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The *Drosophila* homolog of *MCPH1*, a human microcephaly gene, is required for genomic stability in the early embryo

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Summary

Mutation of human microcephalin (*MCPH1*) causes autosomal recessive primary microcephaly, a developmental disorder characterized by reduced brain size. We identified *mcph1*, the *Drosophila* homolog of *MCPH1*, in a genetic screen for regulators of S-M cycles in the early embryo. Embryos of null *mcph1* female flies undergo mitotic arrest with barrel-shaped spindles lacking centrosomes. Mutation of Chk2 suppresses these defects, indicating that they occur secondary to a previously described Chk2-mediated response to mitotic entry with unreplicated or damaged DNA. *mcph1* embryos exhibit genomic instability as evidenced by frequent chromatid bridging in anaphase. In contrast to studies of human *MCPH1*, the ATR/Chk1-mediated DNA checkpoint is intact in *Drosophila mcph1* mutants. Components of this checkpoint, however, appear to cooperate with MCPH1 to regulate embryonic cell cycles in a manner independent of Cdk1 phosphorylation. We propose a model in which MCPH1 coordinates the S-M transition in fly embryos: in the absence of *mcph1*, premature chromosome condensation results in mitotic entry with unreplicated DNA, genomic instability, and Chk2-mediated mitotic arrest. Finally, brains of *mcph1* adult male flies have defects in mushroom body structure, suggesting an evolutionarily conserved role for MCPH1 in brain development.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/120/20/3565/DC1

Key words: *Drosophila*, Embryogenesis, Microcephaly, Cell cycle, Mitosis, DNA checkpoint, BRCT domain

Introduction

*Drosophila melanogaster* is an ideal model organism for study of the cell cycle during development (reviewed by Foe et al., 1993; Lee and Orr-Weaver, 2003). *Drosophila* achieves rapid embryogenesis by using a streamlined cell cycle that is not dependent on transcription or growth. The first 13 embryonic cell cycles are nearly synchronous nuclear divisions without cytokinesis occurring in the shared cytoplasm of the syncytial blastoderm. These cycles differ from canonical G1-S-G2-M cycles in that they have no intervening gaps; instead DNA replication and mitosis rapidly oscillate. Maternal RNA and protein stockpiles drive these abbreviated ‘S-M’ cycles (~10 minutes each). In mammalian embryos, rapid peri-gastrulation divisions that occur later in development share many features and have been proposed to be related by evolutionary descent to early embryonic divisions of flies and frogs (O’Farrell et al., 2004). Thus, advances gained from studies of these streamlined cycles in ‘simple’ model organisms likely have relevance for understanding mammalian cell cycles.

In a genetic screen for regulators of embryonic S-M cycles, we identified the *Drosophila* homolog of a human disease gene, *MCPH1* (microcephalin). Mutation of human *MCPH1* causes autosomal recessive primary microcephaly, a developmental disorder characterized by severe reduction of cerebral cortex size (Jackson et al., 2002). *Mcp1* is highly expressed in the developing forebrain of fetal mice, consistent with its proposed role in regulating the number neuronal precursor cell divisions and, ultimately, brain size (Jackson et al., 2002). Human MCPH1 protein is predicted to contain three BRCA1 C-terminal (BRCT) domains (reviewed by Glover et al., 2004; Huyton et al., 2000), which mediate phosphorylation-dependent protein-protein interactions in cell-cycle checkpoint and DNA repair functions.

Several studies have implicated human MCPH1 in the cellular response to DNA damage. The DNA checkpoint is engaged at critical cell-cycle transitions in response to DNA damage or incomplete replication and serves as a mechanism to preserve genomic integrity (reviewed by Nyberg et al., 2002). Triggering of this checkpoint causes cell-cycle delay, presumably to allow time for correction of DNA defects. When a cell senses DNA damage or incomplete replication, a kinase cascade is activated. Activated ATM and ATR kinases phosphorylate their targets, including the checkpoint kinase Chk1, which is activated to phosphorylate its targets. The first clue that MCPH1 plays a role in the DNA damage response came from siRNA-mediated knockdown studies in cultured mammalian cells demonstrating a requirement for MCPH1 in the intra-S phase and G2-M checkpoints in response to ionizing...
radiation (Lin et al., 2005; Xu et al., 2004). Two recent reports have further implicated MCPH1 in the DNA checkpoint, although puzzling discrepancies remain to be resolved (reviewed by Bartek, 2006). One report indicates that MCPH1 functions far downstream in the pathway, at a level between Chk1 and one of its targets, Cdc25 (Alderton et al., 2006). Another report (Rai et al., 2006) suggests that MCPH1 is a proximal component of the DNA damage response required for radiation-induced foci formation (i.e. recruitment of checkpoint and repair proteins to damaged chromatin).

