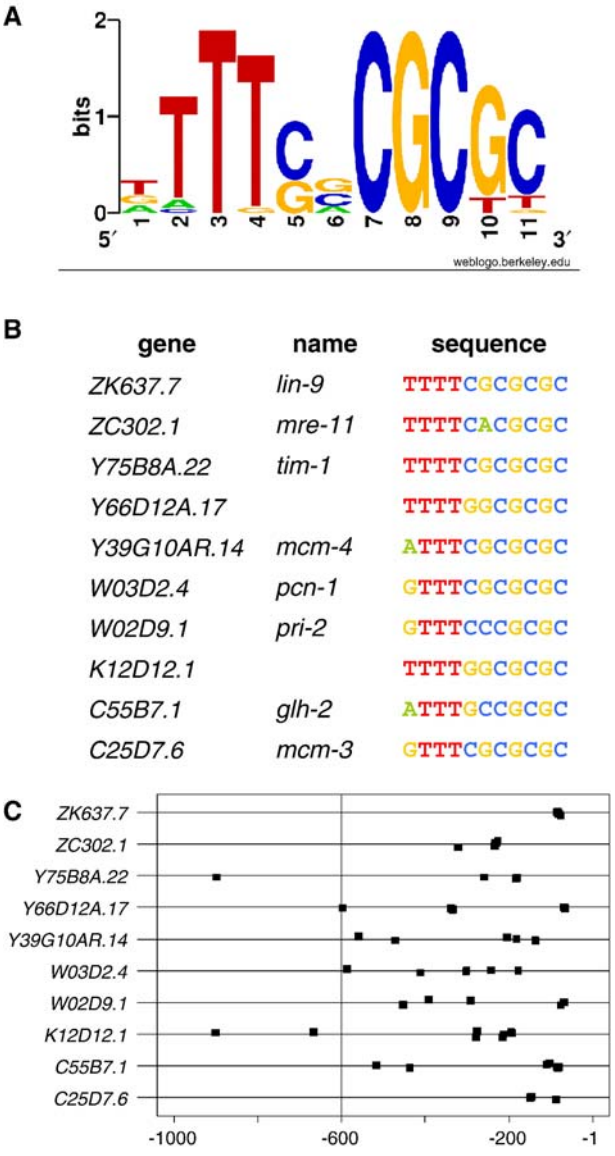


Fig. 3. Refinement of the *C. elegans* E2F binding site motif. The *C. elegans* E2F binding site consensus motif (A) was refined using an unbiased set of conserved genes (see text for details). Example genes (B) show clustering of this motif toward their transcriptional start sites (C).

The motif was found to be significantly ($p<0.01$) over-represented in our list of LIN-35-responsive genes at all three developmental stages (see Materials and methods for criteria), suggesting that this motif has biological significance. Furthermore, a strong correlation was observed between the PWM score and the probability that a given gene would be upregulated in the *lin-35 null* mutant background. For example, amongst the 50 best-scoring LIN-35-responsive genes from the L1 stage, all genes were observed to be upregulated in *lin-35* mutants ($p \ll 0.01$) compared to 70% of genes among all L1 stage responsive genes. In contrast, the presence of a high-

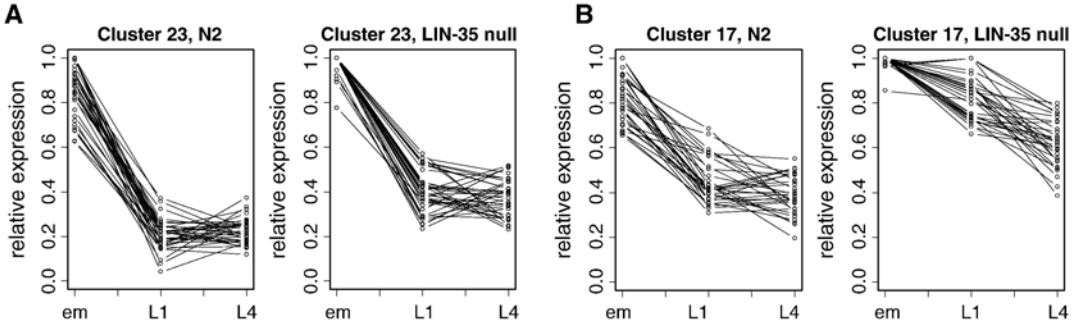
scoring E2F motif was not found to be a reliable predictor of gene behavior in embryos, further indicating that the binding factor(s) acting through this motif is functioning differently at this stage.

