The anaphase promoting complex/cyclosome is required during development for modified cell cycles

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Communicated by Gerald R. Fink, Whitehead Institute for Biomedical Research, Cambridge, MA, July 2, 2002 (received for review March 18, 2002)

Animals and plants use modified cell cycles to achieve particular developmental strategies. In one common example, most animals and plants have tissues in which the cells become polyploid or polytene by means of an S–G cycle, but the mechanism by which mitosis is inhibited in the endo cycle is not understood. The *Drosophila morula* (*mr*) gene regulates variant cell cycles, because in addition to disrupting the archetypal cycle (G_1–S–G_2–M), *mr* mutations affect the rapid embryonic (S–M) divisions as well as the endo cycle (S–G) that produces polyploid cells. In dividing cells *mr* mutations cause a metaphase arrest, and endo cycling nurse cells inappropriately reenter mitosis in *mr* mutants. We show *mr* encodes the APC2 subunit of the anaphase promoting complex/cyclosome. This finding demonstrates that anaphase promoting complex/cyclosome is required not only in proliferating cells but also to block mitosis in some endo cycles. The *mr* mutants further indicate that transient mitotic functions in endo cycles change chromosome morphology from polytene to polyploid.

The regulation of variant cell cycles is a crucial aspect of developmental control, yet many of these cycles are poorly understood. This observation is true for the endo cycle, a modified cell cycle used throughout the plant and animal kingdoms to produce polyploid or polytene cells (for review see ref. 1). In this cycle, DNA replication cycles with a gap phase, but mitosis does not occur. There is, however, variability in endo cycling tissues in the extent to which mitotic functions are repressed. In polytene cells, in which the replicated sister chromatids remain in tight association, it appears that no aspects of mitosis occur. In contrast, in mammalian megakaryocytes sister–chromatid separation and anaphase A movements occur, but anaphase B and cytokinesis are lacking (for review see ref. 2). Oscillations in the levels and activity of cyclin E/cyclin-dependent kinase (CDK) complexes are crucial for endo cycles (for review see ref. 1), but the mechanism by which mitotic functions are inhibited remains to be defined. Somehow, expression of mitotic cyclin proteins is shut off, and they may be destroyed in a regulated fashion. Variation in the control of the destruction of mitotic cyclins and other mitotic activators could explain the differences to which mitotic functions persist in distinct endo cycling cell types.

A pathway for inactivation of mitotic regulators by targeted proteolysis has been delineated (for reviews see refs. 3–5). Polyubiquitination of substrate proteins by a ubiquitin ligase, the anaphase promoting complex/cyclosome (APC/C), targets them for destruction by the 26S proteosome. The APC/C is composed of at least 11 subunits. In the yeast *Saccharomyces cerevisiae* mutations in the APC subunits *cdc16*, *cdc23*, and *cdc27* were identified because they block cyclin ubiquitination and destruction. They cause a failure of release of sister–chromatid cohesion, block the metaphase/anaphase transition, and prevent exit from mitosis. The APC/C is regulated in part by two associated proteins, Cdc20 (FIZZY in *Drosophila*) and Cdh1 (FIZZY-RELATED in *Drosophila*), and these proteins both activate the APC/C with proper timing and provide substrate specificity. The APC/C is activated at the metaphase/anaphase transition by the Cdc20 protein and later in telophase and G_1 by the Cdh1 protein. Mutations in the *Drosophila fizzy* and *fizzy-related* APC/C regulators have been characterized (6–9). Embryos mutant for *fizzy* arrest in metaphase of mitosis, whereas embryos lacking *fizzy-related* fail to cease proliferation at the appropriate stage. Recently, mutations have been described in the *Drosophila APC5* subunit gene and shown to affect mitotic divisions during larval stages (10).

The failure of mitosis to progress beyond metaphase in mutants for APC/C subunits is caused by the failure to degrade substrates whose sequential destruction is needed for steps through mitosis (for reviews see refs. 3–5). At the metaphase/anaphase transition the securin protein family members are ubiquitinated and proteolyzed. Members of this family include the Pds1 protein in *S. cerevisiae*, Cut2 in *Schizosaccharomyces pombe,* and PIMPLES in *Drosophila* (11–13). The securin proteins regulate the separase protease that targets the cohesin complex (for review see ref. 14), and in yeast the Slk19 protein needed for mitotic spindle function (15). Thus, by indirectly activating separase, the APC/C causes the release of sister–chromatid cohesion and events needed for the completion of mitosis. Mitotic cyclins are also targeted for degradation by the APC/C; this shuts off the mitotic cyclin/CDK1 complex to inactivate mitosis-promoting functions and to also permit resetting of the replication origins for another round of DNA synthesis. Additional direct substrates of the APC/C as well as indirect substrates that are cleaved by separase are likely to be involved in the exit from mitosis.

