The C. elegans F-box/WD-repeat protein LIN-23 functions to limit cell division during development

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SUMMARY

In multicellular eukaryotes, a complex program of developmental signals regulates cell growth and division by controlling the synthesis, activation and degradation of G1 cell cycle regulators. Here we describe the lin-23 gene of Caenorhabditis elegans, which is required to restrain cell proliferation in response to developmental cues. In lin-23 null mutants, all postembryonic blast cells undergo extra divisions, creating supernumerary cells that can differentiate and function normally. In contrast to the inability to regulate the extent of blast cell division in lin-23 mutants, the timing of initial cell cycle entry of blast cells is not affected. lin-23 encodes an F-box/WD-repeat protein that is orthologous to the Saccharomyces cerevisiae gene MET30, the Drosophila melanogaster gene slmb and the human gene βTRCP, all of which function as components of SCF ubiquitin-ligase complexes. Loss of function of the Drosophila slmb gene causes the growth of ectopic appendages in a non-cell autonomous manner. In contrast, lin-23 functions cell autonomously to negatively regulate cell cycle progression, thereby allowing cell cycle exit in response to developmental signals.

Key words: Ubiquitin proteolytic pathway, SCF, Hyperplasia, G1 to G0 transition, cul-1, Caenorhabditis elegans

INTRODUCTION

Development of multicellular animals requires the careful interplay of cell division, differentiation, and morphogenesis. In all animals, cell division is regulated to a large extent by the action of cyclin-dependent kinases (CDKs) and their associated cyclin subunits, which are required for CDK activation (Pines, 1995; Edgar and Lehner, 1996). CDK/cyclin complexes are regulated by three general mechanisms (Pines, 1995). First, transcriptional regulation of cyclins and CDK/cyclin inhibitory molecules (cyclin-dependent kinase inhibitors or CKIs). Second, ubiquitin-mediated degradation of cell cycle regulators, in particular cyclins and CKIs. And third, activation and inactivation of the CDK subunit by phosphorylation.

The basic framework of cell cycle regulation, as well as the functions of many cell cycle regulators, has been conserved in all eukaryotes. Nevertheless, there are significant differences between cell cycle regulation in animals and yeast. In particular, for a given cell cycle regulator in yeast there is often an increased number of homologs in animals, presumably to allow greater flexibility in regulating cell division with developmental or tissue-specific events (Pines, 1995; Liu and Kipreos, 2000).

Genetic methods have been instrumental in the identification of genes involved in cell cycle regulation. The majority of cell cycle regulators have been identified through genetic screens in budding and fission yeast with metazoan homologs subsequently studied. To an increasing extent, forward genetic methods are contributing to our understanding of the role of cell cycle regulators in higher eukaryotes, most notably in Drosophila melanogaster (Edgar and Lehner, 1996). Caenorhabditis elegans, with the advantage of invariant, visible cell lineages that allow cell division to be followed at single cell resolution, has only recently begun to be widely used as a model in the study of cell cycle regulation. In most cases, the cell cycle regulators identified in Drosophila and C. elegans have had yeast counterparts that also function in cell cycle regulation, although the cell cycle function of the multicellular eukaryotic homolog has generally evolved to fit a developmental context.

Recessive mutations that affect the proliferation of multiple tissues often identify genes that regulate the cell cycle machinery directly, while tissue-specific proliferation phenotypes are often indicative of genes regulating tissue-specific signal transduction pathways. We have isolated a gene, lin-23, that is required for limiting the cell proliferation of all tissues during development of C. elegans. lin-23 shares homology with the MET30 gene of budding yeast, which has been shown to function in the ubiquitin-mediated degradation of the CDK-inactivating kinase Sbe1 and to indirectly regulate G1 cyclin mRNA stability (Kaiser et al., 1998; Patton et al., 2000). Met30p functions as an F-box component of an SCF ubiquitin-ligase (E3) complex (Patton et al., 1998; Kaiser et al., 1998). SCF complexes contain Skp1, a Cullin, Rbx1, and an E-box containing protein that directly binds the substrate (see Deshaies, 1999).

In higher eukaryotes, lin-23 orthologs have been identified in...
Materials and Methods

Genetic analysis

Alleles e1883 and e1925 were isolated by clonally screening EMS-treated hermaphrodites for self-progeny with extra postembryonic cells. Alleles rh293 and rh294 were isolated in a clonal non-complementation screen of ethylmethanesulfonate (EMS)-mutagenized dpy-10(e128)/let-23(mn22). 3200 F1 animals were screened. F1 clones that failed to segregate Dpy progeny were tested for non-complementation to lin-23(e1883). Alleles e1521, a107, m731, and rh194 were gifts from J. Hodgkin, T. Schedl, M. L. Edgley and J. D. Plenefisch, respectively. lin-23 allele e1883 was mapped by 3-factor cross between lin-4(e912) and dpy-10(e128). A total of 17 recombinants from lin-23(e1883)/lin-4(e912) dpy-10(e128) heterozygotes gave the relative distances: lin-4 (10/17) lin-23 (7/17) dpy-10. The deficiency mnDF30 failed to complement lin-23; and lin-23(e1883)/mnDF30 heterozygotes had a phenotype indistinguishable from lin-23(e1883) homozygotes. Analysis of cell numbers in embryos and larvae was as described by Kipreos et al. (1996).