To generate a list of putative E2F-target genes from our data set, several criteria were applied. First, candidate genes had to have a minimum score of 4 (based on several selective criteria, data not shown) from PWM application. Second, the nucleotides at positions 3, 5, 7, 8 and 9 had to conform to the refined motif (Fig. 3A). Third, the gene had to have at least one consensus E2F site within 600 base pairs of the transcriptional start site, as has been previously shown to return biologically relevant data (Chi and Reinke, 2006). Of the 1949 responsive genes on the list, 452 met all three criteria. The presence of many known E2F targets (including cyclin A, cyclin E, thymidylate synthase and *mcm* genes) demonstrates the validity of this algorithm (see Fig. S7 for a complete list).

The 452 genes identified shared several important characteristics. First, they demonstrated enrichment in cell cycle and DNA maintenance functions. Genes with this consensus site also often contained multiple copies (Fig. 3B) and these motifs were generally biased toward transcriptional start sites (Fig. 3C). Finally, an examination of homologs in *C. briggsae* (a species closely related to *C. elegans*) revealed that 75% of these genes contained at least one E2F binding site in their proximal promoter regions, suggesting that this regulatory pathway is well conserved.

As a means for further analysis, the putative E2F target genes were divided into 25 clusters based on their expression profiles using a K-means/K-medians clustering (KMC) algorithm (see Materials and methods) (Fig. S8). Of these, 197 genes fell into nine clusters strongly enriched for cell cycle genes ($p \ll 0.01$). The gross expression patterns exhibited by these genes in wild-type animals showed a striking similarity between the clusters (Fig. 4), which was attenuated in null mutants. This pattern suggests that LIN-35 is used to repress expression of these genes upon entry into larval stages of development. However, some decrease in the expression of these genes in *lin-35* mutants suggests that other transcriptional regulators could be acting in parallel to *lin-35* in this process.

It is noteworthy that many genes not previously annotated to play a role in cell cycle and replication functions were also located in these nine clusters. For example, *gpr-2*, a protein regulating chromosomal and spindle movements, possesses a number of high-scoring E2F sites and was upregulated at all three observed stages. TIM-1 (timeless), another protein upregulated at both larval stages, participates in the regulation of developmental timing in other systems (Gotter, 2006; Hardin, 2005; Myers et al., 1995). A number of transcription factors, most of which have not been previously connected to cell cycle control (e.g., *cbp-1*, *mdl-1*, *lin-59*, *C18G1.2*), were also found. The existence of nearly two hundred proteins in these clusters potentially expands the network of Rb/E2F cell cycle functions quite considerably.



gene name	gene name	gene name
B0393.3	*gln-5*	*pcn-1*
C25A1.9	*gln-6*	*R107.4*
C35D10.13	*him-10 s*	*spn-4*
C50B6.2	*htz-1*	*T12F5.1*
cbp-1	*icp-1*	*T22C1.4*
cdt-1	*K07A1.2*	*tag-349*
csc-1	*kbp-4*	*Y48A6B.11*
cye-1	*lig-1*	*Y5F2A.2*
dom-6	*lsy-2*	*ZK154.5*
efl-1	*mcm-7*	*ZK643.2*
F11E6.7	*mes-4*	*zyg-9*
F55C5.4	*nhr-3*	

gene name	gene name	gene name
ani-1	*mcm-2*	*ptc-1*
tag-255	*smc-4*	*gpr-1*
cls-2	*mix-1*	*C01H6.9*
chk-1	*unc-71*	*lin-13*
plk-1	*kup-1*	*Y69A2AR.28*
mdf-2	*smo-1*	*C55C3.5*
F25H5.5	*F26H9.4*	*F14B8.2*
cku-80	*T28B11.1*	*T10E9.8*
polk-1	*brc-1*	*M03F8.1*
Y110A7A.4	*lst-4*	*F33H2.3*

Fig. 4. Expression profiles for genes with E2F binding sites. Two representative clusters (A and B) of responsive genes with E2F binding sites that are enriched in genes with cell cycle functions are shown. Graphs on the left of each panel represent relative expression levels for each gene in N2, graphs on the right represent relative expression levels for the same genes in *lin-35* mutants. Also shown are lists of the genes contained in the clusters. Panel A represents the cluster with the best average score when a PWM is applied, panel B represents a cluster with a lower average score. Lines are intended to facilitate visualization of cluster patterns and are not intended to represent proximal chronological steps.

