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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

Jerome Korzelius a,1, Inge The a, Suzan Ruijtenberg a, Vincent Portegijs a, Huihong Xu c, H. Robert Horvitz b, Sander van den Heuvel a,*

a Developmental Biology, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands
b Howard Hughes Medical Institute, Department of Biology, Massachusetts Institute of Technology, Cambridge, MA, USA
c Department of Pathology and Laboratory Medicine, Boston University School of Medicine and Boston Medical Center, 670 Albany Street, Boston, MA, USA

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A B S T R A C T

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 endoreplication 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 midblastulat...

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 unwinds 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 re-replication in *C. elegans* resulted in the discovery of the CUL-4/DBB-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). It is likely that genetic analyses of animal systems will reveal additional mechanisms that connect S-phase control to developmental processes.

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 replicative helicase complex. Our results support a conserved function 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.

**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)/e1466 dpy-5(e61)/szT1[lon-2(e678)]; +/ +szT1, JK2739 lin-6(e1466)/e1466 dpy-5(e61)/ +/ +[lon-2(e397) let-7(q782) qd48]; SV987 cyd-1(he112) rol-1(e91)/ +/ +; heIs30[Prnr-1::cyb-1DesBox::3XSV1055]; SV987 cyd-1(he112) rol-1(e91)/ +/ +; heIs30[Prnr-1::cyb-1DesBox::3XSV1056].

**Expression, animals were washed off the plates, anaesthetized with 50 μM Sodium Azide and mounted on slides with a 2% agar pad. Most images were taken with an Axioplan 2 microscope and Axioscan 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.**

**Molecular cloning of lin-6**

Deficiencies tdJ3, which includes lin-6, and tdJ4, 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 kbp YAC was labeled with γ[32P]-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::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. myo-2::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. Pdp-7::mCherry and Pelt-2::mCherry were created by replacing the mcm-4 promoter from the Pmcm-4::mCherry::mcm-4 3'UTR construct by a 500 bp (dpy-7) or a 5 kb (elt-2) promoter fragment. Constructs were coinjected with sur-5::GFP (50 ng/μl) and myo-2::TdTomato (10 ng/μl) into the CB3475 or MT1442 strain. Pnr::CYB-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 CYB-1DesBox fragment contains a putative destruction box for APC-dependent degradation. The codon usage was altered (optimized) to prevent co-suppression of the endogenous cyb-1 gene. CYB-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)/ +/ +; +/ + and TdTomato as a co-injection marker. Transgenic lines were created by microinjection 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 Axioscan 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-ethynyl-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× 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. We considered two alternative explanations for this possibility, we added the DNA replication inhibitor hydroxyurea (HU) to synchronize growing cultures of L1 animals. Subsequently, we fixed and stained animals for the mitosis–specific phospho-histone H3 Ser10 epitope (pH3S10) at various times during the division of 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. To discriminate between these possibilities, we added the DNA replication inhibitor hydroxyurea (HU) to synchronously growing cultures of L1 animals. We observed 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. 11). 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. 11 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. Interestingly, 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 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 rnr-1::GFP (Boxem and van den Heuvel, 2001). We observed mrr-1::GFP expression in lin-6 larvae but also
quite frequently in starvation-arrested L1 larvae of the \( rnr-1::\text{GFP} \) control strain (data not shown). The latter observation indicates that fluorescence might result from ectopic expression of the \( rnr-1::\text{GFP} \) 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 \( \text{Pmr-1::CYB-1DesBox3XVenus} \) 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 \( \text{lin-6(e1466)} \) L1 larvae expressed this S-phase reporter with similar temporal and spatial control, while the reporter was not induced in \( \text{cyd-1(e112)} \) mutant larvae (Fig. 3). We conclude that cells progress through the G1/S transition in \( \text{lin-6} \) mutants, yet fail to replicate their DNA.

