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DNA replication machinery contributes to development of eye in Drosophila

Hidetsugu Kohzaki[1], Yota Murakami[1]

[1]Department of Cell Biology, Institute for Virus Research, Kyoto University, Sakyo-ku, Kyoto, Japan 606-8507

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1. ABSTRACT

Chromosomal DNA replication machinery is essential for growth of all cells in all organs in multicellular organisms. Its knockdown in several tissues of *Drosophila* led to rough eye phenotype and the loss of bristles in the eye. These results show that the DNA replication machinery contributes to the body development independent of growth.

2. INTRODUCTION

DNA replication machinery is essential for growth of all cells. The origin recognition complex is the platform of DNA replication initiation complex. In *Drosophila*, Cdt1-Mcm2-7 is thought to be the licensing factor that directs the S phase per one cell cycle. A lot of evidence indicates that Mcm2-7 is also a replicative helicase (1). The Cdc45, Mcm2-7, and GINS (CMG complex) is the eukaryotic DNA replication fork complex in *Drosophila* embryo (2). DNA polymerases, including Polα-primase, Polδ and Polε, are the enzymes responsible for the elongation phase of DNA replication. The yeast Cdc6 is a loader of Mcm2-7 onto DNA replication origin. However, higher eukaryotic Cdc6 function has not been clarified.

In researching the availability of RNA interference (RNAi) in *Drosophila* (3), we wanted to compare the phenotypes of flies in which particular genes were knocked down in various tissues and stages (4). Previously, we knocked down DNA replication machinery by *Act5C*- and *tubulin-Gal4* drivers, which express target genes in the whole body. But the knockdown of *Mcm2*, *Mcm4*, *Cdt1*, and *Cdc6* was lethal (4). Instead, we used a tissue-specific RNAi knockdown system in combination with a Gal4-UAS system (5). A transgene of interest, which is expressed with a Gal4-dependent promoter, is introduced into the embryo. By crossing with a fly expressing *Gal4* in a tissue-specific manner (*Gal4* driver), one can obtain flies that express the transgene in a tissue-specific manner. When antisense RNA against the target gene is expressed using the tissue-specific *Gal4* driver system, the RNA transcribed from the target DNA forms double-stranded RNA, which can be destroyed by RNAi machinery, resulting in the depletion of the target gene product.

In *Drosophila* tissues, chromosomal abnormalities, including in gene amplification

and endoreplication, occur in a developmentally regulated manner (6). Recently, we showed that the DNA replication machinery required for *Drosophila* development and chromosome replication in the mitotic cell cycle is needed for gene amplification and endoreplication (4).

We hypothesized that the behavior of Mcm2 would be close to that of the eukaryotic DNA replication initiation complex. In this study, we knocked down the *Mcm2*, *Mcm4*, *Cdt1*, and other subunits of the chromosomal DNA replication licensing factor complex in the *Drosophila* eye disc. First, we showed that the RNAi knockdown of chromosomal DNA replication machinery could induce the abnormal bristle formation. Next, we demonstrated that knockdown of DNA replication resulted in rough eye phenotype, probably through defects in DNA replication. These findings showed that chromosomal DNA replication machinery plays an active role in tissue development in *Drosophila* development, and contributes to body development independent of growth.

3. MATRERIALS AND METHODS

3.1. Fly stocks

Fly stocks were maintained under standard conditions. The RNAi knockdown lines were obtained from the National Institute of Genetics (Mishima, Japan) and Vienna Drosophila RNAi Center (Vienna, Austria). GMR-Gal4 (yw), sp/CyOGFP (yw), pre/TM6BGFP (yw), Gla/CyoGFP (7), rL074 was received from the Bloomington Drosophila Stock Center (Indiana, USA). This line is the Mcm2 enhancer trap line (8) in which b-galactosidase was inserted in mcm2 promoter.All RNAi flies were received from the Vienna Drosophila RNAi Center (VDRC).