Additional functions have been reported for MCPH1. MCPH1− lymphocytes of microcephalic patients exhibit premature chromosome condensation (PCC) characterized by an abnormally high percentage of cells in a prophase-like state, suggesting that MCPH1 regulates chromosome condensation and/or cell-cycle timing (Trimborn et al., 2004). A possible explanation for the PCC phenotype is that MCPH1-deficient cells have high Cdk1-cyclin B activity, which drives mitotic entry; decreased inhibitory phosphorylation of Cdk1 was found to be responsible for elevated Cdk1 activity in MCPH1-deficient cells (Alderton et al., 2006). It is not clear whether MCPH1’s role in regulating mitotic entry in unperturbed cells is related to its checkpoint function; intriguingly, Chk1 has similarly been reported to regulate timing of mitosis during normal division (Kramer et al., 2004). MCPH1 (also called Brit) was independently identified in a screen for negative regulators of telomerase, suggesting that it may function as a tumor suppressor (Lin and Elledge, 2003). Further evidence for such a role comes from a study showing that gene copy number and expression of MCPH1 is reduced in human breast cancer cell lines and epithelial tumors (Rai et al., 2006).

We report here the identification and phenotypic characterization of *Drosophila* mutants null for *mcph1*. We show that syncytial embryos from *mcph1* females exhibit genomic instability and undergo mitotic arrest due to activation of a DNA checkpoint kinase, Chk2. We find that, in contrast to reports of MCPH1 function in human cells, the ATR/Chk1-mediated DNA checkpoint is intact in *Drosophila mcph1* mutants. We propose that *Drosophila* MCPH1, like its human counterpart, is required for proper coordination of cell-cycle events; in early embryos lacking *mcph1*, chromosome condensation prior to completion of DNA replication causes genomic instability and Chk2-mediated mitotic arrest.

**Results**

**Screen for Drosophila cell-cycle mutants identifies absent without leave (awol)**

In an effort to identify genes required for S-M cycles of the early embryo, we previously screened (Lee et al., 2003) a maternal-effect lethal subset of a collection of ethylmethanesulphonate (EMS)-mutagenized lines from Charles Zuker’s lab (Koundakjian et al., 2004). We screened ~2400 lines by examining DAPI-stained embryos of homozygous females. Because early embryonic development is entirely regulated by maternally deposited mRNA and protein, only the maternal genotype is relevant in this screen. We identified 33 lines (12 chromosome II and 21 chromosome III mutants) representing 26 complementation groups in which the majority of embryos from mutant females arrest at the syncytial blastoderm stage. We previously identified two alleles of *giant nuclei*, which prevents excessive DNA replication in S-M cycles (Freeman et al., 1986; Renault et al., 2003), from this collection (Lee et al., 2003). We have now identified alleles of four well-known regulators of the cell cycle from the same screen (supplementary material Table S1). All four genes encode protein kinases with conserved roles in cell-cycle regulation, *wee1*, *grapes*, *telomere fusion* and *aurora* encode *Drosophila* orthologs of *Wee1* (a Cdk1 inhibitory kinase), DNA checkpoint kinases Chk1 and ATM (*ataxia telangiectasia* mutated), and the mitotic kinase Aurora A, respectively (Fogarty et al., 1997; Glover et al., 1995; Oikemus et al., 2004;
Price et al., 2000). Identification of these alleles of bona fide cell-cycle regulators validates our screen. We chose for further study the largest complementation group on chromosome II (comprising ZII-0978, ZII-1861 and ZII-4050) identified in our screen. Females homozygous or transheterozygous for any of these mutations are completely sterile, producing embryos that arrest in a metaphase-like state (~90% of embryos) in cycles 1-8 (the majority in cycles 6-8). Unevenly spaced, asynchronously dividing nuclei and centrosome duplication prior to chromosome segregation are often seen (Fig. 1B-D; Table 1); all of these are consistent with failure of nuclear divisions. Tubulin foci are frequently missing from one or both poles of mitotic spindles, which are typically shorter and more barrel-shaped than those of wild type (Fig. 1E; Table 1). Chromosomes are poorly aligned and occasionally displaced from the metaphase plate (Fig. 1F). Staining for Centrosomin, a core centrosomal component (Li and Kaufman, 1996), revealed that lack of tubulin foci at one or both poles in mutant-derived embryos is due to an absence of centrosomes (Fig. 1H,I; Table 1); we occasionally see ectopic centrosomes within spindle. Telophase spindles were not scored because centrosome duplication normally occurs at this phase in the early embryo.