Double homozygous mutants of lin-23 and sel-10 were obtained from the homozygous lin-23 and homozygous sel-10 strain lin-23(e1883+); sel-10[ar41] lon-3[e12175] and had both Lin and Lon phenotypes. A clonal analysis of progeny from lin-23(e1883)/dpy-10(e128); cul-1(e1756)/unc-69(e587) double heterozygotes was performed with 327 embryos. The progeny were wild type (77 observed/82 expected); Unc (43/41); Dpy (45/41); Lin – both cul-1 and lin-23 homozygotes (85/82); Dpy Unc (19/20); Dpy Lin (19/20); Unc Lin (20/20); and the remainder (19/20), inferred to be Lin Cul, were 5 arrested embryos (pre-comma stage) and 14 L1 and L2 arrested animals with a range of cell division from no postembryonic divisions to hyperplasia.

Mosaic analysis was performed on lin-23(oz107), ncl-1(e1865) homozygotes containing the array ekEx11. The strain was created from lin-23(oz107)/mmC1, ncl-1(e1865) by injection of a mixture of 20 ng/μl cosmid T25H2 (containing lin-23(+) ), 20 ng/μl cosmid C33C3 (containing ncl-1(+) ); Miller and Shakes, 1995), 40 ng/μl plasmid pBK48 (containing GFP driven by the let-585 promoter; Kelly et al., 1997) and 120 ng/μl of N2 wild-type genomic DNA cut with Smal. A homozygous lin-23(oz107), ncl-1(e1865) line, ET84, was obtained. L4-stage ET84 animals were examined by epifluorescence microscopy, with GFP epifluorescence indicating the presence of the lin-23(+) transgenic extrachromosomal array. The point in the lineage of array loss was deduced from the pattern of fluorescing vulval cells, which maintained their normal positions in the vulva. Confirmatory information was obtained by scoring for the Ncl (large nucleoli) phenotype of the neuronal P5.a, P6.a and P7.a descendants for those cases in which the array was lost prior to the creation of the vulval precursor cells (VPCs) (in these neuronal cells the let-585 promoter was not active).

Quantitation of intestine cell ploidy was performed by serial confocal sectioning of propidium iodide-stained adults and L2 stage larvae using hypodermal and neuronal cells as internal 4C and 2C standards, respectively, as described by Feng et al. (1999).

Phylogenetic analysis

Initial protein sequence alignments were made with the CLUSTAL X program (Thompson et al., 1994). The alignment was then optimized by hand to minimize insertions/deletions. Neighbor-joining, maximum likelihood and parsimony methods were used to create phylogenies. For neighbor-joining analysis, a distance matrix, created with the ProtML program of MOLPHY version 2.3 using the JTT model (Adachi and Hasegawa, 1996), was used to create a tree with the NEIGHBOR program of PHYLIP version 3.57c (Felsenstein, 1993). Maximum likelihood analysis was performed with the ProtML program of MOLPHY version 2.3 (Adachi and Hasegawa, 1996), using the exhaustive search method with the JTT model. Parsimony analysis was performed with PAUP* version 4.0b2 (Swofford, 1999) using the exhaustive search method.

Bootstrap support values derive from: parsimony bootstrap analysis with 1000 replicates using default Branch and Bound settings; maximum likelihood RELL bootstrap analysis with 10,000 replicates; and neighbor-joining bootstrap analysis with 1000 replicates.

Antisense RNA analysis

Antisense lin-23 RNA was synthesized from the 1.1 kb 3′ cDNA using the Ambion MEGAScript kit. RNA was diluted to 0.75 mg/ml with diethylpyrocarbonate-treated water, and injected into both ovaries of wild-type adult hermaphrodites (Mello and Fire, 1995). Embryo cell numbers were obtained by counting nuclei of squashed embryos stained with DAPI, as described by Kipreos et al. (1996).

Molecular analysis

Genomic DNA containing lin-23 was identified by complementation of the mutant phenotype. To assay for lin-23(+) activity, 50 μg/ml of cosmid or cosmid subclones along with 150 μg/ml plasmid PRF4, carrying the dominant marker rol-6 (su1006) allele (Mello and Fire, 1995), were microinjected into the distal gonad arms of lin-23(e1883) dpy-10(e128)/lin-4(e912) using published methods (Mello and Fire, 1995). Transformants carrying the injected DNA as stable extrachromosomal arrays were cloned and their progeny were scored for Dpy non-Lin-23 individuals. Rare recombinants with this same phenotype were excluded by testing for Dpy Lin-4 self progeny.

