C. elegans MCM-4 is a general DNA replication and checkpoint component with an epidermis-specific requirement for growth and viability

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C. elegans MCM-4 is a general DNA replication and checkpoint component with an epidermis-specific requirement for growth and viability

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ABSTRACT

DNA replication and its connection to M phase restraint are studied extensively at the level of single cells but rarely in the context of a developing animal. C. elegans lin-6 mutants lack DNA synthesis in postembryonic somatic cell lineages, while entry into mitosis continues. These mutants grow slowly and either die during larval development or develop into sterile adults. We found that lin-6 corresponds to mcm-4 and encodes an evolutionarily conserved component of the MCM2–7 pre-RC and replicative helicase complex. The MCM-4 protein is expressed in all dividing cells during embryonic and postembryonic development and associates with chromatin in late anaphase. Induction of cell cycle entry and differentiation continues in developing mcm-4 larvae, even in cells that went through abortive division. In contrast to somatic cells in mcm-4 mutants, the gonad continues DNA replication and cell division until late larval development. Expression of MCM-4 in the epidermis (also known as hypodermis) is sufficient to rescue the growth retardation and lethality of mcm-4 mutants. While the somatic gonad and germline show substantial ability to cope with lack of zygotic mcm-4 function, mcm-4 is specifically required in the epidermis for growth and survival of the whole organism. Thus, C. elegans mcm-4 has conserved functions in DNA replication and replication checkpoint control but also shows unexpected tissue-specific requirements.

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Introduction

A crucial aspect of the cell division cycle is DNA replication, which takes place during the synthesis (S) phase of the cell cycle (Arias and Walter, 2007; Bell and Dutta, 2002). DNA replication must be highly accurate and tightly controlled to maintain genomic integrity over many rounds of cell division. A developmental context adds additional constraints on S-phase regulation. For instance, in meiosis M phases follow each other without intervening S phases, while in endoreduplication cycles, rounds of DNA replication continue in the absence of M phases. Nonetheless, during the division of most somatic cells, DNA duplication should happen once and only once, and M phase should not initiate until S phase is complete. Stalled replication forks and DNA damage activate a checkpoint that delays cell cycle progression (Ciccia and Elledge, 2010). Activation of this replication/damage checkpoint involves the Chk1 kinase and forms part of normal Drosophila and C. elegans development. Drosophila Chk1 (grapes) is required for decelerating embryonic cell cycles at the midblastula transition (Fogarty et al., 1997; Sibon et al., 1997), while the C. elegans ortholog chk-1 contributes to different cell cycle timing of early blastomeres (Brauchle et al., 2003). Thus, DNA replication and replication checkpoint control have developmental functions that go beyond the duplication of individual cells.

Studies of single-cell eukaryotes, Xenopus egg extracts and mammalian cells in culture have generated substantial insights in the process of DNA replication (Arias and Walter, 2007; Bell and Dutta, 2002). To accomplish the correct duplication of its DNA in each cell cycle, the cell treats the ‘licensing’ of the DNA for replication and the actual start of DNA replication as separate events. In the licensing phase of the cell cycle, pre-replication complexes (pre-RCs) are assembled at future origins of DNA replication. The sequential action of ORC1-6 proteins, Cdc6 and Cdt1 load the MCM2–7 DNA helicase onto the origins during late mitosis and early G1 (Bell and Dutta, 2002). The MCM2–7 complex is thought to act during S-phase as the helicase that unwind the DNA at the replication origins (Aparicio et al., 1997; Labib et al., 2000; Pacek and Walter, 2004). At the onset of S phase, CDK (cyclin-dependent kinase) and DDK (Dbf4 dependent Cdc7 kinase) activity control activation of the MCM2–7 helicase while at the same time preventing new recruitment of MCM2–7 complexes. This way, DNA synthesis is limited to a single round in each cell cycle (Nguyen et al., 2001; Petersen et al., 1999; Piatti et al., 1996; Schwob and Nasmyth, 1993).
Our understanding of the control of DNA replication in an organismal context is less advanced. However, important insights have been obtained from studies of, for instance, endoreduplication and gene amplification in Drosophila (Claycomb and Orr-Weaver, 2005; Lilly and Duronio, 2005). In addition, work from various researchers has demonstrated that conserved molecular modules regulate S-phase entry and DNA replication checkpoint responses in C. elegans (Kipreos, 2005; O'Neil and Rose, 2006; van den Heuvel, 2005). Several studies illustrate the potential for uncovering novel aspects of DNA replication control in C. elegans. For instance, analysis of DNA replication in C. elegans resulted in the discovery of the CUL-4/DDB-1 E3 ubiquitin ligase, which prevents origin re-firing and is conserved in mammals (Arias and Walter, 2006; Kim and Kipreos, 2007; Senga et al., 2006; Zhong et al., 2003). In addition, defects in DNA synthesis were found to cause lineage-specific delays in cell division, through a checkpoint mechanism that also contributes to the normal difference in timing of division between the anterior AB and posterior P1 blastomeres (Brauchle et al., 2003; Encalada et al., 2000).

In this study, we report the molecular and genetic characterization of the C. elegans gene lin-6. We show that lin-6 mutant larvae maintain temporal expression of S-phase and differentiation genes, while the somatic cells are defective in DNA synthesis and lack the G2/M checkpoint that senses incomplete replication. Mapping and cloning revealed that lin-6 is also known as mcm-4 and encodes the C. elegans MCM4 ortholog, a member of the six-subunit MCM2-7 pre-RC and replisome complex. Our results support a conserved function MCM4 ortholog, a member of the six-subunit MCM2-7 pre-RC and replisome complex.