3.2. Knockdown experiments

The eye imaginal disc is a useful system for studying the relationship between the DNA replication initiation complex and the cell cycle or cell growth. We investigated the expression of Mcm2 in the eye imaginal disc of the Mcm2 enhancer trap line, rL074. To investigate the function of Mcm2 in the rear of the eye imaginal disc, we knocked down Mcm2 by GMR-Gal4 by means specific to tissues, time, and space. To knock down components of DNA replication machinery in tissues that undergo mitotic cell cycles, we chose the eyeless-Gal4 driver, which expresses target genes mainly in the eye–antennal primordia and central nervous system in the eye imaginal disc of the late embryo. We performed exhaustive knockdown of chromosomal DNA replication machinery, including Cdc45, GINS (9, 10), Polδ (11), and Polα-primase (2, 12).Next, we researched the genetic interaction between Mcm2 and the chromosomal DNA replication machinery.

3.3. Quantitative reverse transcriptase-PCR

Total RNA was isolated using Trizol Reagent (Invitrogen). Oligi dT primers and a Takara high fidelity RNA PCT kit (Takara, Kyoto, Japan) were used for generation of complementary DNA. Thereafter, real-time PCR was performed using a SYBR Green I kit (Takara) and the Applied Biocystems 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) (13). RNA expression efficiencies decreased to 25% in every case.

3.4. Scanning electron microscope

The eyes of the mutant flies were observed with a VE-7800 scanning electron microscope (Keyence, Osaka, Japan) in the high vacuum mode (13).

3.5. The number of loss of bristles and fusion cells

The number of loss of bristles and fusion cells were counted using the photos of electron microscope.

4. RESULTS

4.1. Mcm2 is expressed strongly in posterior of Drosophila melanogaster eye imaginal disc

We have researched the assembly mechanism of the eukaryotic DNA replication initiation complex using Polyoma virus, budding yeast and Drosophila ovary system.

As the Eye imaginal disc is very useful system for cell cycle, cell development and cell synchronization, we have started to study the relationship between DNA replication initiation complex and cell cycle or cell growth using this system.

At first, we focused on Mcm2. Mcm2 is a subunit of chromosomal DNA replicative helicase, Mcm2-7. In Drosophila, Mcm2-7 is a small part of CMG (CDC45, Mcm2-7 and GINS) complex, which is believed to be the chromosomal DNA replicative fork complex. We hypothesized the behavior of Mcm2 was close to that of the eukaryotic DNA replication initiation complex.

The Mcm2 enhancer trap line, rL074 is available (8). We investigated the expression of Mcm2 in the eye imaginal disc. Surprisingly, the expression of lacZ is limited in the posterior of morphogenic fallow. The endogenious Mcm2 expression is similar to LacZ staining (8). The tissue-specific knockdown of gene