The *Drosophila morula* (*mr*) gene is critical for the inactivation of mitotic functions throughout development in a variety of developmentally-modified cell cycles (16). The initial *mr* alleles, described in 1919 and 1937 by Bridges, are female sterile (17). In these mr^1 and mr^2 mutants, the endo cell cycle of the polyploid ovarian nurse cells is affected (16). The nurse cells initiate the endo cycle, but after several cycles return to mitosis, condensing their chromosomes, assembling mitotic spindles, and arresting in a metaphase-like state. Stronger alleles of *mr* cause lethality late in larval development (16). In these mutant animals, there is a failure to inactivate mitotic functions in proliferating cells. Dividing cells in the larval brain arrest in metaphase. The *mr* phenotypes indicate that *mr* is required to prevent mitosis in some endo cycling cells, but also for the inactivation of mitotic functions and exit from mitosis in dividing cells. These intriguing phenotypes made it important to define the molecular mechanism by which *mr* inhibits mitotic activities.

DEVELOPMENTAL BIOLOGY

Abbreviations: APC/C, anaphase promoting complex/cyclosome; CDK, cyclin-dependent kinase.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AY129297).

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Here we describe a molecular analysis of *mr*. We find that it encodes the APC2 subunit of the APC/C, thus explaining the dual role that *mr* plays in inhibiting mitotic functions in the endo cycle and in promoting mitotic exit. These results uncover a surprising requirement for the APC/C in controlling chromosome morphology in polyploid cells.

Materials and Methods

Southern and Northern Blots. Quantitative Southern blots to map deficiency breakpoints from heterozygous flies were done as described in Bickel *et al.* (18). cDNAs obtained from the Berkeley *Drosophila* Genome Project were sequenced by Research Genetics (Huntsville, AL). RNA from different developmental stages was isolated, Northern blots were prepared, and these were hybridized to the purified insert fragment from the LD21042 cDNA that was labeled by random priming (18). The expression pattern of the *mr* transcript was analyzed during oogenesis by *in situ* hybridization to whole mount ovaries as described (19).

cDNA Rescue Experiments. The cDNA insert in clone LD24965 was excised with *Eco*R1/*Xho*I and subcloned into the pCS2+ vector to acquire desired sites. The fragment was then cut out with *Bam*HI/*Xba*I and subcloned into the same sites of the pUASp vector, which was obtained from P. Rorth (European Molecular Biology Laboratory, Heidelberg). This transposon is called *P[w$^+$ UAS mr]*. Embryo injections and the establishment of transgenic lines was as described by Spradling (20). In two of the lines used for rescue experiments (A1, 6D), *P[w$^+$ UAS mr]* was inserted on the third chromosome, and in line C5 the transposon was inserted on the X chromosome. Two Gal-4 driver lines were used to induce expression of the *mr* cDNA: the actin-Gal-4 line was from the Bloomington Stock Center (Bloomington, IN), and the Nanos-Gal-4:VP16 line was obtained from P. Rorth (21).

DNA Sequencing of *mr* Mutations. To sequence the *mr* mutations DNA was prepared from homozygous animals, the ORF was PCR amplified, and the PCR products were sequenced directly by Research Genetics. For *mr^1* or *mr^2*, homozygous adult females were used. For the lethal alleles *mr^3* and *mr^5*, homozygous mutant larvae were identified from stocks in which the *mr* mutants were in trans to the *CyO* balancer chromosome containing *P[w$^+$, Act-GFP]*. Homozygous *mr^4* mutant larvae were collected from stocks containing the *TSTL* chromosome 2;3 translocation that is marked with the dominant *Tubby* marker, which can be scored in larvae or adults. *mr^4* mutant larvae that were non-Tubby were collected. The DNA sequence was determined for both strands, and the isogenic chromosome on which the *mr^3*, *mr^4*, and *mr^5* mutations were induced was sequenced as a control.

Immunostaining of Ovaries and Embryos. Strains containing extra copies of the *cyclin B* gene were provided by C. Lehner (Univ. of Beyreuth, Beyreuth, Germany). The chromosome morphology of nurse cells was examined after 4′,6-diamidino-2-phenylindole (DAPI) or propidium iodide staining as described (16). Mitotic spindles were examined in embryos after staining with rat anti-α-tubulin antibodies from Accurate Chemical and Accurate Scientific (Westbury, NY) as described by Tang *et al.* (22), except that the embryos were fixed in methanol. Anti-cyclin B staining of egg chambers was done as previously described (16). The monoclonal antibody developed by P. O'Farrell (Univ. of California, San Francisco) was obtained from the Developmental Studies Hybridoma Bank. Microscopy was done on a Zeiss LSM510 confocal laser system mounted on a Zeiss Axiovert 100M microscope with a ×40/1.2 W Korr C-APOCHROMAT water objective. Optical sections were taken and projected onto a single plane.