awol encodes the Drosophila homolog of MCPH1

We localized awol to a region including five genes by a combination of mapping strategies (see Materials and Methods for details). A candidate in this region was the Drosophila homolog of the human disease gene, MCPH1 (Jackson et al., 2002). Sequencing of PCR-amplified mcph1 coding region from homozygous mutant genomic DNA revealed that awolZ0978 and awolZ4050 are distinct missense mutations in mcph1 causing non-conservative amino acid changes and awolZ1861 is a nonsense mutation resulting in severe truncation of the protein (Fig. 2A). Thus, all three EMS-induced awol alleles represent mutations affecting MCPH1 protein. Furthermore, females carrying any of these awol alleles in

Table 1. Mitotic spindle defects in mcph1 embryos and suppression by mnk

<table>
<thead>
<tr>
<th>Genotype</th>
<th>MI*</th>
<th>Centrosome number (% spindles)</th>
<th>Other spindle defects (% spindles)</th>
<th>Barrel</th>
<th>Interacting</th>
<th>Multipolar</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Decreased†</td>
<td>Increased§</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>54.1</td>
<td>0.2</td>
<td>0.0</td>
<td>0.1</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>mcph1Z1861</td>
<td>88.8</td>
<td>43.6</td>
<td>46.0</td>
<td>97.5</td>
<td>0.0</td>
<td>0.2</td>
</tr>
<tr>
<td>mnk</td>
<td>54.2</td>
<td>0.2</td>
<td>0.1</td>
<td>0.0</td>
<td>0.2</td>
<td>0.0</td>
</tr>
<tr>
<td>mnk mcph1Z1861</td>
<td>57.5</td>
<td>0.2</td>
<td>1.2</td>
<td>0.0</td>
<td>15.0</td>
<td>6.0</td>
</tr>
</tbody>
</table>

*Mitotic index=% embryos in mitosis/total number of embryos (>100 embryos scored per genotype). The presence of both condensed chromosomes and a mitotic spindle was used as the criterion for scoring mitotic embryos.
†Spindles with centrosomal detachment at one or both poles.
§Spindles with >1 centrosome per pole (one or both poles) or ectopic centrosomes within spindle. Telophase spindles were not scored because centrosome duplication normally occurs at this phase in the early embryo.

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Fig. 2. mcph1 is the awol gene. (A) The Drosophila mcph1 gene structure. Exons are represented by filled boxes, 5′- and 3′-UTRs by open boxes, and splicing events by thin lines. The gene CG13189 lies within the largest intron of mcph1. Alternative splicing produces transcript mcph1-RA or -RB. Arrows below gene or transcript names indicate direction of transcription. Positions of the point mutations in each of the three EMS-induced alleles of awol and resulting amino acid changes (numbers refer to MCPH1-B) are indicated above the mcph1 gene. Imprecise excision of P-element EY11307 (inverted triangle) generated allele mcph1Exc21 (deleted region indicated by gap). (B) Western analysis reveals trace amounts of or no MCPH1 protein in extracts of awol embryos relative to wild type (loading control: anti-α-tubulin). The excision allele (Exc21) of mcph1 serves as negative control. Df(2R)BSC439, which removes the mcph1 genomic locus.

(C) Comparison of the BRCT domain content (hatched boxes) of the two Drosophila MCPH1 isoforms (MCPH1-A and -B) and human MCPH1 protein (bottom). Positions of the amino acid changes in each of the three EMS-induced alleles of awol are indicated by asterisks. A double-sided arrow indicates the region of MCPH1-B used for antibody production.
trans to a deletion of the mcph1 genomic locus produce embryos with phenotypes indistinguishable from that of homozygous mutant females (data not shown), suggesting that all three Zuker awol alleles behave genetically as nulls.

To confirm that mutation of mcph1 is responsible for the awol phenotype, we generated a null allele (mcph1Exc21) by imprecise P-element excision (Fig. 2A). mcph1Exc21 homozygous females produce embryos with the awol phenotype; similar results were obtained for females carrying this excision in trans to any of the EMS-induced awol alleles or a deletion of the mcph1 genomic locus (data not shown), further confirming that mutation of mcph1 causes the awol phenotype. Importantly, expression of transgenic mcph1 using the UAS-Gal4 system (Brand and Perrimon, 1993; Rorth, 1998) restored fertility to awol20978/awol24050 females, resulting in a hatch rate of ~40% of their embryos (supplementary material Table S2). Thus, mcph1 is the awol gene. We used the MCPH1 isoform that is most abundant in the early embryo for transgenic rescue; it is possible that full rescue of the maternal-effect lethality of awol mutants might additionally require expression of the less abundant isoform (see below for description of MCPH1 isoforms; Fig. 2A and supplementary material Fig. S1B).