Materials and methods

Strains and culturing

Strains were cultured on NGM plates seeded with E. coli OP50 according to standard protocol. Feeding RNAi was performed on NGM plates supplied with 50 μm/ml Ampicillin and 2 mM IPTG. Animals were synchronized by hypochlorite treatment and hatching eggs in M9 medium with 0.05% Tween-20. L1 larvae were then transferred to NGM plates with OP50 and allowed to develop for the appropriate amount of time. Experiments were conducted at 20 °C unless indicated otherwise. Strains used were: N2 Bristol wild-type, CB3475 lin-6(e1466)/szT1[lon-2 (e678)]; +/szT1, MT1442 lin-6(e1466) dpy-5(e61)/szT1[lon-2(e678)]; +/szT1, JK2739 lin-6(e1466) dpy-5(e61)/htT2[blb-4(e937) let-7(q782) qa48], SV987 cyt-1(he112) rol-1(e91)/mmC1; hels30[Prnr-1::cyb-1DesBox::3XVenus], SV1035 mcm-4(e1466) dpy-5(e61)/szT1[lon-2(e678)]; +/szT1, heEx349[PmcM-4::mCherry], SV1032D lin-6(e1466) dpy-5(e61)/szT1[lon-2(e678)]; +/szT1, heEx347[Prnr-1::cyb-1DesBox::3XVenus], SV1055 mcm-4(e1466) dpy-5(e61)/szT1[lon-2(e678)]; +/szT1, heEx358[Pelt-2::MCM-4::mCherry], SV1057 mcm-4(e1466) dpy-5(e61)/szT1[lon-2(e678)]; +/szT1, heEx359[Pelt-2::MCM-4::mCherry], SV1056 mcm-4(e1466) dpy-5(e61)/szT1[lon-2(e678)]; +/szT1, heEx360[Pelt-2::MCM-4::mCherry], SV1047 mcm-4(e1466) dpy-5(e61)/szT1[lon-2(e678)]; +/szT1, heEx361[dpy-7::MCM-4::mCherry], SV1059 mcm-4(e1466) dpy-5(e61)/szT1[lon-2(e678)]; +/szT1, heEx362[bar 10]/sur-5::GFP; myo-2::TdTomato.

Molecular cloning of lin-6

Deficiencies tDf3, which includes lin-6, and tDf4, which does not include lin-6, were used to link the lin-6(e1466) mutation to the physical map. YACs from the region were used in transgenic rescue experiments and Y74C10 was observed to rescue the lin-6 phenotype.

This ~330 kb YAC was labeled with γ32P-ATP and used as a probe to isolate cDNAs from a C. elegans library. Forty-three cDNAs were identified and assigned to at most 9 different genes. RNA interference of one of these genes caused cell cycle defects that closely resembled those of lin-6 mutants. One of the eight independent cDNAs for this gene (clone 6.10) was used as a probe to isolate genomic clones from a C. elegans phage library. Two of the identified clones partially rescued the lin-6(e1466) phenotype, suggesting that this genomic DNA includes the lin-6 gene and that the cDNAs were derived from lin-6. The nucleotide sequences from four independent cDNAs were determined, and genomic DNA from wild-type and e1466 animals was sequenced to determine the exon and intron sequences and nature of the mutation. All cDNAs contained sequences from 7 exons, a 3’ poly(A) tract, and are predicted to encode the MCM-4 protein of 823 amino acids. This protein is likely full-length for three reasons: the first methionine is preceded by a stop three codons upstream, several cDNAs and ESTs start at about the same nucleotide and northern analysis of wild-type mRNA revealed a single transcript consistent with the size of the cDNA.

Reporters, transgenics and microscopy

MCM-4::mCherry (Pmcm-4::MCM-4::mCherry::mcm-4 3’UTR) was generated by amplifying a 5.7 kb fragment encoding MCM-4 and 2.4 kb of promoter sequence from genomic N2 DNA using Phusion polymerase (Finnzymes) and cloning this fragment into the pGEMT vector (Promega). Subsequently, the mCherry coding sequence (a kind gift of R. Tsien) together with the unc-54 3’UTR was amplified by PCR with Phusion and was fused in-frame with the mcm-4 coding sequence and the pGEMT vector. The unc-54 3’UTR was replaced with the 650 bp mcm-4 3’UTR, which was amplified from genomic N2 DNA, to yield the MCM-4::mCherry reporter. Constructs of the MCM-4::mCherry reporter with either the mcm-4 or unc-54 3’UTR were injected into the MT1442 strain at a concentration of 30 ng/μl. GFP and lin-48::GFP were used as co-injection markers, respectively. The transgenes rescued mcm-4(e1466) larval development, resulting in adults that produced dead embryos. Both constructs gave similar expression patterns in four independent transgenic lines. Pdy-7::MCM-4::mCherry and Pelt-2::MCM-4::mCherry were created by replacing the mcm-4 promoter from the Pmcm-4::MCM-4::mCherry::mcm-4 3’UTR construct by a 500 bp (dpy-7) or a 5 kb (elt-2) promoter fragment. Constructs were co-injected with sur-5::GFP (50 ng/μl) and myo-2::TdTomato (10 ng/μl) into the CB3475 or MT1442 strain. Pnr::CYP-1DesBox::3XVenus was created by cloning a tandem C. elegans optimized Venus (a kind gift of T. Ishihara, Kyushu University, Kyushu, Japan) in-frame with an N-terminal fragment of C. elegans cyb-1 cyclin B1 (nucleotides 1–321). This CYP-1DesBox fragment contains a putative destrucution box for AP-1-dependent degradation. The codon usage was altered (optimized) to prevent co-suppression of the endogenous cyb-1 gene. CYP-1DesBox was expressed as a translational fusion with tripleVenus, controlled by the rnr-1 ribonucleotide reductase promoter in the pVT501 vector (a kind gift of V. Ambros). Detailed cloning information and sequence maps are available upon request. This construct was injected into MT1442 mcm-4(e1466) dpy-5(e61)/szT1[lon-2(e678)]; +/szT1 at 40 ng/μl with lin-48::TdTomato as a co-injection marker. Transgenic lines were created by micro-injection as described (Mello et al., 1991). To examine reporter gene expression, animals were washed off the plates, anaesthetized with 10 mM Sodium Azide and mounted on slides with a 2% agar pad. Most images were taken with an Axioplan 2 microscope and Axioquant mRm camera (Zeiss Microscopy). Time-lapse images were acquired with a CSU-X1 Yokogawa spinning disk confocal system mounted on an inverted microscope (Nikon) and using an EMCCD camera (iXON DU-885, Andor Technology). Anterior is left, dorsal up in all figures. Scale bar 10 μm, unless otherwise indicated.
Immunostaining and antibodies