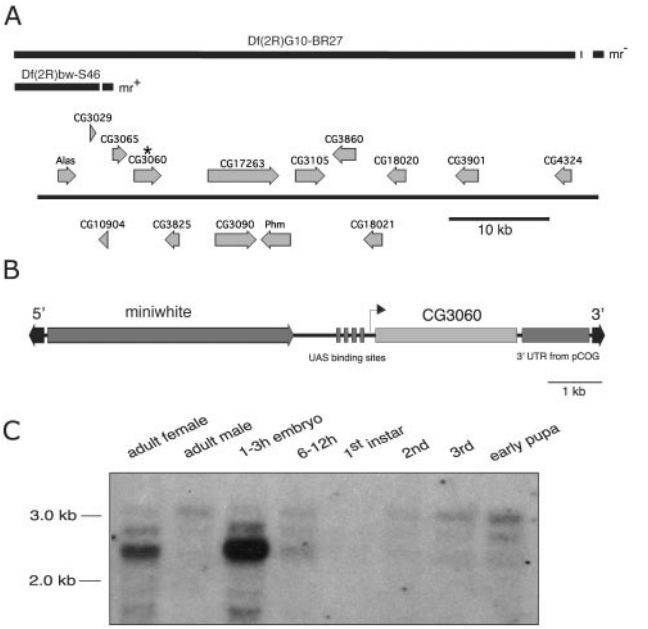

Fig. 1. Isolation and expression of the *mr* gene. (*A*) The genomic region of 60A containing the *mr* gene. The DNA intervals removed by the two crucial deficiencies that are mr$^-$ or mr$^+$ were determined by quantitative Southern blots and are shown by solid lines. The restriction fragments within which each deficiency breaks are drawn as dotted lines to denote that the exact position of the breakpoint within each fragment is not known. The two known genes, *Alas* and *Phm*, and the predicted ORFs are shown by filled arrows whose length is proportional to the size. The arrowheads indicate the 3′ end of each gene. The CG3060 ORF (asterisk) is *mr*. (*B*) The structure of *P[w$^+$ UAS mr]*. The LD24965 cDNA was used. The black arrows at the end of the transposon denote P element sequences. (*C*) Developmental Northern blot of *mr* expression. Poly(A)$^+$ mRNA was isolated from each of the indicated developmental periods, and the Northern filter was probed with the labeled cDNA fragment from LD21042. Three *mr* transcript forms are detected, and these vary in expression level at different developmental stages. See Fig. 5, which is published as supporting information on the PNAS web site, www.pnas.org, for a loading control. The size standards are the 1-kb ladder from GIBCO/BRL.

Results

Identification of the *mr* Gene. We used a positional cloning strategy to recover the *mr* gene. The gene is removed by the deficiency *Df(2R)G10-BR27*, but it is present in *Df(2R)bw-S46* (16). Quantitative Southern blots were used to map the position of the breakpoints of these two deficiencies (data not shown), defining a minimal region of 40 kb that contained the gene (Fig. 1*A*). Within this region, the CG3060 was an ideal candidate for *mr,* because it contains a cullin domain (http://www.fruitfly.org/), and cullin-domain proteins are involved in protein degradation during the cell cycle (3). We sequenced the longest cDNA corresponding to this ORF, LD24965. The sequence analysis confirmed the intron/exon structure predicted by the genome project, with a transcription unit spread over 2.96 kb of genomic DNA producing a processed transcript of 2.53 kb with eight exons.

We tested the ability of this cDNA to rescue the *mr* mutant phenotypes. The insert was cloned into the pUASp expression vector to generate transposon *P[w$^+$ UAS mr]* (Fig. 1*B*). Transformant lines were generated, crossed to *mr* mutants, and expression of the cDNA was induced and examined for phenotypic rescue. To exclude phenotypes from potential background mutations on the *mr* chromosomes, complementation by the transgenes was scored in transheterozygotes with two different *mr* mutant chromosomes. To test for rescue of the female-sterile *mr* alleles, the Gal-4 activator was expressed in the female germ

Table 1. Rescue of *mr* phenotypes by ectopic expression of transgenes

mr genotype	Actin Gal-4 induction				Nanos Gal-4 induction			
	Line A1	Line 6D	Line C5	Control	Line A1	Line 6D	Line C5	Control
mr^3/mr^4	Viable and semifertile	Viable and sterile	Viable and fertile	Lethal				
mr^1/mr^2	Fertile	Fertile	Fertile	Sterile	Fertile	Fertile	Fertile	Sterile

line under the control of the *nanos* regulatory elements. Three independent cDNA transformant lines restored female fertility to mr^1/mr^2 transheterozygotes when Gal-4 was induced but not in uninduced controls (Table 1). Induction of Gal-4 by the ubiquitously expressed *actin* promoter also rescued fertility in these flies (Table 1). The actin-Gal-4 driver was able to restore viability to transheterozygotes of two lethal alleles, mr^3 and mr^4. These experiments revealed differences in each of the transgenic lines, presumably reflecting different levels of expression, in that the $P[w^+\ UAS\ mr]$–C5 line complemented fully to restore both viability and fertility, the $P[w^+\ UAS\ mr]$–A1 line restored viability and partial fertility, whereas the $P[w^+\ UAS\ mr]$–6D line solely rescued viability. The ability of the LD24965 cDNA to rescue both strong *mr* lethal alleles and weaker female-sterile alleles demonstrates that it encodes the structural gene for *mr*.