To further characterize our mcph1 alleles, we generated polyclonal antibodies against an MBP-MCPH1 fusion. Anti-MCPH1 antibodies recognize a major band of ~90 kDa, consistent with the predicted size of MCPH1-B, when used to probe immunoblots of wild-type embryo extracts (Fig. 2B). In contrast, for all mcph1 alleles identified here, we detect greatly reduced or no MCPH1 protein in mutant-derived embryos. Thus, all of these alleles are null (or nearly null) for MCPH1 protein.

MCPH1 isoforms differ in expression pattern and BRCT domain content

Our genetic data revealed that mcph1 null alleles are homozygous viable and that mcph1 is required maternally for early embryonic development. To measure MCPH1 levels throughout Drosophila development, we probed immunoblots of extracts from various developmental stages with anti-MCPH1 antibodies (supplementary material Fig. S1A). As expected, MCPH1 is abundant in ovaries and early embryos, whereas older embryos under zygotic control have relatively low amounts. MCPH1 is present in larval brains and imaginal discs but undetectable in adult brain extracts. Although high levels of MCPH1 are present in adult testes, it is not required for male fertility (data not shown).

Two major isoforms of MCPH1 were detected by immunoblotting: ~90 kDa (predominant in ovaries and embryos) and ~110 kDa (predominant in testes). Both isoforms were detected in larval tissues. The most recent mcph1 gene model annotated by FlyBase predicts two splice variants (A and B) differing at their 5′-ends that encode proteins with distinct amino termini (Grumbling and Strelets, 2006). We compared sizes of recombinant MCPH1-A and -B proteins (produced by in vitro transcription-translation reactions) to that of endogenous MCPH1 isoforms by immunoblotting. We found that the gel mobilities of MCPH1-A and -B closely match that of MCPH1 in testes and ovaries, respectively; thus, MCPH1-A is the ~110 kDa isoform that is abundant in testes, and MCPH1-B is the ~90 kDa isoform that is abundant in ovaries and early embryos (supplementary material Fig. S1B).

We observed a discrepancy between relative sizes of MCPH1-A and -B on our immunoblots (A larger than B; supplementary material Fig. S1B) and as predicted by FlyBase [779 versus 826 amino acids, respectively (Grumbling and Strelets, 2006)]. We were unable to find 3′-end sequence data for mcph1-A on public databases, so we fully sequenced a representative clone (LP15451) and found it to encode a protein of 981 amino acids, which closely matches our estimated size of 110 kDa for endogenous MCPH1-A. Furthermore, our sequencing revealed that mcph1-A contains coding sequence from both mcph1 and CG30038, a gene predicted to overlap the 3′-end of mcph1 (Fig. 2A). Thus, mcph1-A and -B are alternatively spliced at both ends, producing proteins that differ in their N- and C-terminal regions (Fig. 2C), and predicted gene CG30038 comprises alternatively spliced exons of mcph1-A.

MCPH1-A and -B proteins both contain BRCT domains (three or one, respectively). The arrangement of BRCT domains within MCPH1-A (one N-terminal and two C-terminal) resembles that of human MCPH1 (Fig. 2C). Drosophila and human MCPH1 have highest sequence identity in their BRCT domains (37.6%, 52.5% and 26.8% between the N-terminal, first C-terminal, and second C-terminal domains, respectively). The presence of extended amino termini in both Drosophila isoforms relative to human MCPH1 raises the possibility that the reported human sequence (Jackson et al., 2002) may not be full-length.

MCPH1 is a nuclear protein

Because Drosophila MCPH1 contains BRCT domains, we hypothesized that it has a nuclear function. In syncytial embryos, MCPH1 signal localizes to interphase nuclei and disappears in mitosis (supplementary material Fig. S2). As control for antibody specificity, no MCPH1 signal was detected in interphase nuclei of embryos derived from mcph1 null females. Because MCPH1 protein is readily detectable throughout the cell cycle (by immunoblotting of extracts from staged embryos; data not shown), the disappearance of MCPH1 signal in mitosis, as observed by immunostaining, is probably due to its dispersal into the cytoplasm upon nuclear envelope breakdown. Human MCPH1 has been reported to localize to the nucleus (Lin et al., 2005) as well as to centrosomes (Jeffers et al., 2007; Zhong et al., 2006); we observe no centrosomal localization for MCPH1 in syncytial embryos of Drosophila.