Cosmids T25H2, F58F12, and a 10.7 kb subclone of T25H2, T′Sca, that extends from an internal Scal site to the end of the genomic insert, were each found to rescue lin-23(e1883) (Fig. 3). Removing a 1.5 kb Scal-StuI fragment from the end of T′Sca, to produce T′StuI, severely reduced lin-23(+) activity; while removal of a further 1.5 kb StuI-BamHI fragment abolished lin-23(+) activity (Fig. 3). The 9.2 kb T′StuI subclone was used as a hybridization probe to isolate an incomplete, 1.1 kb lin-23 cDNA clone from a phage library.
lin-23 negatively regulates cell division
(Barstead and Waterston, 1989). The missing 5′ region was obtained by PCR amplification from the library using primers to unique lin-23 sequence (GTGCGAA-TCGTGTTATACACT) and vector sequences. PCR products were cloned and two independent isolates were sequenced. The first seven bases of the lin-23 cDNA match the SL1 trans-spliced leader (Krause and Hirsh, 1987), indicating that the cDNA contains the entire 5′ end. Missing 3′ untranslated lin-23 cDNA sequence was obtained by sequencing the EST clone yk22d11 (a kind gift from Y. Kohara). The lin-23 cDNA sequence has been deposited in GenBank (accession number AF275253).

Standard molecular biology methods were used throughout (Sambrook et al., 1989). RNA samples of the various developmental stages were isolated and processed for northern analysis as described by Kipreos et al. (1996). Quantitation of northern signals was performed on a 300A Molecular Dynamics Densitometer. In situ hybridization with lin-23 sense and antisense RNA was performed as described by Feng et al. (1999). Immunofluorescence with monoclonal antibody MH27 (Francis and Waterston, 1991) was performed according to the Freeze-Crack protocol (Miller and Shakes, 1995).

RESULTS

The lin-23 null phenotype

Four lin-23 alleles were isolated in screens for mutants with excess postembryonic cell divisions, and four additional alleles were kind gifts from T. Schedl, J. Hodgkin, M. L. Edgley and J. D. Plenefisch. These eight alleles, which are all recessive, appear phenotypically indistinguishable. Embryogenesis is normal in homozygous lin-23 progeny of heterozygous hermaphrodites. After hatching, all 53 somatic blast cells remain quiescent until stimulated by normal developmental signals. However, upon entering the mitotic cycle, lin-23 blast cells divide excessively (detailed below), generating differentiated cells of appropriate tissue types. None of the 503 postmitotic cells created during embryogenesis was observed to divide in these mutants.

Lateral hypodermoblasts, or seam cells, are hypodermal stem cells that divide nearly synchronously at each larval stage (Sulston and Horvitz, 1977); after each division, non-seam daughters endoreplicate and then fuse with the syncytial hypodermis (Hedgecock and White, 1985) (Fig. 1A). In lin-23, the seam cells divide excessively while retaining their stem cell character and coordination with the larval molt cycle (Fig. 1A). Several other developmental constraints on the seam cell cycle appear unaffected, e.g., the H0 cell remains quiescent and all cell divisions cease at the adult stage (data not shown). There is considerable variation in the precise number and timing of the extra cell divisions among hypodermoblasts, even within the same individual (Fig. 1A, data not shown). This variability is also observed for other lineages (Fig. 1B,C,E, data not shown).

Neuroblasts Q, V5.p, and T.p, arising from the lateral hypodermoblasts, generate a small number of peripheral neurons and neuroglia (Sulston and Horvitz, 1977). At characteristic positions within each neuroblast lineage, the mitotic spindle must shift asymmetrically during anaphase to create daughter cells of unequal sizes. In lin-23, the cell fate decision to generate neuroblasts from hypodermal precursors is maintained. The committed neuroblasts generated, however, produce almost double their normal number of descendants (Fig. 1A,B, data not shown). These cells generally resemble their normal counterparts, e.g., many of the Q descendants are motile, if sometimes misoriented (Fig. 1B). Interestingly, the programmed asymmetry observed in certain neuroblast divisions, e.g., Q.a and Q.p, is reduced or lost (data not shown).

Ventral hypodermoblasts generate several motorneurons plus a hypodermal cell during the L1 stage (Sulston and Horvitz, 1977). The central six hypodermal cells, P3.p-P8.p, form the vulval primordium, while others fuse with the syncytial hypodermis (Fig. 1D). The committed vulval precursors divide during the L3 stage, responding in part to an inductive signal from the uterine anchor cell. In lin-23, ventral hypodermoblasts divide excessively during the L1 stage, producing extra motorneurons, but usually a single hypodermal cell (Fig. 1C). During the L3 stage, these cells divide excessively, generating 42±6 nuclei per vulva (n=21) on average, compared to 22 in wild type. The precursors nearest the uterine anchor cell divide most extensively, suggesting that they remain responsive to its inductive signal (Fig. 1D). Subsequent invagination and differentiation of the vulva appear nearly normal (Fig. 2A,B). Hypodermoblasts G1, G2, W and K also divide excessively (data not shown).