Identification of four potential non-E2F LIN-35 regulatory sites

The remaining 1,497 responsive genes that did not contain consensus E2F binding sites were divided into clusters and subclusters using a KMC algorithm (see Materials and methods). Upstream sequence data from each gene in these subclusters were retrieved, masked and searched for overrepresented motifs (see Materials and methods). In order to identify biologically relevant motifs amongst those returned by the software, a number of significance thresholds ($p<0.01$) were applied (also see Materials and methods). Among these, the motif had to be significantly overrepresented in the cluster compared to the list of non-E2F-responsive genes and also had to be overrepresented in the non-E2F-responsive genes compared to the 452 E2F-responsive genes and to the entire *C. elegans* genome.

This algorithm was used to identify two very precise motifs present exclusively in responsive genes that were downregulated at the L1 stage. The first motif (TWTAGATCTRGGACAGW-GATAA, Fig. 5A) was found in four genes encoding major sperm proteins (MSP). This motif was long, very highly conserved and was found in less than 10 genes in the entire genome. This motif was also located at nearly the same position with respect to the transcription initiation site in all of these genes, a relatively uncommon feature amongst promoter binding sites. The second motif was preferentially located in heat shock proteins (Fig. 5B). This motif (CAYTYGARCTGCTT) was located in twelve genes in the genome and was found in five genes in the subcluster from which it was identified. These five genes were the only responsive genes containing this motif. This motif was also highly conserved and seemed to show a bias toward the −1 promoter position. The identification of both a significant proportion of the genes in the genome as well as the clustering together of all responsive genes containing these motifs demonstrates the validity of using this sequential clustering technique.

A more general motif (AYTGATAAVA) with a GATA core was identified in a large number of the responsive genes (Fig. 5C). This motif, independently discovered by an unbiased search of clusters (as described above), nearly perfectly matches

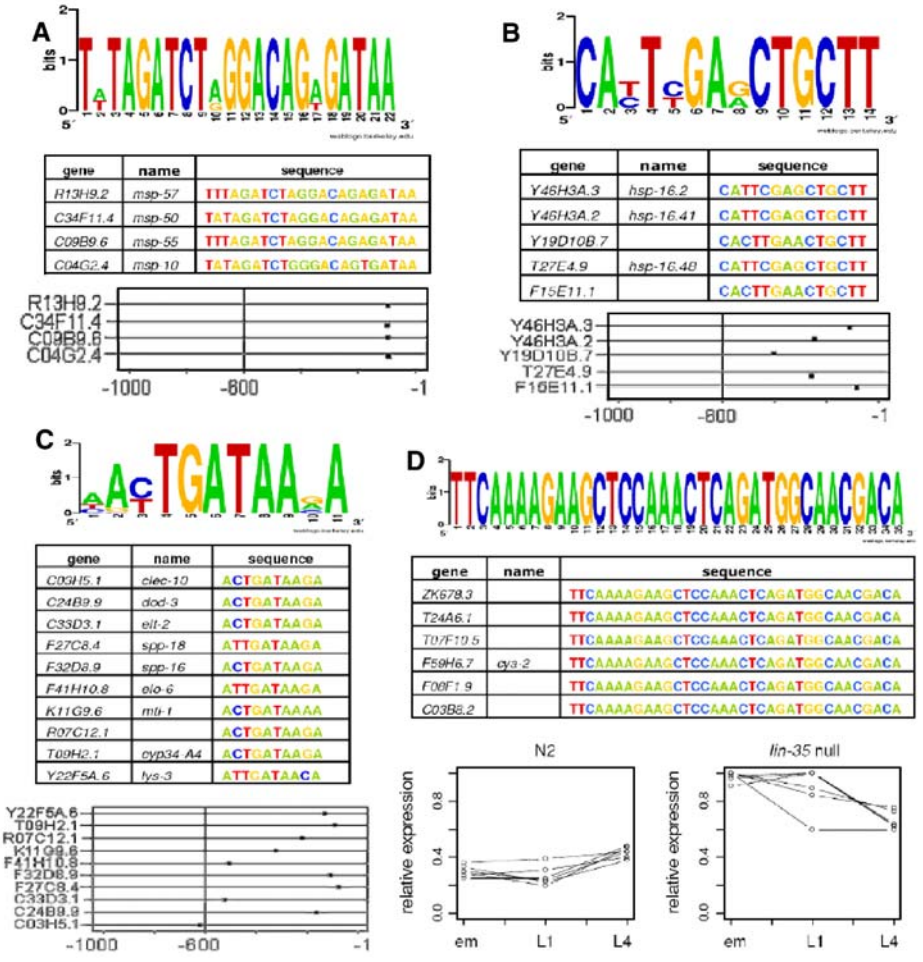


Fig. 5. Identification of non-E2F binding elements in *lin-35*-responsive genes. (A) A motif enriched in four major sperm proteins that were downregulated at the L1 stage. (B) A motif enriched in heat shock proteins that were downregulated at larval stages. (C) A motif with a GATA core that was identified in 528 genes, examples of which are listed. This motif is frequently located in intestinal genes and is clustered toward transcriptional start sites. (D) A motif that was identified in six cyclin A-like that were strongly upregulated in embryos but did not show a localization bias. Lines in panel D are intended to facilitate visualization of cluster patterns and are not intended to represent proximal chronological steps.