Mitotic arrest in mcph1 syncytial embryos is a consequence of Chk2 activation

The defective mitotic spindles of embryos derived from mcph1 females (hereafter referred to as ‘mcph1’ embryos’) exhibit key features reminiscent of Chk2-mediated centrosomal inactivation. In particular, these spindles are short, barrel-shaped, anastral, and associated with poorly aligned chromosomes (Fig. 1). Late syncytial embryos of Drosophila use a two-stage response to DNA damage or replication defects (Sibon et al., 2000). The DNA checkpoint mediated by Meiotic 41 (MEI-41) and Grapes (GRP), the Drosophila orthologs of ATR (ATM-Rad3-related) and Chk1 kinases, respectively, delays mitotic entry via inhibitory phosphorylation of Cdk1 to
allow repair of DNA damage or completion of replication (Sibon et al., 1999; Sibon et al., 1997). When this checkpoint fails, a secondary damage-control system operating in mitosis is activated; resulting changes in spindle structure block chromosome segregation, presumably to stop propagation of defective DNA (Sibon et al., 2000; Takada et al., 2003). This damage-control system, known as centrosomal inactivation, is mediated by the checkpoint kinase Chk2 (Takada et al., 2003).

Loss of γ-tubulin from centrosomes of mitotic spindles is another characteristic feature of Chk2-mediated centrosomal inactivation. We detected decreased γ-tubulin staining of centrosomes during mitosis in mcph1 embryos compared to wild type (supplementary material Fig. S3). We typically observe complete detachment of centrosomes from spindles in mcph1 embryos. High levels of DNA damage induced by intense laser illumination can similarly cause complete centrosomal detachment from spindle poles of wild-type embryos (Takada et al., 2003), suggesting that the spindle changes we observe in mcph1 embryos represent an extreme form of centrosomal inactivation.

To determine whether mitotic defects in mcph1 embryos are due to Chk2-mediated centrosomal inactivation, we created lines doubly mutant for mcph1 and maternal nuclear kinase (mnk), also known as loki, which encodes Drosophila Chk2 (Abdu et al., 2002; Brodsky et al., 2004; Masrouha et al., 2003; Xu et al., 2001). A similar approach has been used to demonstrate Chk2-mediated centrosomal inactivation in grp, mei-41 and wee1 embryos (Stumpff et al., 2004; Takada et al., 2003). Null mnk mutants are viable and fertile, but they are highly sensitive to ionizing radiation (Xu et al., 2001). Remarkably, we found that mnk suppresses many of the mitotic defects of mcph1 embryos (Fig. 3A-D; Table 1). Mitotic spindles are restored to near-normality: in contrast to the short, barrel-shaped, anastral spindles of mcph1 embryos, mnk mcph1 embryos have elongated spindles with attached centrosomes. Thus, Chk2 activation contributes significantly to the mcph1 phenotype in syncytial embryos.

In addition to suppressing the mitotic spindle defects of mcph1 embryos, mnk strikingly suppresses their developmental arrest (Fig. 3G-K). Whereas mcph1 embryos uniformly (100%) arrest in early to mid-syncytial cycles (cycles 1-8), most (>95%) mnk mcph1 embryos complete syncytial divisions, cellularize, and cease developing near gastrulation. Thus, Chk2 activation causes mcph1 embryos to arrest at the syncytial stage. Cellularized mnk mcph1 embryos show irregularities in cell size and shape and intensity of DNA staining; gastrulation is grossly aberrant. We conclude that mutation of mnk removes the ‘brakes’ from mcph1 embryos, allowing further nuclear divisions and development in the face of DNA defects, which eventually become so severe that embryos die peri-gastrulation.

mcph1 syncytial embryos exhibit a high frequency of chromatin bridging

We sought to understand the primary defects leading to Chk2 activation in mcph1 embryos. Known triggers of Chk2-mediated centrosomal inactivation are mitotic entry with incompletely replicated or damaged DNA (Sibon et al., 2000; Takada et al., 2003). Although mnk suppresses many of the cell-cycle defects of mcph1 embryos, we occasionally observe abnormal DNA aggregates shared by more than one spindle and multipolar spindles in mnk mcph1 embryos that progress beyond the usual mcph1 arrest point (Fig. 3E; Table 1). These defects are not observed in mnk embryos, suggesting that they are due to a lack of mcph1. In whole mounts of both mnk mcph1 and mcph1 embryos, we frequently observe chromatid bridging, which represents a physical linkage of chromosomes that prevents their segregation to opposite poles at anaphase (Fig. 3F; data not shown); this bridging could result from mitotic entry with unreplicated, damaged, and/or improperly condensed chromosomes. We were prohibited from quantifying this phenotype, however, as yolk proteins obscure nuclei that lie deep within the interior of early syncytial embryos. We circumvented this problem by adapting a larval
Chromatin bridging probably represents a primary defect of \textit{mcph1} because it occurs at a similar frequency (81\%) in \textit{mcph1} and wild-type embryos. This assumption is strengthened by a recent study showing that \textit{mcph1} embryos undergo Chk2-mediated arrest, we sought to determine if MCPH1 is required for the DNA checkpoint in \textit{Drosophila}.