The phenotypes of the *mr* mutations indicated that the gene is required for cell cycle regulation throughout development: during adult oogenesis, in the early S–M embryonic cycles, in larval endo cycles, and in mitotically dividing larval tissues. We examined the expression pattern of the gene by hybridizing the insert from a *mr* cDNA (LD21042) to a Northern blot with RNA isolated from different developmental stages (Fig. 1*C*). This experiment showed that the *mr* gene is expressed throughout development, but, interestingly, three different transcript forms are present, and these show different developmental regulation. There is an abundant transcript of ≈2.5 kb present in adult females and early embryos, most likely the form expressed during oogenesis and deposited into the developing oocyte. In larval development, transcripts of 2.9 and 3.2 kb become more prevalent, and in adult males solely the 3.2-kb transcript is detectable. The cDNAs recovered by the genome project from embryonic libraries all encode one protein form and are likely to represent the transcript that experimentally measures 2.5 kb. Additional analyses will be required to determine whether the three transcript forms arise from distinct promoters or alternative processing, and whether these result in alternative forms of the protein.

The MORULA Protein Is the Ortholog of APC2. BLAST searches of the predicted ORF of the LD24965 cDNA showed that the protein is closely related to the APC2 subunit of the APC/C (23, 24). APC2 contains a cullin domain, but MR shows sequence conservation throughout the protein sequence, not solely within the cullin domain. Overall, MR is 36% identical to human APC2 and shares 56% homology (Fig. 2). MR is more distantly related to the APC2 subunit from *S. cerevisiae* (Fig. 2).

To understand the basis of the lethal and sterile phenotypes in *mr* mutants, we sequenced the five *mr* mutations. The mr^3 mutant has the most severe phenotype in larval brains, and the molecular analysis confirms that this is the strongest allele. The mr^3 strain contains a nucleotide substitution that is predicted to change Trp-282 to a stop codon, truncating the protein to approximately one-third of its length and removing the cullin domain (Fig. 2). The mr^4 and mr^5 alleles were phenotypically characterized as strong alleles because they cause lethality, and these too have pronounced molecular changes. Both alleles share the same nucleotide substitution that would alter a splice acceptor site after the sixth intron (Fig. 2). If the intron were not spliced, the protein would be expected to be missing the C