During the L1 stage, mesoblasts Z1 and Z4 each generate 5 presumptive gonadoblasts plus a motile distal-tip cell that determines the shape of the ovary and regulates germline proliferation by maintaining the distal germ cells in a mitotic state (Kimble and Hirsh, 1979; Kimble and White, 1981). During the L3 stage, the committed gonadoblasts divide to produce the ovary, spermatheca and uterus. In lin-23, Z1 and Z4 often undergo one or more extra divisions during the L1 stage, generating extra gonadoblasts and distal-tip cells (Fig. 1E). In particular, about 38% of these gonads (94/250) have one extra distal-tip cell, arising from either Z1 or Z4, while another 2% (6/250) have two extra cells, one from each mesoblast. These extra distal-tip cells direct the formation of extra gonadal arms and are competent to keep distal germ cells mitotic (Fig. 2C). During the L3 stage, the committed gonadoblasts divide excessively, producing an epithelium with normal regional differentiation, including spermathecae with the characteristic high concentration of tight junctions (Fig. 2D,E). The non-gonadal mesoblast M also divides excessively, producing extra coelomocytes, body wall muscles and sex muscles (data not shown).

Intestinal cells int3-int9 generally undergo nuclear division, i.e., mitosis without cytokinesis, at L1 lethargus, followed immediately by endoreplication, while the anterior intestinal cells int1 and int2 do not enter mitosis and instead undergo a single endoreplication at the L1 lethargus (Sulston and Horvitz, 1977; Hedgecock and White, 1985). Additional endoreplications occur for all intestine cells at each subsequent molt, so that the adult intestine has binucleate cells with a 32C DNA content in each nucleus. In lin-23 mutants, int3-int9 cells undergo two successive nuclear divisions at the L1 lethargus, becoming tetraneurale, while int2 cells often undergo a single nuclear division (Fig. 1F). Typically in lin-23 adults, int1 cells are mononucleate with 32C DNA content (32.1±5.1C, n=10), while binucleate int2 and tetraneurale int3-9 cells have 16C DNA content per nucleus (16.9±2.9C, n=10), indicating that the absence of lin-23 does not cause excess endoreplicative cycles and that the extra divisions in lin-23 at the L2 lethargus are conversions of endoreplicative cycles to mitotic cycles.

In contrast to somatic blast cell divisions, germ cell number is not affected in lin-23 mutants. A count of germ cell number in newly hatched young adults showed no differences between
lin-23 negatively regulates cell division

lin-23 and wild type, with 665±126 and 684±133 germ cells (n=10), respectively.

**lin-23 maternal product**

lin-23 hermaphrodites are sterile, producing only a few, inviable embryos. These embryos appear normal as early blastula, but eventually arrest, usually with excess cells and no overt morphogenesis (Fig. 2F,G). Using DNA squashes, we have counted over 850 cells in lin-23 embryos compared to the 558 cells present in wild-type embryos at hatching ( Sulston et al., 1983). To determine whether lin-23(+)* is required within the germline for normal embryonic development, we injected lin-23 antisense RNA (derived from lin-23 cDNA, see below) into the germ cell syncytia of wild-type hermaphrodites.

For many embryonically acting genes in *C. elegans*, antisense and double-strand RNAs have been shown to eliminate both maternal and zygotic products (Fire et al., 1998). Eggs fertilized in the first few hours after injection of lin-23 antisense RNA completed embryogenesis, but exhibited postembryonic hyperplasia characteristic of lin-23 larvae. Eggs fertilized later invariably arrested during embryogenesis, phenocopying embryos from homozygous lin-23 hermaphrodites (data not shown). We conclude that lin-23(+)* is required for development beyond blastula, but that maternal products suffice through the end of embryogenesis.

**Molecular analysis of lin-23**

lin-23 was mapped between lin-4 and dpy-10 on chromosome II. Genomic clones from this region were assayed as transgenes for their ability to complement lin-23(e1883). lin-23(+)* activity was localized to a 9.2 kb fragment of cosmid T25H2 and this fragment was used to isolate cDNA clones (Fig. 3). The lin-23 cDNA hybridizes to a single transcript of approx. 2.8 kb with the highest level of expression in embryos and gravid adults (Fig. 4A). In situ hybridization revealed high levels of lin-23 mRNA in the germ line of adults and early embryos (Fig. 4B,C). Zygotes have high levels of lin-23 mRNA indicating the presence of maternally provided mRNA (Fig. 4C). The level of lin-23 mRNA decreases during embryogenesis and was undetectable above background levels in larvae (Fig. 4C, data not shown).

The full-length lin-23 mRNA is predicted to encode a cytosolic protein of 665 amino acids. Six lin-23 mutations were mapped by chemical cleavage of mismatched bases (Cotton et al., 1988) and sequenced. Each allele has a single GC to AT transition creating a missense codon or nonsense codon (Fig. 5). Allele rh294, which terminates at codon 12, is evidently a molecular null. All of the alleles appear to be functional nulls, as each allele is phenotypically indistinguishable from rh294.