Because MEI-41 and GRP are required during larval stages for the DNA checkpoint (Brodsky et al., 2000; Jaklevic and Su, 2004), we tested whether MCPH1 is required. In response to ionizing radiation (IR), eye-antennal imaginal disc cells of wild-type larvae undergo G2 arrest. We found that \textit{mcph1} larvae also exhibit IR-induced G2 arrest under conditions in which \textit{mei-41} larvae fail to arrest (Fig. 5A). We next tested the intra-S phase response to IR in larval brain cells. \textit{mcph1} brains exhibited IR-induced intra-S phase arrest similar to that of wild type, whereas no arrest was seen in \textit{mei-41} brains (Fig. 5B). We also tested sensitivity of \textit{mcph1} larvae to hydroxyurea (HU), which blocks DNA replication. Under conditions in which no \textit{mei-41} larvae survived, \textit{mcph1} larvae were HU resistant, surviving at near-Mendelian ratios (Fig. 5C). We conclude that MCPH1 is not required for the DNA checkpoint in larval tissues. We also found that \textit{mcph1} larvae, in contrast to \textit{mei-41}, survive normally following low-dose IR exposure (Fig. 5D), indicating that MCPH1 is not required for DNA repair (Jaklevic and Su, 2004).

The MEI-41/GRP-mediated DNA-replication checkpoint is also developmentally activated at the midblastula transition (MBT) (Sibon et al., 1999; Sibon et al., 1997). Rapid S-M cycles of the early embryo are under maternal genetic control, and the switch to zygotic control occurs at the MBT after cycle 13. During late syncytial cycles (11-13), titration of a maternal DNA-replication factor is thought to induce a \textit{mei-41/grp} checkpoint that causes Cdk1 inhibitory phosphorylation. Mitotic entry is thereby slowed, presumably to allow time to complete replication. Embryos from \textit{mei-41} or \textit{grp} females fail to lengthen interphase in late syncytial cycles and undergo extra S-M cycles (Sibon et al., 1999; Sibon et al., 1997).

We asked if MCPH1 is required for the MEI-41/GRP-dependent DNA-replication checkpoint at the MBT. \textit{mcph1} embryos undergo arrest due to Chk2 activation prior to their reaching cortical divisions (cycles 10-13). Thus, to test whether \textit{mcph1} is required for cell-cycle delay at the MBT, we performed live analysis of cortical divisions in \textit{mnk mcph1} embryos that lack a functional Chk2-mediated checkpoint. We reasoned that any primary defects in cell-cycle timing due to mutation of \textit{mcph1} would still be apparent in \textit{mnk mcph1} embryos. This assumption is strengthened by a recent study showing that \textit{mnk grp} embryos that progress through the MBT due to lack of Chk2-mediated arrest retain the cell-cycle timing defects of \textit{grp} embryos (Takada et al., 2007). We monitored timing of nuclear envelope breakdown and reformation by differential interference contrast microscopy (DIC) and found no significant differences in interphase or mitosis lengths in \textit{mnk mcph1} and wild-type embryos (Fig. 6A).

To further confirm that the DNA-replication checkpoint is

\begin{table}
\centering
\begin{tabular}{|c|c|c|}
\hline
Genotype & Chromatin bridging & Embryos & Late A/T figures (n) \\
\hline
wild type & 2.9 & 12 & 549 \\
\textit{mcph1} & 68.3 & 14 & 60 \\
\textit{mnk} & 10.6 & 14 & 113 \\
\textit{mnk} \textit{mcph1} \textit{Z1861} & 80.8 & 10 & 468 \\
\hline
\end{tabular}
\caption{\textit{mcph1} is required for the DNA checkpoint in \textit{Drosophila}.}
\end{table}

\textit{mcph1} is not required for the DNA checkpoint in \textit{Drosophila}.