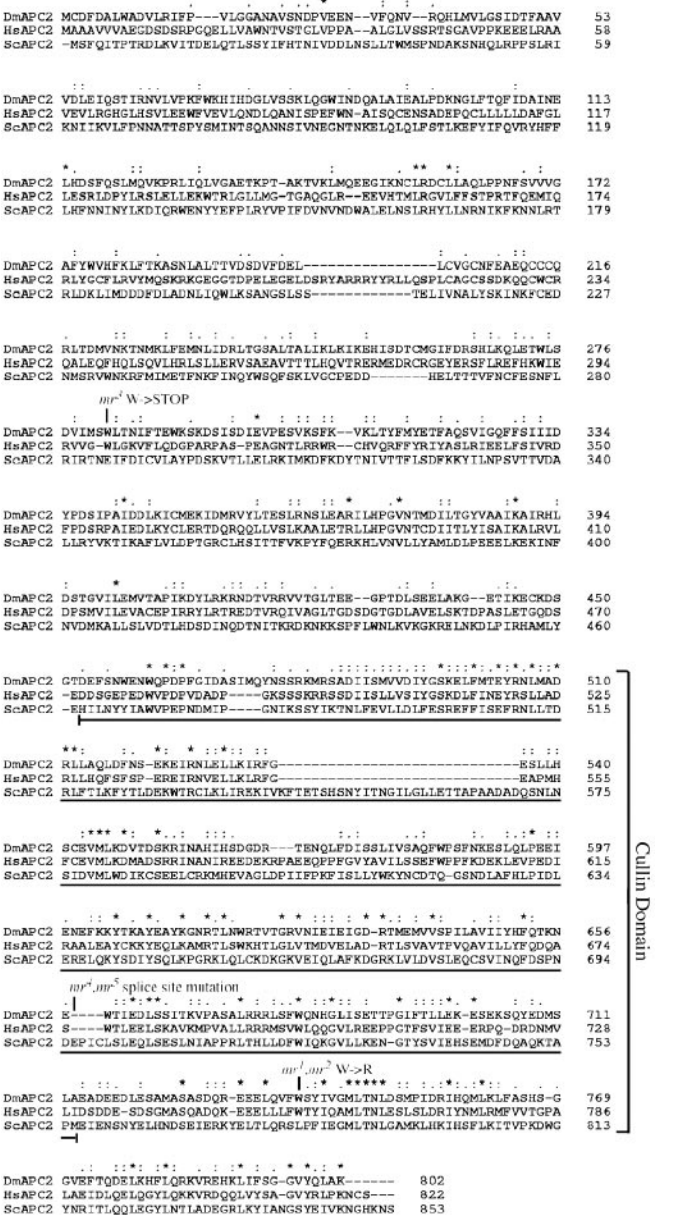

Fig. 2. The *Drosophila mr* gene is an ortholog of APC2. The translated *mr* cDNA sequence (Dm) is aligned with the APC2 coding region from human (Hs) and *S. cerevisiae* (Sc). The cullin domain is underlined and indicated by brackets. Residues conserved in all three species are highlighted by asterisks. Double dots indicate that one of the following groups is fully conserved: STA, NEQK, NHQK, NDEQ, QHRK, MILV, HY, or FYW. A single dot represents conservation of groups with less similarity: CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, FVLIM, or HFY. The dashed lines show where the alignment program introduced gaps to maximize homologous alignment. The changes present in the *mr* mutants are also indicated. In the mr^3 mutant Trp-282 is changed to a stop codon. The mr^4 and mr^5 mutants have a nucleotide substitution at a splice acceptor site that would cause remove the C terminus of the protein from Glu-657 on. The sole change found in mr^1 and mr^2 strains was a substitution of Trp-739 to Arg.

Table 2. Enhancement of *mr* lethality by increased *cyclin B*$^+$ genes

Progeny	Cross 1	Cross 2
Balancer siblings (mr^1 or $mr^3/SM6a$)	210	104
mr mutants (mr^1/mr^3)	39	0

Cross 1, control: $mr^3/SM6a \times mr^1\ sp/SM6a$; cross 2: increased cyclin B$^+$: $mr^3\ sp/SM6a \times mr^1\ sp/SM6a$; $[CycB^+] \times 2$.

terminus, including part of the cullin domain. The mr^4 and mr^5 were recovered from the same ethyl methanesulfonate screen and likely represent repeat isolates from the same premeiotic mutation event. The mr^1 and mr^2 alleles were isolated from natural populations about 20 years apart, and thus could contain the same mutation (17). Indeed, both have a single nucleotide change predicted to cause a Trp to Arg amino acid substitution (Fig. 2). This change is C-terminal to the cullin domain. This Trp is conserved in mammalian APC2 subunits, but not in the budding yeast protein. This flexibility in amino acid sequence may explain why these are the weakest of the *mr* mutations.

The APC/C Is Required for the Repression of Mitotic Functions in Some Endo Cycles. The identification of MR as APC2 readily explains the metaphase arrest observed in proliferating tissues from *mr* mutants and establishes that APC2 is essential for APC/C activity. This identification is significant also for demonstrating that the APC/C is necessary during endo cycles to inhibit mitotic functions and is consistent with the previous observation that levels of cyclin B are inappropriately high in *mr* mutant nurse cells (16). Our finding that APC/C is required for endo cycles raised the question of whether increased levels of cyclin B were responsible, at least in part, for the larval *mr* mutant phenotypes. To address this question, we tested whether increased levels of cyclin B could enhance *mr* phenotypes. The transheterozygous combination of the mr^1/mr^3 mutant alleles provided a sensitized test because these transheterozygotes produce viable adults, though at only 50% the number predicted for a fully viable combination (Table 2). We increased the copy number of wild-type *cyclin B* genes by two, thereby increasing the level of cyclin B protein (25, 26). We found that the increased cyclin B enhanced the lethal phenotype such that in the presence of extra copies of the *cyclin B* gene, no viable mr^1/mr^3 adults were recovered (Table 2). These results provide *in vivo* confirmation that levels of cyclin B affect the *mr* phenotype and contribute to the lethality of strong *mr* mutants.

We tested also for enhancement of the female-sterile phenotype of the mr^1/mr^2 alleles by increased levels of cyclin B to examine the requirements for APC/C function during specific differentiation aspects of the nurse cell endo cycle (see schematic in Fig. 3*A*). The five initial endo cycles of the nurse cells produce polytene chromosomes in which the replicated sister chromatids remain in tight association. After cycle 5, the chromosomes condense, and then the replicated copies partially disperse so that in subsequent endo cycles the chromosomes appear polyploid rather than polytene (27). A striking feature of the mr^1/mr^2 phenotype is that the first five nurse cell endo cycles appear normal (16). The *mr* defect is not manifested until the polytene/polyploid transition, when in *mr* mutant nurse cells the chromosomes condense more fully than in wild type, spindles are formed, and the condensed chromosomes remain arrested in a metaphase-like state. This phenotype showed the same time of onset in nurse cells mutant for the lethal mr^5 mutation, generated by germline clones (16). This finding raised the possibility that the polyteny/polyploidy transition involves a cell cycle change to

