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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.

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 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 (Dbf-4 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).

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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/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). 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 *mcm-4* in replication licensing, DNA synthesis and the replication checkpoint. In addition, *mcm-4* is essential for normal larval growth and viability, which reflects a surprisingly specific MCM-4 requirement in the outermost epithelial cell layer known as hypodermis or epidermis.

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)/hT2[bli-4(e937) let-?(q782) qIs48]*, SV987 *cyd-1(he112) rol-1(e91)/mnC1; heIs30[Prnr-1::cyb-1DesBox::3X-Venus]*, SV1035 *mcm-4(e1466) dpy-5(e61)/szT1[lon-2(e678)]; +/szT1; heEx349[Pmcm-4::MCM-4::mCherry]*, SV1032 *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]*, SV1056 *mcm-4(e1466) dpy-5(e61)/szT1[lon-2(e678)]; +/szT1;heEx359[Pelt-2::MCM-4::mCherry]*, SV1057 *mcm-4 (e1466) dpy-5(e61)/szT1[lon-2(e678)]; +/szT1;heEx360[Pdpy-7:: MCM-4::mCherry]*, SV1058 *mcm-4(e1466) dpy-5(e61)/szT1[lon-2(e678)]; +/szT1;heEx361[Pdpy-7::MCM-4::mCherry]*, SV1059 *mcm-4(e1466) dpy-5 (e61)/szT1[lon-2(e678)]; +/szT1;heEx362[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 γ^{32}P-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 in 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. *Pdpy-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. *Prnr::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)/szT1[lon-2(e678)]; +/szT1)* at 40 ng/μl with *lin-48::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 Axiocam 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.