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**Fig. 2.** Hyperplasia in lin-23 mutants. (A-C,F,G) Differential interference contrast and (D,E) immunofluorescence micrographs. Developing vulval, v, and uterine, u, epithelia in (A) wild-type and (B) lin-23(e1883) L4 hermaphrodites. (C) Gonad with three distal arms (arrowheads) in lin-23(e1883) L3 hermaphrodite. Spermathecae (arrowheads) in (D) wild-type and (E) lin-23(e1883) adult hermaphrodites stained with monoclonal antibody MH27 to zonula adherens (Francis and Waterston, 1991). Extra spermathecae in lin-23 mutants are associated with the extra gonadal arms. (F) Wild-type embryo near hatching (approx. 700 minutes) and (G) arrested lin-23 embryo (>700 minutes). Scale bars are 10 μm.
LIN-23 contains an F-box motif (Bai et al., 1996; Kumar and Paietta, 1995) followed by seven tandem WD repeats (Neer et al., 1994) (Figs 5, 6A). The F-box motif is found in a number of cell cycle regulators and has been shown capable of mediating protein-protein interaction (Bai et al., 1996). WD repeats are found in proteins with diverse functions (Neer et al., 1994) (Figs 5, 6A). Another C. elegans gene, sel-10 (Hubbard et al., 1997), has a higher degree of homology to CDC4 than to MET30, with 31% identity to Cdc4p and 23% identity to Met30p in the WD-repeat region (Fig. 6A). Another C. elegans gene, sel-10 (Hubbard et al., 1997), has a higher degree of homology to CDC4 than to MET30, with 31% identity to Cdc4p and 23% identity to Met30p in the F-box and WD-repeat regions (Fig. 6A). Phylogenetic analysis suggests that sel-10 is more evolutionarily related to CDC4, while lin-23 is more related to MET30 (Fig. 6B). As both the S. cerevisiae and C. elegans genomes have been sequenced, and there are no other C. elegans genes that are more closely related to MET30 and CDC4; it appears that lin-23 and MET30 are orthologs, and that sel-10 and CDC4 are orthologs. Both Met30p and Cdc4p form SCF complexes containing the cullin Cul53p (see Deshaies, 1999). The C. elegans homolog of CDC53, cul-1, has a hyperplasia mutant phenotype similar to...
lin-23 negatively regulates cell division

lin-23 (Kipreos et al., 1996); and the cul-1, lin-23 double homozygote arrests development earlier than either single homozygote.

To test whether lin-23 functions redundantly with the CDC4 ortholog sel-10, we constructed a heterozygous lin-23, homozygous sel-10 strain. sel-10 encodes a suppressor of lin-12 loss-of-function alleles (Sundaram and Greenwald, 1993). sel-10 homozygous null animals are essentially wild type with only mild impenetrant phenotypes that are attributable to mild overactivity of the lin-12 receptor, e.g., 1% of animals lack an anchor cell and 4% of animals have gonad migration defects (Hubbard et al., 1997). The double lin-23, sel-10 homozygote progeny of this strain appeared to have a phenotype identical to lin-23 homozygotes, with the minor exception that the number of animals with extra distal tip cells decreased relative to lin-23 homozygotes from 40 ± 7% (n = 250) to 6 ± 2% (n = 102). This experiment suggests that there is no significant functional redundancy between lin-23 and sel-10.

lin-23 functions cell autonomously

Loss-of-function mutations of the Drosophila ortholog of lin-23, slmb, also result in excessive proliferation (Jiang and Struhl, 1998; Theodosiou et al., 1998). However, the proliferation does not arise in slmb(−) cells, but occurs in surrounding tissues due to the ectopic expression of the morphogens Decapentaplegic (Dpp) and Wingless (Wg; Jiang and Struhl, 1998; Theodosiou et al., 1998). To test whether lin-23 functions in a cell autonomous or non-autonomous manner, we carried out mosaic analysis. Our analysis focused on the vulva, as the most is known about the regulation of cell proliferation in the vulva relative to other C. elegans tissues. During the L3 stage, the vulval precursor cells (VPCs) are induced to proliferate in 1°, 2°, or 3° cell lineage patterns based on an initial inductive signal secreted by the anchor cell of the uterus (Greenwald, 1997). Mosaic analysis was performed on a lin-23, ncl-1 homozygous line that contained an extrachromosomal array containing the lin-23(+) gene, the ncl-1(+) gene, and a plasmid in which the let-858 promoter drove the expression of green fluorescent protein (GFP). Cells that had the array were lin-23(+), ncl-1(+), and had green epifluorescence. Cells that lost the array were lin-23(−), ncl-1(−) with large nucleoli, and had no epifluorescence.