EdU (5-ethyl-2′-deoxyuridine) labeling and staining were performed according to a protocol developed by S. Crittenden and J. Kimble using the Click-IT EdU Alexa Fluor 594 kit (Invitrogen). In short, MG1693 (Thy-deficient) bacteria were grown in 100 ml of minimal medium containing 20 mM EdU. Worms were fed on NGM + ampicillin plates with these bacteria for the appropriate time. For fixation, worms were freeze-cracked on poly-L-lysine coated slides in liquid nitrogen and subsequently immersed in methanol (5 min at −20 °C) and acetone (20 min at −20 °C). Slides were washed 1 x in PBS with 0.1% Tween-20 and incubated with PBS + 0.1% Triton X-100 for better permeabilization. Slides were dried and the animals encircled with a PAP liquid blocker pen. The Click-IT reaction was subsequently performed on slides according to the manufacturer’s instructions. Afterwards, slides were blocked using 10% donkey serum and 1% BSA and stained with monoclonal mouse anti-GFP (1:100, Sigma). Donkey anti-mouse FITC (Jackson ImmunoResearch Laboratories) was used as a secondary antibody. Slides were mounted in Prolong Anti-Fade Gold (Invitrogen) according to the manufacturer’s instructions. Afterwards, slides were observed using a confocal microscope. Immunostaining of C. elegans embryos was performed as previously described (van der Voet et al., 2009).

Antibodies were raised against an MCM-4 internal 336 amino acid peptide (Xhol-BamHI fragment) supplied with an N-terminal 6xHis tag and purified from E. coli on Ni2+ beads. Anti-rabbit MCM-4 (Rabbit/Bleed 62-3) sera were either used crude (1:100, Fig. S4) or after affinity-purification with the same protein-fragment blotted onto nitrocellulose membranes (1:20, Fig. 6). Other primary antibodies used: anti-Nuclear Pore Complex mAb414 (Abcam, 1:100), anti-P-granule antibody K67 (Developmental Studies Hybridoma Bank, 1:2), anti-AJM-1 antibody MH27 (Developmental Studies Hybridoma Bank, 1:20), and rabbit anti-dsRed (Clontech, 1:100). Secondary antibodies used were: goat anti-mouse Alexa-488 (Invitrogen, 1:250), goat anti-rabbit Alexa-568 (Invitrogen, 1:250). Fluorescent images were taken with an Axioplan 2 microscope and Axiocam mRM camera (Zeiss Microscopy). Anterior is left, dorsal up in all figures. In both immunostaining and live imaging experiments, a minimum of n = 10 animals were examined for each treatment. Scale bar 10 μm, unless otherwise indicated.

Results

lin-6 mutants enter mitosis without DNA replication

The lin-6(e1466) mutation was identified in the first systematic search for mutants with defects in the normally invariant postembryonic cell lineages of C. elegans (lin mutants) (Horvitz and Sulston, 1980; Sulston and Horvitz, 1981). lin-6(e1466) mutant animals were reported to initiate mitosis while DNA replication was absent outside of the germline. This phenotype suggested a checkpoint defect and prompted us to investigate the cell cycle defects in lin-6 mutants in more detail.

Heterozygous lin-6(e1466)+/+ animals developed as normal (Fig. 1A and C and data not shown), indicating that the lin-6(e1466) allele is recessive. We generated trans-heterozygotes of lin-6(e1466) and the deficiency tdF3, which spans the entire lin-6 locus, and found them indistinguishable in phenotype from homozygous lin-6(e1466) mutants (Table 1). Thus, e1466 is probably a strong loss of function or null allele of lin-6. In the presence of maternal wild-type product, lin-6(e1466) homozygous animals completed embryogenesis and hatched as normal first-stage larva (L1). Starting in the first larval stage, cell divisions failed, yet the animals continued to grow at a rate that is reduced and more variable than that of wild-type animals (Figs. 1A-D and 7). The divisions of all postembryonic blast cells outside the gonad were defective and formed abnormally small nuclei (Fig. 1F, arrowhead). Some of these nuclei remained in close contact, probably as a result of incomplete mitosis and cytokinesis, while others fully separated (Figs. 1A-F and 7C and D). Germline development was slow but frequently continued until the L4 stage. As a result, lin-6 mutants that reached the adult stage generally had extensive gonads, with reflexed arms and sperm cells, but without oocytes (Fig. 1G). We examined the presence of germ-cell specific P-granules by immunohistochemistry (Strome and Wood, 1983). Gonads of lin-6 animals showed extensive staining of P-granules, which demonstrates the proliferation of precursor germ cells during larval development (Fig. 1H). Thus, starting in the L1 stage, somatic blast cell undergo abnormal divisions in lin-6(e1466) larvae, while germline development continues until late larval development.

We used incorporation and immunohistochemical detection of the thymidine analogue BrdU as a sensitive assay for DNA synthesis. Wild-type animals grown in the presence of BrdU from 0 to 14 h of larval development incorporated BrdU in the postembryonic blast cell lineages (Fig. 1I). In lin-6 mutants, only cells in the germline continued DNA replication (Fig. 1J). Occasionally, limited DNA synthesis could be detected in the blast cells that initiate postembryonic division soon after hatching. These cells include the neuroblasts QR and QL, the mesoblast M and the epidermal nuclei V5R and V5L (Fig. 1J, arrowhead and data not shown). We did not detect any BrdU incorporation in the precursor cells of the ventral nerve cord (P cells) (compare Fig. 1I and J). We used DIC microscopy to follow the development of progeny from heterozygous parents from the early L1 stage onward. Homozygous lin-6 mutant larvae initiated postembryonic blast cell divisions at the same time as their heterozygous and wild-type siblings (data not shown). The 12 P precursor cells produced on average 27 ± 3.1 SD daughters, confirming that some even went through a second round of division in the absence of DNA replication. Together, these observations confirm that postembryonic somatic blast cells fail to replicate their DNA but initiate mitosis without delay in lin-6 mutant animals.

lin-6 is required for the checkpoint that couples M phase entry to S-phase completion