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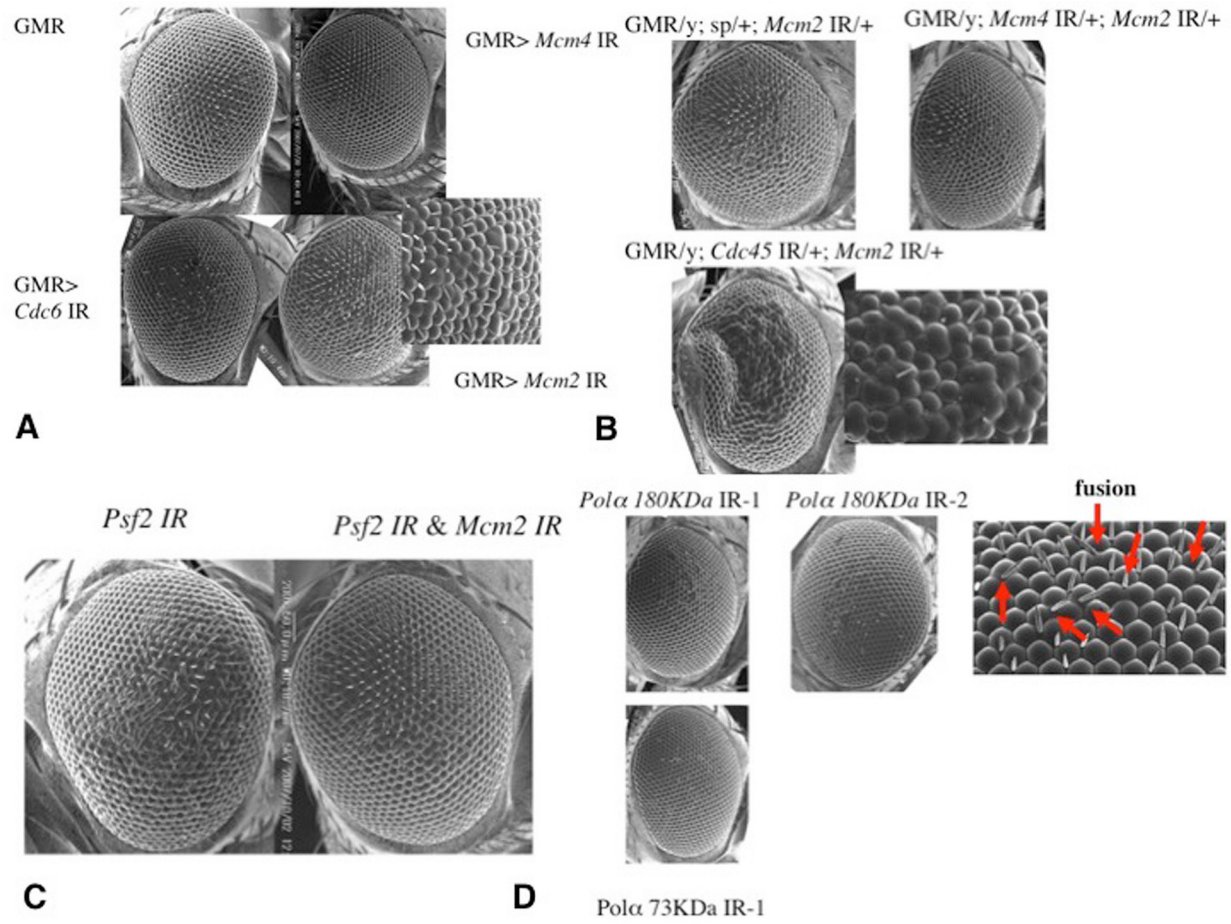

Figure 1. The knockdown of *Mcm2* by GMR-Gal4 driver induced rough eye phenotype. A. *GMR-Gal4, Mcm2-IR (UAS-mcm2)*; ">": two lines were crossed. B. Genetic interaction between Mcm2 and Mcm4 or Cdc45. C. Genetic interaction between *Mcm2* and *Psf2*, a subunit of GINS. D. The knockdown of Pola-primase by GMR-Gal4 driver induced loss of bristles and rough eye phenotype. The red arrows showed the fusion.

expression in RNAi knockdown flies resembled the Cre-loxP system in mice (3, 4, 5). In *Drosophila*, various flies temporally expressing Gal4 in various specific tissues (Gal4 driver) have been stocked. A fly having UAS-shRNA interference transgene construct crosses to the Gal4 driver. The mRNA of the target gene of UAS-shRNA was eliminated temporally and spatially in a tissue-specific manner (3, 4, 5).

In the initial strategy for researching RNAi availability in *Drosophila* (3), we aimed to compare the phenotypes of the flies in which the particular genes were knocked down in the embryonic stage and whole body and their mutants (4).

Previously, we performed knockdown of DNA replication machinery by *Act5C*- and *tubulin*-Gal4 drivers, which express target genes in whole body. The knockdown of Mcm2, Mcm4, Cdt1, and Cdc6 resulted in lethality (4).

4.2. The knockdown of Mcm2 by GMR-Gal4 leads to rough eye phenotype but not Cdc6 and Mcm4

To investigate the function of Mcm2 in the posterior of the eye imaginal disc, we perform the knockdown experience of RNAi interference of Mcm2 by GMR-Gal4 in tissue, time and space specific manner. As indicated in Figure1A, GMR>>Mcm2 IR showed the rough eye phenotype. On the other hand, though GMR>>Mcm4 IR showed no phenotype, GMR>>Cdc6 IR leads to the loss of bristles.