The uterine precursor cells arise from the somatic gonadal precursors Z1 and Z4, which both arise from the embryonic MS blast cell. The VPCs P5.p, P6.p, and P7.p arise from the embryonic blast cell ABp. Loss of the array in MS produces uterine Fig. 5. lin-23 mRNA and protein sequence. The first seven nucleotides of the lin-23 cDNA derive from a trans-spliced SL1 leader (overlined) and indicate that the cDNA is full-length (Krause and Hirsh, 1987). lin-23 exon junctions are marked by triangles. The predicted LIN-23 protein sequence is shown below the nucleotide sequence. The F-box sequence at residues 87 to 127 is boxed. The seven WD-repeat sequences are denoted by brackets below the protein sequence. A potential bipartite nuclear localization sequence (Dingwall and Laskey, 1991) at residues 160 to 174 is underlined. Alleles in which GC to AT transitions create terminator codons are circled: rh294 (Q12 to TAG), e1925 (W327 to TGA), e1883 (W450 to TGA), oz107 (W450 to TAG), and rh194 (W499 to TAG). Allele rh293, in which a GC to AT transition created a missense mutation (G411 to R), is boxed.
Fig. 6. (A) Sequence comparison of *C. elegans* LIN-23 and SEL-10, *H. sapiens* βTRCP, *D. melanogaster* slmb, and *S. cerevisiae* Met30p and Cdc4p (Hubbard et al., 1997; Spevak et al., 1993; Jiang and Struhl, 1998; Theodosiou et al., 1998; Thomas et al., 1995; Yochem and Byers, 1987). The alignment begins with the extended F-box of Kumar and Paietta (1995) (stars above sequence), and extends through the seven WD-repeats. The standard F-box motif of Bai et al. (1996), is boxed and the seven WD repeats are shown in dashes. Sequences were aligned using the Clustal W program (Thompson et al., 1994) and then adjusted manually. Residues identical in three or more proteins are shown in bold. (B) Phylogenetic analysis using the sequences above plus the *H. sapiens* βTRCP2 protein, which is 92% identical to βTRCP in the region aligned above. Regions of the alignment that were used in the phylogeny are underlined in the SEL-10 sequence. Rooted at its midpoint, this cladogram depicts the tree obtained by Parsimony, Maximum Likelihood, and Neighbor-joining methods. Exhaustive search methods were used for both Parsimony and Maximum Likelihood, indicating that this is the best tree for both methods. Bootstrap support values are given in the nodes for Parsimony, Neighbor-joining, and Maximum Likelihood methods, respectively (separated by slash marks).

hyperplasia, while loss of the array in ABp produces VPCs that lack *lin-23*(+) and that exhibit hyperplasia of the vulva. Loss of the array in the uterus did not promote vulval hyperplasia, in particular, in 27 of 27 cases in which the vulva was *lin-23*(+) and the uterus was *lin-23*(–). The vulva had normal cell proliferation and morphogenesis (Fig. 7A,B). In two cases with a *lin-23*(+) vulva and *lin-23*(–) uterus, we observed a multivulva phenotype in which an extra vulva was created distant from the normal site, apparently the result of an extra anchor cell (AC) being created in the uterus (data not shown). Finally, we observed 12 animals showing mosaicism among the VPCs or their descendants. In all cases, descendants lacking *lin-23* exhibited hyperplasia, while those with *lin-23*(+) expression had normal cell divisions (Fig. 7C-E). These results indicate that *lin-23* functions cell autonomously to regulate cell proliferation.

**DISCUSSION**

In this paper we present evidence that the *lin-23* gene functions as a negative cell cycle regulator. From heterozygous parents, *lin-23* maternal product, which is provided at least in part as mRNA, is sufficient for normal embryogenesis. In the absence of maternal product, embryos arrest development with excess cell numbers, suggesting that *lin-23* is required to restrain cell proliferation during embryonic development. *lin-23* maternal

Fig. 7. Analysis of *lin-23* vulval mosaics. Mosaic analysis was performed on *lin-23(oz107); ncl-1(e1865)* homozygous animals carrying an extrachromosomal array, *ekEx11*, that contains the *lin-23*(+) gene, the *ncl-1*(+)gene, and a plasmid with GFP expressed by the *let-858* promoter. Cells containing the extrachromosomal array were scored for the presence of epifluorescence and absence of Ncl phenotype (large nucleoli). (A,B) Loss of the array in the uterus does not affect vulva development. (A) DIC image of the vulva and uterus in a late-L4 stage *lin-23(oz107); ncl-1(e1865)* hermaphrodite mosaic for array *ekEx11*. The number of nuclei in each half of the vulva are given; note that wild type have 11 nuclei in each half. (B) Epifluorescent image of the same animal, with the number of cells fluorescing in each half of the vulva; not all cells are visible in this focal plane. The uterus, which lacks the array, exhibits hyperplasia. (C-E) Cells that lose the array within the vulva exhibit hyperplasia. The lineages of the VPCs P5.p, P6.p, and P7.p (Sulston and Horvitz, 1977) are shown (C, top). To demonstrate the location of the VPC descendants in the L4 vulva, lines connect the terminal vulval cells of the lineage with the location of their nuclei in the L4 vulva (Sulston and Horvitz, 1977) (C, bottom). The position of the filled circles next to the lineage indicates the VPC or VPC descendant in which the *ekEx11* extrachromosomal array was lost; in one case the array was apparently lost in a precursor to both P6.p and P7.p. In every case, cells that lost the array produced extra cells (6.0±4.2 extra cells per loss of array, n=12). In all cases, neighboring vulva cells that did not lose the array had normal numbers of cell divisions. (D,E) An example of loss of the *ekEx11* array in P5.p. (D) DIC image of the uterus and vulva of a mid-L4 stage *lin-23(oz107); ncl-1(e1865)* hermaphrodite that is mosaic for array *ekEx11*. The number of cells for each half of the vulva are given: 17 in the anterior and 11 in the posterior. (E) Epifluorescent image of the same animal. The number of fluorescent cells for each half of the vulva are given; the 4 fluorescent cells in the anterior were found in the location of wild-type P6.pa descendants. The 13 cells without the array were in the region of the vulva normally populated by P5.p descendants. Scale bar is 10 μm.
product appears to be exhausted by the L1 larval stage. In the L1 and subsequent larval stages, blast cells divide supernumerary times to produce excess cells. All tissue types are represented among the blast cells that divide excessively in lin-23 mutants, indicating that lin-23 is required to restrain mitotic cell division irrespective of tissue type.