Replication defects are expected to trigger a checkpoint that delays mitotic entry (Hook et al., 2007). Thus, it is remarkable that somatic cells in lin-6 mutants enter mitosis at the normal time in the apparent absence of DNA replication. We considered two alternative explanations for this aspect of the Lin-6 phenotype: lin-6 is required for DNA synthesis and also to activate the checkpoint that monitors completion of DNA replication, or alternatively, incomplete S phase cannot be sensed by a checkpoint in C. elegans larvae. To discriminate between these possibilities, we added the DNA replication inhibitor hydroxyurea (HU) to synchronously growing cultures of L1 animals. Subsequently, we fixed and stained animals for the mitosis-specific phosphorylated histone H3 Ser10 epitope (pH3S10) at various times of L1 development. Treatment of wild-type animals with HU delayed onset of mitosis for prolonged times (Fig. 2C, compare with A), demonstrating that initiation of mitosis is indeed dependent on the completion of DNA synthesis in C. elegans. Initiation of mitosis was not delayed in lin-6 mutants treated with HU, indicating that mitotic entry is not coupled to DNA synthesis in these mutants (Fig. 2B and D). Thus, lin-6 is required for DNA replication as well as for the checkpoint that restrains mitosis until completion of S phase.

lin-6 is not required for G1 progression and differentiation

Similar to lin-6, cdk-4 Cdk4/6 and cyclin D are also required for DNA synthesis during larval development (Boxem and van den Heuvel, 2001; Park and Krause, 1999). Postembryonic blast cells in cdk-4 and cyclin D mutants arrest in G1 and do not activate the S-phase transcriptional reporter mrl-1::GFP (Boxem and van den Heuvel, 2001). We observed mrl-1::GFP expression in lin-6 larvae but also
quite frequently in starvation-arrested L1 larvae of the rnr-1::GFP control strain (data not shown). The latter observation indicates that fluorescence might result from ectopic expression of the rnr-1::GFP transgene or from GFP lingering from previous divisions. To create a more reliable marker for the G1/S transition, we fused a CYB-1 cyclin B1 N-terminal fragment (CYB-1DesBox), which directs APC/C-dependent protein degradation in mitosis, in-frame with triple Venus (3XVenus) and expressed this translational fusion protein from the rnr-1 promoter (see Materials and methods). This Pmr-1::CYB-1DesBox3XVenus reporter is highly specific for cells in division, as fluorescence appeared during S phase, accumulated in the presence of HU and disappeared at the time of M phase completion or soon thereafter. Again, wild-type and lin-6(e1466) L1 larvae expressed this S-phase reporter with similar temporal and spatial control, while the reporter was not induced in cyd-1(he112) mutant larvae (Fig. 3). We conclude that cells progress through the G1/S transition in lin-6 mutants, yet fail to replicate their DNA.

Interestingly, CYB-1DesBox3XVenus expression disappeared more slowly in lin-6 mutants than in wild-type animals (Fig. S1). As postembryonic blast cells in lin-6 mutants enter mitosis at the appropriate time, based on the timing of nuclear envelope breakdown, the prolonged presence of CYB-1DesBox3XVenus likely indicates a delay.

Fig. 1. lin-6 mutant animals are defective in DNA replication and cell division. A–D: Differential interference contrast (DIC) microscopy images of lin-6(e1466) heterozygous larvae (A,C) and lin-6(e1466) homozygous mutants (B,D). lin-6/+ larvae have normal cell divisions. Indicated are P cell descendants that form the vulva (A,C, arrowheads) and an intestinal cell after nuclear division (A, bracket). In contrast, lin-6(e1466) homozygous mutant animals lack postembryonic cell division in these tissues (B,D, arrows and arrowhead). Note the significant size of the gonad in the lin-6 homozygous larva (D, gonad outlined with dotted line). E,F: DNA staining with propidium iodide (PI, red) illustrates division defects of the ventral cord precursor cells P in late L1 larvae. Descendants of the P cells fill the ventral cord in the wild type (E, arrow), while in the lin-6(e1466) mutant (F) defective P cell divisions create gaps (arrow) and small fragmented nuclei (arrowhead). G,H: The somatic gonad and germline continue DNA synthesis and considerable numbers of cell divisions during larval development of lin-6(e1466) mutants. (G) DNA stained with PI (red), the dashed line outlines a gonadal arm. Note the presence of sperm in the spermatheca (arrow). (H) Precursor germ cells are visualized by immunostaining of P-granules (red dots) in a L3-stage larva, DNA is stained with DAPI (blue). I,J: Detection of DNA replication. L1 larvae grown in the presence of the thymidine analogue BrdU from 0 to 14 h of larval development followed by anti-BrdU staining. (I) BrdU incorporation in a wild-type animal demonstrates DNA replication in various postembryonic lineages. Labeled cells include the descendants of the P cells (P, arrowhead) and mesoblast (M, arrow). (J) lin-6 mutant larva, which shows postembryonic DNA replication only in the gonad (asterisk). Descendants of the Mesoblast (arrow), the V5 seam cells and Q neuroblasts (not visible) occasionally show limited DNA replication. Anterior is left, dorsal up in all figures. Scale bar, 10 μm.
in exit from mitosis. Pmr-1::CYB-1:DesBox3XVenus was also detected at later times of development, in particular in the epithelial lateral seam cells and posterior daughters of the P (Pn,p) cells (data not shown). This expression cannot be explained by perdurance of the fluorochrome and indicates that cells continue their program of cell cycle entry, even when previous divisions failed.

In addition, induction of differentiation also continued in the absence of DNA replication. For instance, the formation of incomplete adult alae in part of the animals indicates completion of epithelial differentiation. Previous dye-filling studies demonstrated that lin-6 mutants miss the PDE ciliary neuron in the postdeirids, a lateral neuronal structure formed during the first larval stage from posterior descendants of the V5 seam cells (Sulston and Horvitz, 1981). We introduced the Pdat-1::GFP transgene in the lin-6(e1466) background as a reporter for PDE cell fate determination (Nass et al., 2002). Expression of this transgene was seen in 20/20 lin-6 animals assayed at the L4 larval stage (Fig. S2). Division of the precursor cells of the postdeird was defective in lin-6 mutants (Fig. S2, arrow), which probably prevents formation of a functional PDE ciliary neuron (Sulston and Horvitz, 1981). However, the Pdat-1::GFP-positive neurons sometimes formed axonal projections in lin-6 animals that look similar to the projections seen in wild-type Pdat-1::GFP-positive neurons. These data suggest that cell type specification and differentiation can continue extensively in developing lin-6 larvae even in cells that underwent aberrant division.