4.3. The knockdown of the chromosomal DNA replication machinery by GMR-Gal4 leads to rough eye phenotype and the loss of bristle

Though the phenotype of GMR>>Mcm2 IR is different from those of Cdc6 and Mcm4, we performed the exhaustive knockdown experience of chromosomal DNA replication machinery including

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Table 1. The number of loss of bristles and fusion cells in the knock-downed eye

Gene	Loss of bristle	Fusion cells
Mcm2	42/81 (51.85%)	43/81 (53.09%)
Polα180KDaIR-1	296/296 (100%)	22/24 (91.6.7%)
Polα 180KDaIR-2	298/304 (98.0.3%)	21/24 (87.5.%)
Polα 73KDaIR-1	141/162 (87.0.4%)	0 / 8 (0%)
GMR-Gal4	8/162 (4.93%)	0/162 (0%)

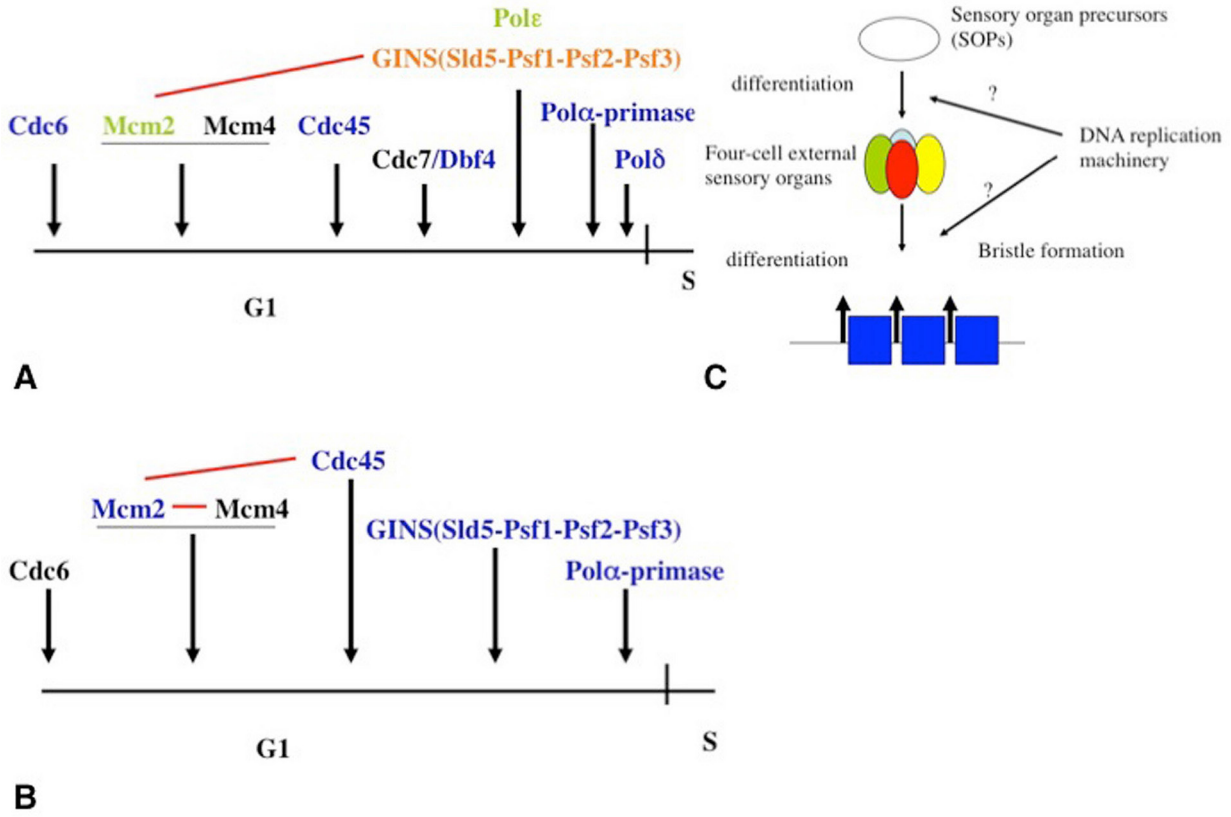

Figure 2. The genetic interaction. A. The genetic interaction at the bristle formation. Blue: proteins induced the loss of bristles; Green: proteins decreased the loss of bristles; Orange: proteins induced the abnormal bristles; Red bar: genetic cross. B. The genetic interaction at through eye phenotype. Blue: proteins induced rough eye; Red bar: genetic cross. C. The knockdown of DNA replication machinery by GMR-Gal4 driver induced the loss of bristles.