a recently refined consensus ELT-2 GATA motif (McGhee et al., 2007). ELT-2 is known to be the major regulator of intestinal development in *C. elegans* (Fukushige et al., 1999). As transcription factors that bind GATA sites are known to be capable of enhancing transcription from distances much farther than one kilobase, upstream regions up to three kilobases were examined for this site in the complete set of 1,949 responsive genes. A total of 528 genes were identified containing this consensus motif. Searching a serial analysis of gene expression (SAGE) library prepared from intestinal tissue of dissected *glp-4* adult *C. elegans* worms (SWAG1) returned approximately half of these genes in common (McGhee et al., 2007), and 57% of the putative *C. briggase* orthologs of these genes were found to contain the GATA consensus site. Furthermore, the observed overlap between the two lists is particularly significant since different developmental stages were examined in the different experiments (embryos and L1 and L4 larvae in this study and adults in the SAGE library). These results are also consistent with the observed enrichment of genes located in an intestinal mount at all examined stages (see Fig. S4). These findings suggest that LIN-35 may act through ELT-2 to control intestinal-associated gene expression. Finally, this is also consistent with a demonstrated role for LIN-35/Rb in pharyngeal development (Fay et al., 2004), as a statistically significant overlap ($p < 0.05$) between our list of differentially expressed genes and genes involved in foregut development was observed (Gaudet et al., 2004).

In addition to the motifs already mentioned, a fourth, very specific, motif was located by clustering genes that were responsive at the embryonic stage (Fig. 5D). Although the thirty-five nucleotide motif obtained (TTCAAAAGAAGCTCCAAACTCAGATGGCAACGACA) was completely conserved, it did not show a location bias toward the transcriptional start site (data not shown). Six of the seven genes in the genome containing this site showed a response in *lin-35* mutants at the embryonic stage. Five of these genes were annotated as cyclin A-like genes and the sixth, annotated as an uncharacterized protein, returned cyclin A as the best result from a BLAST search. Expression levels of these genes were very dissimilar in N2 and *lin-35 null* worms, with expression of these genes being significantly higher in the mutants. Additionally, only two of these genes contain consensus E2F binding sites. The absence of consensus binding sites in the remaining genes, combined with their expression patterns in wild-type and mutant worms, strongly suggests that these genes are being regulated by LIN-35, but that this regulation is largely taking place through a non-E2F transcription factor. As these genes are annotated as cyclins, this suggests that LIN-35 may play a role in cell cycle regulation in the embryo independently of E2F.

Discussion

In this study, a total of 1949 genes were found to be responsive to a null mutation in *lin-35*, the sole pocket protein family member in *C. elegans*. While it is somewhat surprising that these organisms can function with misregulation of upwards of five hundred genes at each developmental stage, they appear to suffer little more than a developmental lag. Their ability to survive this misregulation is possibly due to compensatory changes in the regulated expression of other genes, alterations in protein activities and differences in polypeptide clearance. This is in marked contrast with other developmental models, such as mouse and fruit fly, where null alleles of pRb and its fly homolog, dRbF1, are lethal (Clarke et al., 1992; Du and Dyson, 1999; Jacks et al., 1992; Lee et al., 1992).

Despite the lack of an overt phenotype, our analysis demonstrates a remarkable conservation of function between LIN-35/pRb and pocket proteins from other organisms. LIN-35/pRb regulates a large number of genes involved in cell cycle progression, replication and mitosis, most of which are upregulated in the *lin-35 null* mutant background (Fig. 2; Figs. S1 and S3), similar to findings in humans and flies (Balciunaite et al., 2005; Black et al., 2003; Dimova et al., 2003; Finocchiaro et al., 2005; Markey et al., 2002; Muller et al., 2001). In fact, the greater overlap observed between the mammalian pocket protein targets and LIN-35 versus *Drosophila* RbF attests to the strength of this system for analyzing pRb family functions (Fig. S5).