lin-6 encodes the MCM4 subunit of the MCM2-7 replication pre-initiation complex

We cloned the lin-6 gene through a combination of genetic mapping and yeast artificial chromosome (YAC) rescue (see Materials and methods, Fig. S3A). Conceptual translation of lin-6 cDNAs predicted a protein of 823 amino acids (Fig. S3B). Analysis of the corresponding DNA sequences in lin-6(e1466) revealed a G:C-to-A:T transition, which is predicted to change the Gln88 codon to an amber stop codon. Termination of translation this early is likely to result in strong or complete inactivation of LIN-6 function, in agreement with strong or complete inactivation of LIN-6 function, in agreement with previous studies showed that replication defects delay progression through the cell cycle of early blastomeres (Encalada et al., 2000), through activation of an at-1 ATR-dependent checkpoint pathway (Brauchle et al., 2003). In agreement with these studies, we found that inhibition of DNA replication by exposing adults to the ribonucleotide reductase inhibitor HU (100 mM) or rnr-1 RNAi resulted in delayed mitotic entry in the one-cell embryo (Fig. 4A). Following the delay, spindle duplication continued without chromosome segregation, and the DNA remained present in the center of the embryo as a single or two separate masses (derived from the paternal and maternal pronucleus) in 49/50 embryos (Fig. 4D, compare to 4B, 4C, 4E, arrows). In contrast, mitotic entry was not delayed after mcm-4(RNAi) (Fig. 4A and C). The DNA was segregated to opposite poles and cycles of chromosome segregation followed by cell division continued, thereby reducing the amount of DNA in each blastomere (Fig. S4B, compare to DNA staining of polar bodies (arrowheads)). The DNA became fragmented (50/50 embryos, compared to none in the wild-type control), and ultimately these mcm-4(RNAi) embryos arrested with up to 30 nuclei and very little DNA in each nucleus (Fig. 4B).

RNAi of mcm-4 substantially decreased the delay induced by rnr-1 inhibition (Fig. 4A), further supporting that mcm-4 is required for the

Table 1

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<td>Emb (n)</td>
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<td>lin-6/+ X lin-6/+</td>
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<td>0.0% (12)</td>
<td>25.8% (332)</td>
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<td>lin-6(e1466)/dpy-5(e61)</td>
<td>4.3% (52)</td>
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Fig. 2. Absence of a replication checkpoint response in lin-6 mutants. Wild-type (A) and lin-6 mutant (B) larvae at 10 h of L1 development contain mitotic P cells in the ventral cord, which stain positive for the mitosis-specific histone H3 phospho-Ser10 epitope (pH3S10) (A,B, arrows). C: Wild-type animal treated with the DNA-replication inhibitor hydroxyurea (HU). P cells have migrated into the ventral cord but remain phospho-H3S10 negative, indicating that cells arrest before mitosis (arrowheads). D: In the HU-treated lin-6 animal, P cells failed to arrest before mitosis and show phospho-H3S10 positive staining (arrows) and a metaphase plate (arrowhead).
S-phase checkpoint that delays M-phase entry. However, mcm-4 (RNAi) was not fully epistatic to rnr-1 inhibition, which indicates either residual mcm-4 function or an mcm-4 independent partial delay (Fig. 4A and E). RNAi of mcm-5, which encodes another MCM subunit, resulted in a phenotype similar to that of mcm-4 (48/50 embryos). Genome fragmentation has also been reported for cdt-1(RNAi) and cdc-6(RNAi) embryos (Kim et al., 2007; Zhong et al., 2003). In contrast, inhibition of div-1, which encodes the DNA polymerase α primase B-subunit, resembled rnr-1 inactivation and triggered mitotic delay (Encalada et al., 2000). We conclude that the MCM replicative helicase, which forms part of the DNA replication pre-initiation complex, is required for both DNA synthesis and the delay of cell cycle progression when replication is incomplete.

MCM-4 shows cell cycle-dependent expression and localization

How cells commit to and withdraw from the division cycle are important developmental questions. As a subunit of the DNA replication pre-initiation complex, MCM-4 should be present at the time of S-phase onset. Moreover, replication licensing is separated in time from activation of origin firing at G1/S, and as such MCM-4 might be expressed well before S phase or even remain present at the end of mitosis. To examine the temporal and spatial expression of MCM-4, we created an MCM-4::mCherry reporter construct and generated antibodies that recognize MCM-4.

The reporter transgene contains 2.4 kb of genomic DNA upstream of the predicted ATG translation initiation codon, the predicted mcm-4 exon and intron sequences, and 650 bp downstream of the stop codon, including the predicted poly(A) signal. Coding sequences for mCherry were inserted just before the termination of the mcm-4 open reading frame (see Materials and methods). Expression of this combined promoter and C-terminal translational fusion construct rescued the mcm-4(e1466) mutation. Specifically, mcm-4(e1466) animals with an extrachromosomal Pmcm-4::MCM-4::mCherry array appeared healthy and viable and showed normal cell division in the ventral cord, intestine and seam, formed a normal vulva and occasionally produced a few embryos (data not shown). Moreover, single copy integration of this mcm-4::mCherry transgene fully rescued the mcm-4(e1466) embryonic and postembryonic defects (JK and SvdH, manuscript in preparation). As MCM-4::mCherry functionally substitutes for MCM-4, its expression and localization likely resembles the endogenous protein.

We did not detect MCM-4::mCherry in starvation-arrested L1 animals. However, expression was specifically induced in all postembryonic blast cell lineages well before mitotic entry, at the expected time of S-phase induction (Fig. 5A–H). The fusion protein localized to the cell nucleus until degradation of the nuclear envelope in prometaphase, at which point MCM-4 became diffusely localized through the cell. This diffuse localization indicates that MCM-4 is not chromatin-associated in mitosis (Fig. 5H, arrow). MCM-4::mCherry did not disappear upon completion of mitosis but was segregated to both daughter cells. Even cells that permanently withdrew from cell division, such as the motor neurons of the ventral nerve cord, initially retained MCM-4::mCherry expression (data not shown). However, this expression subsequently disappeared in differentiated cells as well as in cells that temporarily arrested cell division, such as the Pn.p vulval precursor cells in the ventral cord. These experiments indicate that mcm-4 is transcriptionally activated at approximately the time of G1/S transition and that MCM-4 protein is segregated to both daughter cells in mitosis.