Cdc45, GINS (9, 10), Polδ (11) and Polα-primase (2, 12) (Figure.1B, C, D). As shown in Figure.1, the knockdown of the chromosomal DNA replication machinery leads to rough eye phenotype and the loss of bristles (Table.1). These results suggested that the machinery participates, directly or indirectly, in eye and bristle formation.

4.4. Genetic interaction between Mcm2 and CMG complex in rough eye phenotype

Next, we researched the genetic interaction between Mcm2 and the chromosomal DNA replication machinery. GMR>>Mcm4 IR, Mcm2 IR rescued the rough eye phenotype of GMR>>Mcm2 IR. GMR>>CDC45 IR, Mcm2 IR increased the phenotype (Figure 2A, B). Surprisingly, GMR>>CDC6 IR, Mcm2 IR is similar to the phenotype of GMR>>Mcm2 IR. Mcm2 didn't interact with Cdc6 genetically. Interestingly, though GMR>>Psf2 IR induced abnormal bristle formation and rough eye phenotype, GMR>>Psf2 IR, Mcm2 IR showed almost normal (Figure 1C, 2B, C). Finally, we couldn't find adult flies having GMR;+; rL074/Mcm2 IR. These data showed that Mcm2 would have unique function in nervous system.

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5. DISCUSSION

5.1. The knockdown of DNA replication machinery by GMR Gal4 drivers disturbed eye and bristle development

Previously, Tower *et al* have found the growth abnormality of the bristles of the back by analyzing Chiffon (dbf4 mutant) (14). Though we did not study the knockdown of Chiffon this time, it is expected to similar with the mutation. We've expected DNA replication machinery can also affect the hair morphogenesis. In this paper, we've just showed them artificially at the first time (Figure.2).

The tissue-specific knockdown of gene expression in the flies resembled the *Cre-loxP* system in mice (3, 4, 5). In a cross between a *Gal4* driver line and a line with a UAS small hairpin RNAi transgene construct, the RNAi target mRNA was eliminated temporally and spatially in a tissue-specific manner (3, 4, 5). We found that the knockdown of a number of components of the DNA replication machinery caused rough eye phenotype (10, 13) and bristle dysplasia (15). Among them, *Mcm2* is likely to function not only during the growth phase, but also during differentiation, and to have function in DNA replication.

At the Growth differentiation transition (GDT) point (16), differentiation signals are expected to enter into chromosomal DNA replication machinery (17). *Mcm2* may be the endpoint of these signals. Screening and the use of various mutants will clarify this.

Interactions of DNA replication machinery have been revealed by the analysis of *in vitro* analysis, such as *S. cerevisiae* or cell culture system, but not in the morphogenesis of higher eukaryotes. We uncovered these interactions in *Drosophila in vivo* through phenotype (Fig. 2C) (15). This shows that the DNA replication machinery functions as a complex during development and differentiation. We will extend our analyses to other tissues.

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We dedicate this work to Dr. Maki Asano. Present address of Dr Hidetsugu Kohzaki: Faculty of Allied Health Science, Yamato University, Suita, Osaka 564-0082, Japan, Present address of Dr Yota Murakami: Department of Chemistry, Faculty of Science, Hokkaido University, Kita-ku, Sapporo, Hokkaido 060-0810, Japan.

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Send correspondence to: Hidetsugu Kohzaki, Faculty of Allied Health Science, Yamato University, Faculty of Allied Health Science, Yamato University, Suita, Osaka 564-0082, Japan, Tel: 81-6-6384-8010, Fax: 81-6-6384-8010, E-mail: charaznable.k@gmail.com

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