Our results also strongly indicate that LIN-35/Rb carries out cell cycle functions largely by acting through E2F family transcription factors, consistent with findings in other organisms (Du and Pogoriler, 2006; Korenjak and Brehm, 2005; Stevaux et al., 2005). Interestingly, the observed misregulation of genes with cell cycle functions in *lin-35* mutants is much more prevalent during larval stages than in embryos, where expression levels of genes containing E2F binding motifs remain relatively unchanged, suggesting that LIN-35/Rb-E2F complexes are largely superfluous for this role in embryos. In accordance with these observations, CyclinD, which is known to act with cyclin-dependent kinases to inhibit pocket protein activity, is dispensable in worm and mouse embryos (Lukas et al., 1995; Park and Krause, 1999). Most interestingly, our results also provide compelling evidence that LIN-35/Rb may control the expression of certain cell cycle genes, specifically those encoding cyclin A family members, during embryogenesis through a mechanism that is independent of E2F binding (Fig. 5D). We also note that similar to embryos, recent microarray studies have shown LIN-35/Rb to play only a minor role in cell cycle regulation in the germline (Chi and Reinke, 2006; Wikenheiser-Brokamp, 2006). Collectively, these findings indicate that cell cycle regulation occurs via distinct mechanisms in different tissues of *C. elegans* and at different times in development.

Since the E2F family of transcription factors is one of the most thoroughly studied groups of pRb binding factors, we undertook an in-depth analysis of the LIN-35/Rb-responsive genes in order to further delineate the *C. elegans* E2F motif and to generate a high-confidence list of over 450 putative targets of the LIN-35/Rb-E2F complex in *C. elegans*. Of these genes, nearly 200 were clustered groups that were strongly enriched in genes with cell cycle functions. Many of these genes are directly involved in cell cycle control and replication and division processes, such as CDKs, cyclins and helicases, and regulators of mitosis. Interestingly, a substantial number of the differentially regulated genes containing this site have no previous connection to these processes, and their identification may serve

as a valuable resource for finding genes with previously unappreciated roles in cell cycle control and progression. It is also important to note that some known pRb/E2F-regulated genes did not make our list E2F targets. For example, ribonucleotide reductase (*rnr-1*) failed to make the list of responsive genes because its fold-change was only 1.4, which was below our cutoff. Furthermore, *bub-1*, another known pRb/E2F target that was identified as being responsive to *lin-35*, failed to make the list because its E2F site did not match sufficiently close to the derived consensus. We contend that the existence of false negatives among our data set was a necessary tradeoff in order to minimize the occurrence of false positives.

In addition to a role in cell cycle regulation, our findings indicate that LIN-35/Rb may play a role in neurological development and neurotransmission. This hypothesized function seems to be important predominantly in embryos since the proportion of genes annotated with these functions drops precipitously by the L1 stage. While this function was somewhat unexpected, pocket proteins have been previously implicated in the differentiation of neural cells in mammals and amphibians (Batsche et al., 2005; Ferguson and Slack, 2001; Slack and Miller, 1996), suggesting that a role in the nervous system may be conserved.

Our analysis also yielded four additional motifs that may constitute target sites for LIN-35 regulation. One of these motifs was located in the upstream regions of major sperm proteins. Another motif is found in the promoter regions of heat shock proteins, which is consistent with reports of pRb playing a role in stress erythropoiesis in mammals (Spike et al., 2004). As discussed above, a third motif was located in genes differentially regulated in embryos that were annotated to be cyclin-A related. A fourth motif, containing a GATA core, has recently been identified as the binding site for ELT-2 (McGhee et al., 2007), the major regulator of intestinal development in worms. This finding is unlikely to be the result of LIN-35/pRb–E2F transcriptional regulation of the *elt-2* gene, however, as the upstream regulatory region is devoid of consensus E2F binding sites. In agreement with this finding, an E2F-independent role of pRb in intestinal differentiation in mice has been observed (Haigis et al., 2006). Further, a region of pRb overlapping the E2F binding site (Dick and Dyson, 2003) has been shown to be tethered to another GATA transcription factor, GATA-1, resulting in repression of its activity (Rekhtman et al., 2003). Thus, a role in the regulation GATA targets by pocket proteins may also be conserved.

While we do not currently know whether the identified role of LIN-35/pRb in regulating genes involved in intestinal and neuronal differentiation is direct, future experiments will address this question, as well as verifying the roles of some of the other genes predicted to function in cell cycle regulation. Regardless of whether this regulation is direct, similar findings in mammals and amphibians suggest that this role of LIN-35/pRb is biologically relevant and is highly conserved across widely disparate species. This fact, combined with the recapitulation of the regulation of homologous genes by pocket proteins in both worms and in mammals, demonstrates the utility of *C. elegans* as a model for determining pocket protein functions with whole organisms in vivo.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.02.021.

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