Detection of endogenous MCM-4 confirmed these observations. We generated rabbit polyclonal antibodies against an extended C-terminal MCM-4 fragment as well as mouse polyclonal antibodies that recognized an N-terminal domain. Antisera directed against either fragment recognized a protein with an apparent MW of ±105 kD in total worm lysates (Fig. 6D and data not shown). This protein is likely MCM-4, based on its MW and absence in mcm-4(e1466) mutants, which contain an early nonsense mutation. We found MCM-4 expressed in dividing cells during all stages of development in wild-type animals. Embryos showed the highest levels of MCM-4 expression, in agreement with the fact that more than half of the somatic cells are formed during embryogenesis (Fig. 6D, lane 1). Interestingly, MCM-4 was reduced but clearly detectable in developmentally arrested L1 animals that hatched in the absence of food (Fig. 6D, lane 2). Even dauer larvae that had been arrested in cell division for 2 weeks still contained detectable MCM-4 protein levels (Fig. 6D, lane 7). These results suggest that a pool of MCM-4 is retained during prolonged periods of quiescence, so that MCM-4...
might function in the re-initiation of DNA synthesis when conditions improve.

Immunostaining of wild-type animals for MCM-4 showed strong nuclear staining in the gonad, embryos and postembryonic lineages (Fig. 6A and C, Fig. S5). This staining was MCM-4-specific, as RNAi of mcm-4 eliminated the nuclear signal in the germline and embryos, and mcm-4(e1466) larvae did not show staining (Fig. 6B and data not shown). MCM-4 was detectable in sperm and accumulated during oocyte maturation in the nucleus but did not show overlap with the condensed chromosomes in diakinesis of meiotic prophase (Fig. S5 and data not shown). MCM-4 was not chromatin-associated during Meiosis I of the fertilized oocyte, and the first polar body did not contain MCM-4 (Fig. 6A). This finding is consistent with the absence of S phase between Meiosis I and II. The second polar body and maternal pronucleus received some MCM-4. Subsequently, embryonic cells in interphase showed strong nuclear staining (Fig. 6C, but not after inhibition of mcm-4 (D” and D”).

Fig. 4. mcm-4(RNAi) embryos lack DNA replication yet continue mitotic DNA segregation. A: Inhibition of mcm-4 by RNAi does not delay mitotic entry and relieves the checkpoint induced by mrr-1 inhibition. RNR-1 inhibition by RNAi or exposure of adults to HU (100 mM, 2–4 h) substantially delays mitotic entry (approximately 10–13 min, respectively). Combination with mcm-4 knockdown diminishes this delay, which further indicates contribution of MCM-4 in an S-phase checkpoint over mitotic entry. Time indicates seconds between meeting of the maternal and paternal pronucleus in the posterior and nuclear envelope breakdown (NEB). Migration of the maternal pronucleus to the posterior is also delayed after mrr-1 inhibition (not included). For proper comparison with mrr-1 mcm-4 double RNAi, control (ctl: gpd-2) dsRNA was added to the mcm-4 and mrr-1 injection mix. B–E: Images from time-lapse spinning disk confocal microscopy illustrate that chromosome segregation continues in the absence of mcm-4 (C C”), but not after inhibition of mrr-1 (D–D”). Note that the zygotic DNA remains present in the center of the embryo, independent of the spindle and cell division (arrows, D” and D”).
4 remained cytoplasmic at the onset of anaphase; however, chromatin association became apparent in late anaphase (Fig. 6A, Fig. S5A, C, and E, compare arrows with arrowheads). These data show that chromosome association of MCM-4 is tightly controlled, consistent with origin licensing taking place at the end of mitosis and disappearing during S phase.

Similar observations were made during larval divisions. Matching the MCM-4::mCherry reporter, endogenous MCM-4 expression was detectable prior to and during mitosis (Fig. 6C). Staining of synchronized L1 animals revealed the timing of MCM-4 expression, which in general preceded mitosis by 1–2 h. After 5 h of L1 development at 20 °C, MCM-4 immunostaining was predominantly detected in the epithelial seam cells, Q neuroblast daughters and gonad primordium. The somatic gonad precursor cells Z1 and Z4 showed nuclear staining, while the mitotically arrested germline precursor cells Z2 and Z3 showed diffuse cytoplasmic staining. At 6 hours of L1 development, the mesoblast (M) also stained strongly as well as the most anterior ventral cord precursors cells (W, P1 and P2). Subsequently at 7 h, additional P cells showed nuclear MCM-4 expression, which became apparent prior to migration of the nucleus into the ventral nerve cord (data not shown). At 8 h of L1 development, the intestinal nuclei showed MCM-4 expression (Fig. 6C), which preceded nuclear division by at least 4 h. At subsequent time points, daughter cells that continued division, such as the Pn.a and M descendants, retained strong nuclear staining. L2 animals stained at 16 h of larval development showed strong MCM-4 expression in the gonad, the H1.a, H2.p, V1-6.p and T.ap seam cells and, weakly, the intestinal nuclei (data not shown).

Importantly, MCM-4 staining did not overlap with DNA in prophase and metaphase, while in late anaphase co-localization with the chromosomes was clearly detectable (Fig. 6C). Similar to our observations with the MCM-4::mCherry reporter, we could not detect any asymmetry in MCM-4 segregation. Thus, even if only one daughter cell continued cell division, both daughters received a similar amount of MCM-4 in mitosis. Furthermore, the MCM-4 protein became undetectable during quiescence, i.e. the P3.p-P8.p daughter cells that resume DNA replication in the L3 stage did not show detectable expression in the L2 stage. Altogether, our reporter gene and antibody staining analysis show that MCM-4 is dynamically expressed and localized during larval development as well as during different phases of the cell cycle. The strong induction of MCM-4 in cells that re-enter the cell cycle after quiescence suggests that MCM-4 expressed in G1/S can contribute origin-licensing and/or replicative helicase activity.