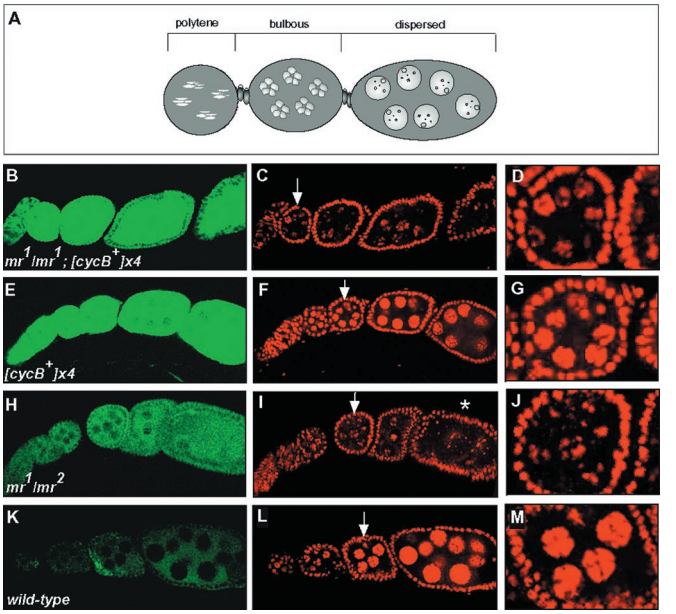

Fig. 3. The *mr* mutant nurse cell phenotype and the effect of increased cyclin B protein. (*A*) Schematic diagram of the changes in nurse cell chromosomes during stages 4–6 egg chamber development. The nurse cell chromosomes are polytene through stage 4; they then condense and take on a bulbous appearance before dispersing to be polyploid. (*B–M*). The effect of *mr* mutations and increased cyclin B as visualized by propidium iodide staining of the DNA (red; *C*, *D*, *F*, *G*, *I*, *J*, *L*, and *M*) and immunolabeling of cyclin B (green; *B*, *E*, *H*, and *K*). In *mr* mutants the nurse cells revert to mitosis at stage 5, shown by an arrow in *C*, *F*, *I*, and *L* and enlarged in *D*, *G*, *J*, and *M*. The onset of mitosis in mutant stage-5 egg chambers is evidenced by the appearance of condensed chromosomes (*D* and *J*) compared with the interphase appearance of wild type (*G* and *M*). Increased cyclin B did not cause the onset of mitosis to occur earlier in nurse cell development. Increased cyclin B did not result in the onset of mitosis in wild-type nurse cells, even when levels were higher than in *mr* mutants. In *mr* mutants the nurse cells in egg chambers after stage 7 frequently became pycnotic, as shown by the egg chamber with the asterisk in *I*.

a transient mitotic state and that, at this point, *mr* mutant nurse cells are vulnerable to reenter mitosis fully.

Consistent with the proposal that the onset of the *mr* phenotype reflects cell cycle changes in the nurse cells at the polytene/polyploid transition, we found that increased levels of cyclin B protein did not cause an earlier appearance of mitosis in the *mr* mutant nurse cells (Fig. 3 *B*, *C*, *H*, and *I*). We did observe an increase in the number of later stage egg chambers with pycnotic or degenerating nurse cells in the presence of increased cyclin B (data not shown). We also found that elevation of cyclin B protein in a wild-type background was insufficient to cause nurse cells to revert to mitosis (Fig. 3 *E* and *F*). It remains possible that increasing the levels of other APC/C substrates would cause an earlier endo cycle defect.

We examined the levels of *mr* transcript during egg chamber development by *in situ* hybridization and found that the transcript was present in the nurse cells throughout oogenesis (see Fig. 6, which is published as supporting information on the PNAS web site). There was not a detectable induction of *mr* transcript at the polyteny–polyploidy transition, as expected given that APC/C activity is controlled posttranscriptionally (5, 28). The *mr* transcript levels were increased in stage-10 egg chambers, a time when nurse cells undergo maximal gene expression.

APC/C Function Is Necessary for S–M Cycles and Centrosome Attachment. The *mr* mutants permitted us to analyze the requirements for APC/C function in two other variant cell cycles, meiosis and the embryonic S–M cycles. Although many egg chambers de-

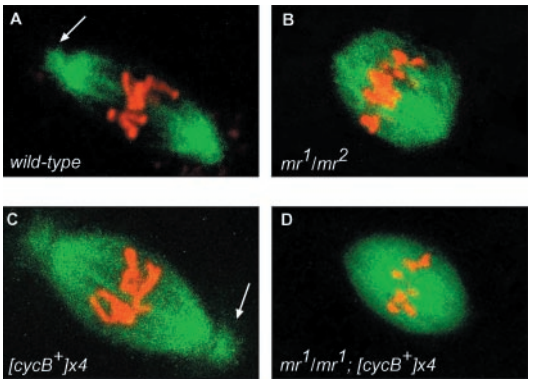

Fig. 4. Spindle and chromosome morphology in *mr* mutant embryos. Embryos were collected from mothers that were wild type, mr^1/mr^2, wild type with four extra copies of *cyclin* B^+, or mr^1/mr^2 with four extra copies of *cyclin* B^+. The fixed embryos were stained with propidium iodide to visualize DNA (red) or anti-tubulin antibodies to visualize the spindle (green). (*A*) A metaphase nucleus in an embryo from a wild-type mother has asters of microtubules at each spindle pole, revealing functional centrosomes (arrows). (*B*) An example of a metaphase figure from an embryo from mr^1/mr^2 mutant mothers. In these mutant embryos the spindles are wide, with broad poles, they lack asters, and the chromosomes are hypercondensed. (*C*) Increased cyclin B in an embryo from a wild-type mother does not result in broad, anastral spindles or increased chromosome condensation. (*D*) Increased cyclin B in embryos from mr^1/mr^2 mutant mothers does not alter the mutant spindle and chromosome morphology.