In lin-23 mutants, cells appear to require normal signals to start cell division. Only blast cells and their descendants divide in lin-23 larval stages, while postmitotic non-blast cells do not divide. Blast cells do not divide precociously, but rather wait until the appropriate developmental stage to divide. Further, among the VPCs, those closest to the AC, which secretes an inductive/proliferative signal (see Greenwald, 1997), divide more than those further from the AC, as is true for wild type. Final differentiation states of lin-23 cells appear normal. Distinct neuronal, hypodermal, somatic gonad, muscle, and intestine phenotypes are readily observed by DIC microscopy. The spermathecae in lin-23 mutants expresses adherens junctions, as revealed by staining with the MH27 antibody, similar to differentiated wild-type spermathecae. Finally, the extra distal tip cells produced in lin-23 mutants are both competent to lead out the developing gonadal arm and to keep distal germ cells in a mitotic state.

Morphogenesis is disorganized in several larval tissues in lin-23 mutants relative to wild type, e.g., spermathecae and uterus, and in embryos lacking lin-23 maternal product. lin-23 may be required for morphogenic processes, alternatively, the disorganization may be the byproduct of excess cell proliferation. In both cki-1 and cul-1 loss-of-function animals, which also exhibit hyperplasia, there is a similar disorganization of larval tissues and lack of clear morphogenesis in embryos (Kipreos et al., 1996; Feng et al., 1999; Hong et al., 1998; data not shown).

In contrast to mitotic cell cycles, lin-23 is not required for restraining purely endoreplicative cycles. The ploidy of intestine cells, which endoreplicate in wild type to achieve 32n, does not increase beyond 32n in lin-23 mutants. The first endoreplication for most wild-type intestine cells is preceded by a nuclear division (segregation of chromosomes but no cytokinesis). Interestingly, the first endoreplication after the nuclear division is converted to a second nuclear division in lin-23 mutants, suggesting that lin-23 is required to allow these cells to bypass mitosis. Later endoreplications are not converted to nuclear divisions in lin-23 mutants, suggesting that mitotic bypass is maintained by a LIN-23-independent mechanism, potentially via the downregulation of mitotic cyclin, as is observed in Drosophila endoreplicative cycles (Edgar and Lehner, 1996).

We cloned lin-23 and determined that it encodes a putative protein with an F-box motif and seven WD repeats. lin-23 is expressed at its highest levels in embryos and the adult germline, but has some expression in all developmental stages. The complete S. cerevisiae genome has just two F-box/WD-repeat proteins, Cdc4p and Met30p (Bai et al., 1996). Both Cdc4p and Met30p function as components of an SCF E3 complex to facilitate the recognition of substrates by a ubiquitin-conjugating enzyme (E2) for ubiquitin-mediated proteolysis. Met30p and Cdc4p function as the substrate recognition subunits of different SCF complexes, SCF\textsuperscript{Met30} and SCF\textsuperscript{Cdc4}. Each complex also includes Skp1p, the cullin Cdc53p, and Rbx1p/Roc1p/Hrt1p, and interacts with the ubiquitin-conjugating enzyme Cdc34p (Deshaies, 1999). Both Cdc4p and Met30p are involved in the degradation of cell cycle regulators. Cdc4p is required for the degradation of G1 cyclins (Cln1p and Cln2p), cyclin-dependent kinase inhibitors (Sic1p and Far1p), and the DNA replication protein Cdc6p (see Deshaies, 1999). Met30p is required for the degradation of the CDK inhibitory kinase Swe1p, and for the repression of the sulfur network genes by inactivating the transcription factor Met4p (Kaiser et al., 1998; Thomas et al., 1995). Increased activity of Met4p in met30 mutants produces a G1 arrest with increased turnover of the mRNA for the G1 cyclins CLN1, CLN2 and PCL2 (Patton et al., 2000).