MCM-4 is required in the epidermis for organismal growth and viability

How cell division is coordinated with organismal growth is an important question in developmental biology. mcm-4 mutants grow slowly and remain smaller and slimmer than wild-type animals (Fig. 7A and B). In addition, mcm-4 mutants quite frequently arrest at the larval molts and subsequently die. mcm-4 mutant larvae that were synchronized at hatching and subsequently were allowed to develop for 120 h had a 12% lethality rate (n = 97), while the wild-type and heterozygous mcm-4(e1466)/+ siblings all developed into healthy gravid adults (n = 300). In contrast, mutations in cyd-1 or cdk-4 cause slow growth without associated lethality. As cyd-1 and mcm-4 mutants both fail to replicate DNA in postembryonic somatic cells, we assumed that DNA replication is needed for normal growth, while the lack of an S-phase checkpoint in mcm-4 mutants probably underlies the lethality. To examine in which cell type mcm-4 is required to promote growth and viability, we expressed mcm-4 specifically in the epidermis or intestine. These two tissues go through endoreduplication cycles, which have been implicated in cellular growth, during larval development (Hedgecock and White, 1985; for review see Edgar and Orr-Weaver, 2001). Furthermore, the larval lethality of mcm-4 mutants seemed to coincide with a molting defect, which suggested a requirement for MCM-4 function in the epidermis.
Expression of MCM-4::mCherry under the control of the intestine specific elt-2 promoter (Hawkins and McGhee, 1995) restored intestinal nuclear divisions and endoreduplication cycles in mcm-4 animals (Fig. S6). This result confirms that mcm-4 acts cell-autonomously in DNA synthesis and endoreduplication cycles. However, expression of MCM-4::mCherry in the intestine did not rescue the size or lethality of the mcm-4 mutants. In contrast, expression of MCM-4::mCherry from the epidermal sur-5::GFP marker without the Pdpy-7::MCM-4::mCherry rescue construct contained few epidermal nuclei (Fig. 7C, arrows). Such nuclei were often small and fragmented, probably because of aberrant mitosis without DNA replication (Fig. 7D, arrowheads, compare with Fig. 1F). Staining with MH27 antibodies, which recognize the apical junction-localized AJM-1 protein in C. elegans epithelia (Francis and Waterston, 1991), demonstrated that the structure of the seam cells in the worm epidermis is abnormal in mcm-4(e1466) animals (Fig. S7B). We noticed that transgenic mcm-4(e1466)/+ animals with a Pdpy-7::MCM-4::mCherry extrachromosomal array produced few animals with a typical Mcm-4 phenotype. However, sterile animals with a vulval protrusion (Pvl) were present that together with the Mcm-4 larvae formed approximately 1/4 (17/59) of the offspring. Examination of mCherry fluorescence revealed that the sterile Pvl animals all carried the extrachromosomal array, while the Mcm-4 animals had lost the array. Thus, expression of the Pdpy-7::MCM-4::mCherry transgene substantially rescues the Mcm-4 phenotype. We observed complete rescue of the small body length and width of the mcm-4 mutants (Fig. 7B and data not shown). Moreover, Pvl animals carrying the Pdpy-7::MCM-4::mCherry transgene did not arrest during molting but developed into healthy, but sterile adults (n = 124 transgenic animals examined). Furthermore, the Pdpy-7::MCM-4::mCherry transgene restored cell division of the epidermal seam cells (Fig. 7E) as well as the structure of the seam cells in mcm-4(e1466) mutants (Fig. S7C). We also noticed some rescue of cell division of the epithelial posterior P cell descendants, consistent with data suggesting dpy-7 promoter activity in the Pn.p cells (A. Saffer and H.R. Horvitz, personal communication). Other tissues, such as the intestine and gonad, developed as in mcm-4 mutants and the transgenic animals were fully sterile. These data indicate that mcm-4 function in the epidermis is sufficient to restore normal growth and viability in mcm-4 mutant animals.

**Discussion**

Studies of simple animals such as *C. elegans* allow for a functional analysis of cell division genes in a developmental context. As part of such an approach, we characterized the e1466 mutation, which defined the *C. elegans* gene *lin-6* (Horvitz and Sulston, 1980). The *lin-6* mutant phenotype combines absence of DNA replication with continued mitosis, substantial development of the gonad and germ-line, variable growth and partly penetrant larval lethality. We found that the *lin-6(e1466)* mutation affects the gene *mcm-4*, which encodes the single *C. elegans* MCM-4 subunit of the MCM-2-7 replicative helicase. MCM-2-7 function has been poorly characterized in *C. elegans*, with *mcm-5(fl652)* as the only other reported mutant (Wang et al., 2007). Many aspects of the *mcm-4(e1466)* phenotype agree with a function as an MCM-2-7 subunit, as determined in other systems. However, the tissue-specific effects of *mcm-4* inactivation are surprising for a basic component of the DNA replication machinery.

*mcm-4* plays a key role in DNA replication and the replication checkpoint, but mutants continue gonad development

The absence of DNA synthesis in mcm-4 mutants is consistent with the critical function of MCM-4 in DNA replication licensing and origin unwinding (Aparicio et al., 1997; Ying and Gautier, 2005; You et al., 1999). However, in mcm-4 mutants DNA replication and cell division continue in the germline during larval development, even allowing the formation of sperm. We expect that perdurance of maternal product and a strong checkpoint response permit continued division cycles in the mcm-4 gonad. The precursors of the somatic gonad and germline (Z1/Z4 and Z2/Z3, respectively) are formed and set aside in the larvae (Horvitz and Sulston, 1980). The *lin-6* mutation affects the gene *mcm-4*, which encodes the single *C. elegans* MCM-4 subunit of the MCM-2-7 replicative helicase. MCM-2-7 function has been poorly characterized in *C. elegans*, with *mcm-5(fl652)* as the only other reported mutant (Wang et al., 2007). Many aspects of the *mcm-4(e1466)* phenotype agree with a function as an MCM-2-7 subunit, as determined in other systems. However, the tissue-specific effects of *mcm-4* inactivation are surprising for a basic component of the DNA replication machinery.
C. elegans MCM proteins by RNAi makes animals hypersensitive to an otherwise non-inhibitory dose of HU (Woodward et al., 2006). Because of the normal excess of MCM proteins, depletion of maternal MCM-4 pools might not be harmful for several rounds of replication, in particular if the time in S phase can be extended through a checkpoint that monitors the completion of DNA synthesis. We expect that the presence of a robust S-phase checkpoint response in the germline (Gartner et al., 2000) contributes to the continued replication with limiting amounts of MCM-4.