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

![Fig. 1.](attachment:figure1.png)
in exit from mitosis. Prmr-1::CBY-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 postdeirid 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 a stop codon. Termination of translation this early is likely to result in

<table>
<thead>
<tr>
<th>Genotype crosses (n = 4)</th>
<th>Progeny phenotype</th>
<th>Gonad cell count</th>
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<tbody>
<tr>
<td></td>
<td>Emb (n)</td>
<td>Stu (n)</td>
</tr>
<tr>
<td>lin-6/+ X lin-6/+</td>
<td>0.9% (12)</td>
<td>25.8% (332)</td>
</tr>
<tr>
<td>lin-6/+ X lin-6(Df)/+</td>
<td>1.0% (14)</td>
<td>23.2% (313)</td>
</tr>
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45% amino acid identity with human and Xenopus laevis MCM4 (Fig. S3C). First discovered in yeast, the MCM genes are essential for DNA replication in all eukaryotes studied (Bell and Dutta, 2002). Six different genes of this family, MCM2 to 7, are present in S. cerevisiae, and a single member of each subfamily appears to be conserved in other eukaryotes. The six MCM proteins form a hexameric complex, which licenses origins for DNA replication as a critical component of the DNA replication pre-initiation complex and acts as the replicative helicase during DNA replication. Because of the high degree of conservation, the C. elegans genome project already assigned the name mcm-4 to the lin-6 gene defined by mutation. We propose to adopt mcm-4 as the common name and will use this name throughout the rest of this manuscript.

The replication pre-initiation complex is needed to restrain M phase

Analysis of the mcm-4(RNAi) phenotype further confirmed the double function of mcm-4 in DNA synthesis and activation of a replication checkpoint that inhibits progression into mitosis. 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 rmr-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, Fig. S4C, 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 up to 30 nuclei and very little DNA in each nucleus (Fig. 6B).

RNAi of mcm-4 substantially decreased the delay induced by rnr-1 inhibition (Fig. 4A), further supporting that mcm-4 is required for the
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 P[mcm-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. 6A, Fig. S5 left panel). In prophase, MCM-4 localization did not overlap with the condensing chromosomes (Fig. 6A, Fig. S5). Upon nuclear envelope degradation, MCM-4 became diffusely localized throughout the cell and clearly did not co-localize with the metaphase-aligned chromosomes (Fig. 6A, arrowhead). MCM-
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 germ line 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. 6A). 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 promoter (Hawkins and McGhee, 1995) rescued the size or lethality of the mcm-4(e1466)/+ animals (Fig. S7B). 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 Pdp-7::MCM-4::mCherry transgene did not arrest during molting but developed into healthy, but sterile adults (n = 124 transgenic animals examined). Furthermore, the Pdp-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. 7C). We also noticed some rescue of cell division of the epithelial posterior P cell descendants, consistent with data suggesting dpv-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[fqw7] as the only other reported mutant (Wang et al., 2007). Many aspects of the mcm-4(e1466) phenotype agree with a function as an MCM2-7 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 sperms. 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 germ-line (Z1/Z4 and Z2/Z3, respectively) are formed and set aside in early embryogenesis (Sulston et al., 1983). Hence, Z1/Z4 might use residual maternal product to continue replication in larval development. Similarly, cd-1 and cdk-4 mutants show fully penetrant arrest of postembryonic somatic blast cell divisions, while some divisions of the gonadal precursors continue (Boxem and van den Heuvel, 2001; Park and Krause, 1999). At the same time, gonad development in mcm-4 mutants is severely retarded compared to that of the wild type. We expect that this difference reflects a gradual decrease in the number of active replication origins. In *Xenopus*, it has been estimated that 10–20 times more MCM2-7 molecules are loaded onto DNA in G1 than the number of origins that are used for DNA replication (see Edwards et al., 2002; Takahashi et al., 2005). This excess of MCM proteins is expected to license origins that are normally dormant, but allow full DNA synthesis during replicative or genotoxic stress (Ibarra et al., 2008; Woodward et al., 2006). This process is likely to play a role in *C. elegans* replication control as well. Partial inactivation of 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 Pdp-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 Pdp-7::MCM-4::mCherry transgene did not arrest during molting but developed into healthy, but sterile adults (n = 124 transgenic animals examined). Furthermore, the Pdp-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. 7C). We also noticed some rescue of cell division of the epithelial posterior P cell descendants, consistent with data suggesting dpv-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.

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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/DBD-1 E3 ubiquitin ligase-dependent manner (Kim et al., 2007; Kim and Kipreos, 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 (Grewal and Edgar, 2003), was severely retarded in mcm-4 mutants. Furthermore, mcm-4 mutant animals frequently died during the larval molt, cyd-1 and cdc-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 cause 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 (Hedgecock 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. S7B; (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 SNA-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, cyd-1 and cdc-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.

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