Chk2-mediated centrosomal inactivation can be triggered in \textit{Drosophila} syncytial embryos by DNA damaging agents, the DNA-replication inhibitor aphidicolin, or mutation of DNA checkpoint components (MEI-41 or GRP) or WEE1, a kinase that prohibits mitotic entry via inhibitory phosphorylation of Cdk1 (Sibon et al., 2000; Stumpf et al., 2004; Takada et al., 2003). Human MCPH1-deficient cells show defective G2-M and intra-S phase checkpoint responses following DNA damage (Alderton et al., 2006; Lin et al., 2005; Xu et al., 2004). In light of these studies linking human MCPH1 to the ATR/Chk1 pathway and our results that \textit{Drosophila mcph1} embryos undergo Chk2-mediated arrest, we sought to determine if MCPH1 is required for the DNA checkpoint in \textit{Drosophila}.
intact in \textit{mnk mcph1} embryos, we assessed the extent of inhibitory phosphorylation of Cdk1 and found it to be comparable to that of wild type (Fig. 6B). We also found wild-type levels of Cyclin B and Cyclin A in \textit{mnk mcph1} embryos (Fig. 6C; data not shown). Low levels of Chk1 protein have been reported in \textit{MCPH1} siRNA human cells (Lin et al., 2005; Xu et al., 2004), but we detected normal levels of Grapes (Chk1) in \textit{mcph1} and \textit{mnk mcph1} embryos (Fig. 6D). Our data and a previous report of defective Cyclin A proteolysis in pre-cortical \textit{grp} embryos (Su et al., 1999) have established a role for \textit{grp} in regulating the cell cycles of early syncytial embryos. We also found that \textit{mcph1} dominantly enhances a weak \textit{mei-41} phenotype to a degree similar to that of \textit{grp} (Fig. 7C). Intriguingly, by immunoblotting, we consistently observe an upward mobility shift in MCPH1 in \textit{grp} or \textit{mei-41} embryonic extracts (Fig. 7D). Taken together, these data suggest that MCPH1 cooperates with MEI-41 and GRP to regulate the cell cycles of the early embryo via a mechanism independent of Cdk1 phosphorylation.

**Fig. 5.** \textit{mcph1} larvae have intact DNA checkpoints and normal sensitivity to DNA-damaging agents. (A,B) Cell-cycle checkpoints in \textit{mcph1} larvae. Bars, 50 μm. (A) G2-M checkpoint. Eye-antennal imaginal disks were dissected from untreated (left) or irradiated (right) larvae, fixed, and stained with antibodies against phosphorylated Histone H3 (anti-PH3), a marker of mitotic cells. Lack of anti-PH3 staining post-IR indicates G2 arrest. Representative disks are shown (with at least twelve discs scored per genotype). (B) Intra-S phase checkpoint. Brains were dissected from untreated (left) or irradiated (right) larvae and labeled with BrdU. Decreased BrdU staining in brain lobes (arrows) post-IR indicates intra-S phase arrest. Representative brains are shown (with at least six brains scored per genotype). (C,D) Survival of \textit{mcph1} larvae following exposure to DNA-damaging agents. (C) Sensitivity to hydroxyurea (HU). Larvae were grown on food minus or plus HU and allowed to develop. For each genotype, the ratio of homozygous mutant to total progeny is expressed as a percentage with total number of adult flies scored shown in parentheses. (D) Sensitivity to IR. Third instar larvae were untreated or exposed to low-dose irradiation and allowed to develop. For each genotype, the ratio of eclosed adults to total pupae is expressed as a percentage with total pupae shown in parentheses.
mcph1 males exhibit defects in adult brain structure

On the basis of the reduced brain size of patients with mutation of mcph1, we tested whether mutation of Drosophila mcph1 affects brain development. We did not observe an obvious change in overall brain size, but we did observe morphological defects in central brain structures. The mushroom bodies (MBs) of the Drosophila adult brain are bilaterally symmetrical structures required for olfactory memory and other complex adaptive behaviors (de Belle and Heisenberg, 1994). MB structure is stereotyped, and gross morphological brain defects often uncover structural defects in MBs. The 2500 intrinsic neurons in each MB can be subdivided into at least three morphologically well-defined subsets (αβ, α′β′ or γ) based on bundling of their axonal projections in the region of the MBs called the lobes (Crittenden et al., 1998). Each MB neuron contributing to the αβ subdivision bifurcates and sends one axon branch vertically to the α lobe and one horizontally to the β lobe. Anti-Fasciclin II (FasII) antibodies strongly

![Fig. 6. Intact DNA-replication checkpoint and normal Cyclin B levels in mcph1 embryos.](image)