Parsimony analysis suggests that LIN-23 is more closely related to Met30p, while another C. elegans protein SEL-10 is more closely related to Cdc4p (Fig. 6). SEL-10 functions to negatively regulate LIN-12 activity and has been found to physically interact with the intracellular domain of LIN-12, suggesting that SEL-10 functions in the turnover of LIN-12 (Hubbard et al., 1997). Surprisingly, a null allele of sel-10 has only minor, impenetrant phenotypic consequences in a wild-type lin-12 genetic background (Hubbard et al., 1997). sel-10 is the apparent ortholog of the yeast cell cycle regulator CDC4. While the sel-10 mutant phenotype does not indicate a role in cell cycle regulation, it is possible that another gene functions redundantly with sel-10 to effect cell cycle regulation. However, a double mutant of lin-23 and sel-10 failed to uncover synthetic cell cycle phenotypes, suggesting that lin-23 does not share critical functions with sel-10. Finally, the dissimilar mutant phenotypes of lin-23 (defective cell cycle exit) and met30 (cell cycle arrest) further suggests that the cellular functions of SCF complexes have not been conserved between yeast and metazoa.

lin-23 has a mutant hyperplasia phenotype similar to that of the cul-1 gene, which also encodes an SCF component (Kipreos et al., 1996). Orthologs of lin-23 and cul-1 function together in SCF complexes in both yeast and humans, making it likely that LIN-23 and CUL-1 also form an SCF complex in C. elegans. The phenotypes of cul-1 and lin-23, while similar, do show differences. Whereas lin-23 maternal products perdure only through embryogenesis, cul-1 maternal products can suffice through the L1 stage. Despite the later onset of the cul-1 mutant phenotype, cul-1 larvae arrest development earlier with a more severe hyperplasia, e.g., cul-1 mutants exhibit on average twice as much vulval hyperplasia as lin-23 mutants (Kipreos et al., 1996; this study).

Cullins have been found to interact with multiple F-box proteins to target different substrates. The phenotype of inactivation of the cullin component should be equivalent to the sum of the inactivation of the separate F-box proteins with which it functions (assuming that the F-box proteins do not have independent functions). Our data therefore predicts that an SCF complex containing CUL-1 and LIN-23 contributes part of the negative cell cycle regulatory capability of CUL-1, but that CUL-1 (with a more severe hyperplasia phenotype) will also function with other F-box protein(s) to negatively regulate the cell cycle. Higher eukaryotic orthologs of lin-23 have been identified in Xenopus (\textit{BTRCP}), humans (\textit{BTRCP} and \textit{BTRCP2}), and Drosophila (\textit{smb}) (Spevak et al., 1993; Margottin et al., 1998; Suzuki et al., 1999; Jiang and Struhl, 1998). The human ortholog, h-BTRCP, was found to be co-opted by the Human Immunodeficiency Virus Vpu protein to target the CD4 receptor for degradation (Margottin et al., 1998); and it also functions in
the degradation of IκBα and β-catenin (Yaron et al., 1998; Shirane et al., 1999; Winston et al., 1999b; Hart et al., 1999; Latres et al., 1999; Fuchs et al., 1999; Kroll et al., 1999; Hatakeyama et al., 1999; Spencer et al., 1999; Suzuki et al., 1999; Tan et al., 1999; Wu and Ghosh, 1999; Vuillard et al., 1999).

Cells lacking the Drosophila ortholog of lin-23, slmb, ectopically express the morphogens Wg and Dpp (Jiang and Struhl, 1998; Theodosiou et al., 1998). Ectopic intersection of Wg- and Dpp-expressing cells in the same compartment of imaginal discs induces formation of ectopic appendages (see Serrano and O’Farrell, 1997). Animals with mosaic patches of slmb(−) cells have extra appendages that are attributable to the ectopic expression of Wg and Dpp (Jiang and Struhl, 1998; Theodosiou et al., 1998). The excess cell proliferation that is observed in slmb mosaic animals derives from the non-cell autonomous growth of wild-type imaginal disc cells that are induced to create ectopic appendages by the secretion of morphogens by the slmb mutant cells. Interestingly, clones of cells with null slmb alleles, although initially present in the imaginal disc, are not observed in the ectopic appendage that they later induce, suggesting either decreased cell proliferation or cell lethality (Theodosiou et al., 1998).

Hyperplasia in lin-23 mutants does not appear to be due to morphogen secretion, as the hyperplasia occurs throughout development in all tissue types, and mosaic analysis indicates that lin-23 mutant hyperplasia occurs through a cell autonomous mechanism. It is currently unclear why the two orthologs have such different modes of action in Drosophila and C. elegans. It is tempting to speculate that both genes evolved to negatively regulate cell proliferation, however, differences in the way cell proliferation is regulated in Drosophila, via the action of morphogens to pattern surrounding cells, compared to C. elegans, where cell intrinsic decisions are much more important, have shaped the mechanism of action of the two gene products.

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lin-23 negatively regulates cell division 5081