generate in the female-sterile mr^1 and mr^2 alleles after stage 7 because of attempted mitosis in the nurse cells (Fig. 3 *H* and *I*), some egg chambers complete oogenesis. This number is affected by genetic background (16). We previously observed that, in embryos produced by mr^1/mr^2 mutant mothers, the zygotic nuclei were arrested in metaphase. We reexamined mature oocytes and embryos from these mothers in more detail to determine whether meiosis was completed, whether pronuclear fusion occurred, and whether spindle structure was affected. Mature *Drosophila* oocytes were arrested in metaphase I, and the metaphase I arrest was properly maintained in all of the mature oocytes examined from mr^1/mr^2 mutant females ($n = 169$). We examined embryos to test whether meiosis was completed in mr^1/mr^2 mutants. Thirty-three embryos from mr^1/mr^2 mutant females that had been stained with antibodies against tubulin and a DNA stain were analyzed by confocal microscopy. Meiosis was completed in all of these embryos (data not shown). There was not a meiosis I or a meiosis II spindle present, and this can be readily seen in mutants blocked in the meiotic divisions (29).

Although meiosis is completed in these *mr* mutants, two striking features were that all of the zygotic nuclei, and frequently the polar bodies, were arrested on metaphase spindles that were anastral and had broad poles (Fig. 4 *B* and *D*). An additional phenotype was that the chromosomes were hypercondensed in the embryos from *mr* mutant mothers (compare Fig. 4 *A* with *B*). This phenotype was observed previously in metaphase-arrested neuroblasts, cells that are undergoing the canonical cell cycle (16). The excessive condensation seen in metaphase-arrested embryonic nuclei indicates that during the S–M cycles as well as the normal cell cycle the chromosomes continue to undergo condensation if they remain arrested in metaphase. To determine whether these phenotypes were the consequence of increased levels of cyclin B protein, we attempted to phenocopy these affects by increasing cyclin B levels using strains with four extra copies of the *cyclin B* gene in a wild-type background. Embryos produced from these mothers did not exhibit the *mr* phenotypes (Fig. 4*C*). These observations complement those of Wakefield *et al.* (30), who showed that increasing levels of cyclin B protein did not cause centrosomes to dissociate from the mitotic spindles. Increased levels of cyclin B did not worsen phenotypes in embryos from mr^1/mr^2 mutant mothers (Fig. 4*D*), suggesting these defects may be caused by increased levels of other APC/C targets.

Discussion

The identification of the *Drosophila mr* gene as APC2 demonstrates the essential role of the APC/C in developmentally modified cell cycles as well as the archetypal mitotic cycle. In particular, it is striking that APC/C function is crucial for endo cycles in which it appears that mitosis does not occur. The *mr* phenotypes reveal an unexpected and intriguing role for the APC/C in setting the parameters of the endo cycle that affect the chromosome structure of the replicated sister chromatids. These results are significant also in establishing an essential role for the APC2 subunit in metazoans.

The Roles of APC/C in Endo Cycles. The endo cycle can produce polytene or polyploid chromosomes. In the former case, the replicated sister chromatids remain tightly associated, whereas they are dispersed in polyploid cells (for review see ref. 1). The *mr* results provide clues into possible cell cycle differences in endo cycles leading to polyteny versus polyploidy. In *Drosophila*, most cells are polytene, and the nurse cells are rare in becoming polyploid. We did not observe an endo cycle failure in any larval polytene tissue in *mr* mutants except the ring gland, which begins the endo cycle late in development (16). In polytene cells, APC/C activity may be required only at the initial transition from the mitotic cycle to the endo cycle to remove any remaining mitotic regulators. The majority of larval tissues undergo the transition to the endo cycle late in embryogenesis (31). Once entrenched in the endo cycle with expression of mitotic cyclin genes shut off, the APC/C would be dispensable. Consistent with this hypothesis, in embryos homozyous for a deletion that removes the *fzr* gene, the onset of the first S phase of the endo cycle is inhibited in several tissues (9). These observations indicate that APC/C is required during embryogenesis, but it is likely that maternal stockpiles of MR protein are present to permit the onset of the endo cycle. It remains possible that *mr* is essential not only for the onset but also for the maintenance of polytene endo cycles throughout development and that the maternal pools persist during larval development and into adult stages. The molecular identification of MR permits the generation of reagents to distinguish whether this is the case.