Our data show that MCM-4 is required for replication checkpoint activation in somatic cells. During larval development of mcm-4 mutants, somatic blast cells entered mitosis without delay and independent of the presence of HU. In addition, divisions in mcm-4 (RNAi) embryos continued without DNA replication, resulting in a fragmented genome. These results agree with studies of other organisms, which clarified the requirement of the MCM complex in activation of the DNA damage and replication checkpoints. Critical in checkpoint activation is the recruitment of Replication Protein A (RPA) to single-stranded DNA (Zou and Elledge, 2003). The helicase activity of MCM proteins generates ssDNA, through unwinding the DNA at the replication fork. When replication forks are stalled, e.g., because of treatment with HU, the MCM helicase activity becomes uncoupled from DNA polymerase activity (Byun et al., 2005). Consequently, fork stalling leads to an accumulation of ssDNA, which recruits additional RPA and causes activation of the checkpoint kinases ATR and Chk1. Both replication fork formation and ssDNA generation require MCM4 function; when MCM4 function is absent, DNA synthesis cannot initiate and the replication checkpoint cannot be activated.

MCM-4 is dynamically regulated throughout the cell cycle and development

In agreement with observations of other eukaryotes, our data indicate that DNA licensing takes place in late M phase and possibly continues in G1. MCM-4 started to co-localize with the DNA in late anaphase in both embryonic and larval cell divisions (Figs. 5 and 6 and Fig. S5). However, upon temporary arrest of cell division or terminal differentiation MCM-4 decreased below detection by immunostaining or MCM-4::mCherry fluorescence. In contrast, Western blotting experiments showed reduced but clearly detectable MCM-4 levels in arrested L1 animals and dauer larvae (Fig. 6). Thus, a relatively low amount of origin-bound MCM2-7 might drive DNA synthesis in cells...
that exit from arrest. Alternatively, MCM proteins newly synthesized in G1 might carry out this function.

We observed strong induction of MCM-4 expression around the time of S-phase onset. The transcription of MCM2-7 genes is inhibited by lin-35 Rb together with E2F transcription factors and activated by CYD-1/CDK-4 and CYE-1/CDK-2 cyclin/CDK complexes (J.K. and S.v.d. H, in preparation, (Kirienko and Fay, 2007). Thus, MCM expression is likely induced at the G1/S transition, close to the switch from origin licensing to origin firing. An important question is whether the newly synthesized MCM-4 can contribute origin-licensing and helicase activities immediately in S phase, while origin re-firing is prevented. In mammalian cells released from quiescence, MCM loading has been shown to occur in late G1 (Mukherjee et al., 2009). Cdt1 and Cdc6 are essential loading factors for the MCM-2 complex in various eukaryotes, and their inactivation prevents origin re-firing during S phase (Arias and Walter, 2007). The C. elegans orthologs CDT-1 and CDC-6 are both inactivated during S phase in a DNA replication and CUL-4/DBB-1 E3 ubiquitin ligase-dependent manner (Kim et al., 2007; Kim and Kirekos, 2007; Korzelius and van den Heuvel, 2007; Zhong et al., 2003). Thus, MCM proteins expressed in late G1 might contribute to a single round of origin firing, as determined by CDT-1 and CDC-6 availability.

**Loss of mcm-4 reveals a remarkable uncoupling between cell cycle progression and organismal development and growth**

One of the striking aspects of the mcm-4 phenotype is that it exposes an uncoupling between cell cycle progression and developmental processes such as differentiation. During cell cycle progression, checkpoints ensure that earlier events are completed before later events initiate. Such feedback mechanisms do not appear to exist for cell division and cell fate acquisition. Based on reporter gene expression and cell morphology, successful mitosis is not needed for induction of subsequent S phases or induction of differentiation (Fig. 3 and Figs. S1 and S2). In this light, it is surprising that growth, which can occur quite independently of cell division and cell fate acquisition. Based on reporter gene expression and cell morphology, successful mitosis is not needed for induction of subsequent S phases or induction of differentiation (Fig. 3 and Figs. S1 and S2). In this light, it is surprising that growth, which can occur quite independently of cell division (Grewal and Edgar, 2003), was severely impaired in mcm-4 mutants. Furthermore, mcm-4 mutant animals frequently died during the larval molt, cd1-1 and cdk-4 mutants also lack DNA replication and grow slowly, but these mutants do not display any larval lethality (Boxem and van den Heuvel, 2001). Thus, the continued mitosis in the absence of DNA replication, which is specific for mcm-4 mutants, might have caused the reduced viability.

Surprisingly, mcm-4 function in the epidermis was sufficient for normal viability and restored larval growth to wild-type levels. In C. elegans, only the intestine and epidermis undergo rounds of endoreduplication during larval development (Hedgeschock and White, 1985). A positive correlation between ploidy of the C. elegans epidermis and volume of the adult animal has provided a strong argument for growth control by endoreduplication (Flemming et al., 2000; Lozano et al., 2006). Intestinal expression of MCM-4 restored DNA replication and nuclear division in mcm-4 larvae but did not rescue the growth and viability defects. In contrast, epidermal expression of MCM-4 rescued the larval lethality as well as the reduced length and thin appearance of mcm-4 mutants. As lethality coincides with the molt, mcm-4 mutants might die from reduced integrity of the cuticle. Expression of mcm-4 in the epidermis of mcm-4 mutants prevents the loss of seam cells that normally contribute to cuticle formation (Fig. 5B; see Fritz and Behm, 2009 and references therein). Moreover, mcm-4 expression in the syncitial epidermis restores endoreduplication, which may promote growth as well as cuticle secretion.

DBL-1 TGFβ has been shown to control postembryonic growth through regulation of SMA-3 in the epidermis (Wang et al., 2002). This effect has been linked to the control of DNA replication (Lozano et al., 2006; Wang et al., 2002). cye-1, ccyd-1 and cdk-4 mutants all show reduced growth, in contrast to cdk-1 mutants that arrest cell division in G2 (Boxem et al., 1999; Boxem and van den Heuvel, 2001; Lozano et al., 2006). In particular, the cye-1 G1 cyclin was found to be crucial for epidermal polyploidization (Lozano et al., 2006). Further studies will be needed to determine if the DBL-1 TGFβ/SMA-3 pathway induces G1 cyclin expression to promote DNA replication and growth of the animal.

Taken together, our findings highlight a remarkable independence among DNA replication, differentiation and cell cycle progression. In addition, our results show that a component of the basic DNA replication machinery can have distinct tissue-specific requirements in growth and viability, which makes it a potential target for regulation by developmental control pathways.

**Supplementary materials**

Reference materials related to this article can be found online at doi:10.1016/j.ydbio.2010.12.009.

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