Even though the APC/C does not appear to be required for the maintenance of polytene endo cycles, it plays a critical role in the parameters of endo cycles that produce polyploid chromosomes. In polytene cells, APC/C may need to be inactive so that securin remains constitutively active and that the cohesin complex and sister–chromatid cohesion contribute to the tight alignment of replicated sister chromatids. In polyploid cells, degradation of securin by the APC/C could lead to the separation of sister chromatids as a result of separase activity. This activity would explain why the APC/C becomes crucial in the nurse cells when the transition from polyteny to polyploidy occurs. In addition, a low level of transient induction of cyclin B/CDK1 activity, so far undetectable by immunolabeling methods, could account for the chromosome condensation observed at this transition. This hypothesis is supported by the presence of cyclin B protein in *mr* mutant nurse cells at this time. Overexpression of cyclin B does not, however, induce the change from polyteny to polyploidy at an earlier developmental stage, and this would be consistent with other mitotic activities such as the separase protease being necessary. Elimination of securin and separase activity in the nurse cells by making mutant clones might permit a test of this hypothesis.

The requirement of APC/C activity for the endo cycle leading

to polyploid chromosomes that we observe in *Drosophila* may be a characteristic feature of endo cycles in many organisms. In alfalfa the expression of a Cdh1-like gene is increased in nodules that have cells undergoing endo cycles (32). Overexpression of an antisense RNA reduced the ploidy of polyploid cells in the petioles, hypocotyls, and roots (32). These results are consistent with a role for the APC/C in the maintenance of the endo cycle in polyploid plant cells, though effects on the onset of the endo cycle were not addressed by these analyses. Elimination of mitotic cyclin protein is necessary for endo cycles in plants, because ectopic expression of cyclin B1;2 in *Arabidopsis* trichome cells causes these cells to undergo mitosis rather than endo cycles (33).

Functions for APC/C in Archetypal, S–M, and Meiotic Cycles. The *mr* mutant effects on the canonical G_1–S–G_2–M cycles are consistent with mutant phenotypes described for the budding yeast *S. cerevisiae*. An increased number of mitotic cells is seen in brains from mutant larvae, and the majority of these are arrested in metaphase (16). Interestingly, many of these are polyploid, revealing that the metaphase arrest is not indefinite and the cells re-replicate. It appears that sister–chromatid separation is occurring before this replication, because the extra chromosome copies are separate and not attached at their centromeres as in the *pimples* securin mutant (34). Thus, either sufficient *mr* function is present even in the lethal alleles (possibly from maternal pools) to allow eventual exit from mitosis, or an APC/C independent pathway for sister separation and resetting of replication origins may exist.

The regulation of mitotic exit during the syncytial S–M cycles of early *Drosophila* embryogenesis requires localized degradation of mitotic cyclins in the vicinity of each nucleus (35). In *mr* mutant embryos the initial S–M cycles arrest in metaphase; this observation combined with the metaphase arrest seen in maternal-effect *fzy* alleles (6) demonstrates that APC/C function is required for mitotic exit during the S–M cycles.

Mutations in APC/C subunits in *Caenorhabditis elegans* have been demonstrated to block the metaphase I/anaphase I transition and completion of meiosis (36–38). In contrast in *Xenopus* oocytes, inactivation of the APC by injection of antibodies to either the Cdc27 APC subunit or the Fzr activator or injection of inhibitory peptides does not affect the completion of meiosis I but causes a metaphase II block (39). Both meiotic divisions are completed in the *mr* mutant eggs. This does not exclude a role for APC/C either in the separation of homologs in meiosis I or sister chromatids in meiosis II, because the *mr* mutations that produce eggs are weak alleles and residual activity may be sufficient for the completion of meiosis.

Analysis of APC function during metazoan development, here exemplified by the phenotypes of *Drosophila mr* mutants, defines the role of this ubiquitin ligase in cells undergoing an archetypal cell cycle but also illustrates its use in modified cell cycles. The role of the APC in meiosis requires further investigation, but its activity in the embryonic S–M cycles is clear. In addition to demonstrating a critical role for APC/C in endo cycles, the *mr* mutants uncover an intriguing use of mitotic activities to alter chromosome morphology in polytene and polyploid cells.

We thank Christian Lehner for providing the strains with extra *cyclin* B^+ genes, Pernille Rorth for the pUASp vector and the Nanos-Gal-4:VP16 driver line, the Berkeley *Drosophila* Genome Project for sequence information, and the Bloomington *Drosophila* Stock Center for stocks. The cyclin B monoclonal antibody was from the Developmental Hybridoma Bank developed under the auspices of the National Institute of Child Health and Development and maintained by the University of Iowa. We acknowledge Linda Hall for providing unpublished information about the 60A genomic interval. We are grateful to Angelika Amon, Gerry Fink, and Jan-Michael Peters for insightful comments on the manuscript, Jan-Michael Peters for helpful discussions, Doug Fenger for advice on DNA sequencing and genomic analysis, Robert Latak for help with sequence alignment, and Nicki Watson for help with microscopy. The confocal microscope used was in the W. M. Keck Foundation Biological Imaging facility at the Whitehead Institute. B.H.R. was a postdoctoral fellow of the Life Sciences Research Foundation, sponsored by Advanced Cancer Studies, Inc. This work was supported by National Institutes of Health Grants GM39341 and GM57960 (to T.L.O.-W.).

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