**Fig. 6.** mcph1 males exhibit defects in adult brain structure.

(A) Quantification of cell-cycle timing during cortical divisions of early embryogenesis. No significant differences in interphase (I) or mitosis (M) lengths were observed for mnk mcph1Z1861 embryos compared to wild-type or mnk controls, whereas shorter interphases were apparent in mei-41 embryos (cycles 12 and 13). Average times with standard deviations (error bars) are shown. Numbers of embryos scored for each genotype are shown in parentheses. (B) Western analysis using phospho-specific antibodies against Cdk1 reveals wild-type levels of pY15-Cdk1 in extracts of mnk mcph1Z1861 embryos (1-2 hours). Control grp embryos have reduced pY15-Cdk1 levels. (C) Western analysis reveals normal Cyclin B levels in mnk mcph1Z1861 embryos (1-2 hours). (D) Western analysis reveals normal GRP levels in mcph1 and mnk mcph1Z1861 embryos (1-2 hours unless otherwise indicated). Loading controls: anti-α-tubulin or anti-GAPDH.

![Fig. 7. mcph1 cooperates with mei-41 and grp in the early embryo.](image)

**Fig. 7.** mcph1 cooperates with mei-41 and grp in the early embryo.

(A) Mitotic spindle from a pre-cortically arrested grapesZ5170 embryo resembles awol-type spindles of mcph1 embryos. Microtubules are in green and DNA in red. Scale bar: 10 μm. (B) Quantification of mcph1-like arrest in grp embryos (2-4 hours). (C) mcph1 dominantly enhances mei-41 embryonic lethality. Introduction of one copy of mcph1Z1861 into a semi-sterile mei-41 background (mei-41RT1/mei-41D5) reduces embryonic hatch rate more than threefold. (D) Immunoblotting shows slower gel mobility of MCPH1 in mei-41RT1 or grp+ embryos (1-2 hours) relative to wild type.
our initial analysis revealed obvious morphological MB defects in brains of mcph1<sup>l<sup>21861</sup></sup> and mcph1<sup>l<sup>Exc21</sup></sup> male flies (Fig. 8A). The nature of the MB defects was variable, ranging from missing or malformed lobes to complete absence of lobes, and defects were often asymmetric. For unknown reasons, we never observed MB defects in brains of female mcph1 flies (data not shown). Quantification revealed defects in 22% of mcph1<sup>l<sup>21861</sup></sup> and 13% of mcph1<sup>l<sup>Exc21</sup></sup> male brains (Fig. 8B). We similarly found defects in 11.5% of brains from males carrying mcph1<sup>l<sup>21861</sup></sup> intrans to a deletion of the mcph1 genomic locus; no defects were found in control heterozygous (mcph1<sup>l<sup>21861</sup>/</sup>+<sup>)</sup>) male brains. These data establish a role for mcph1 in Drosophila brain development.

**Discussion**

We identified *Drosophila mcph1*, the homolog of the human primary microcephaly gene *MCPH1*, in a genetic screen for cell-cycle regulators and have shown that it is required for genomic stability in the early embryo. Three additional primary microcephaly (MCPH) genes have been identified in humans: *ASPM, CDK5RAP2*, and *CENPJ* (reviewed by Cox et al., 2006). Much of our understanding of the biological functions of the proteins encoded by human MCPH genes has come from studies of their *Drosophila* counterparts. Mutation of abnormal spindle (asp), the *Drosophila* ortholog of *ASPM*, results in cytokinesis defects and spindles with poorly focused poles (do Carmo Avides and Glover, 1999; Wakefield et al., 2001). The *Drosophila* ortholog of *CDK5RAP2*, centrosomin (*cnn*), is required for proper localization of other centrosomal components (Li and Kaufman, 1996; Megraw et al., 1999). *Sas-4*, the *Drosophila* ortholog of *CENPJ*, is essential for centriole production, and the mitotic spindle is often misaligned in asymmetrically dividing neuroblasts of *Sas-4* larvae (Basto et al., 2006). Whereas all of these primary microcephaly genes are critical regulators of spindle and centrosome functions, mitotic defects in *Drosophila mcph1* mutants are largely secondary to Chk2 activation in response to DNA defects; thus, *mcph1* probably represents a distinct class of primary microcephaly genes.

*MCPH1* is a BRCT domain-containing protein, suggesting that it plays a role in the DNA damage response. Conflicting models of MCPH1 function, however, have emerged from studies of human cells as it has been proposed to function at various levels in this pathway: upstream, at the level of damage-induced foci formation (Rai et al., 2006) and further downstream, to augment phosphorylation of targets by the effector Chk1 (Alderton et al., 2006). The phenotype of embryos from null *mcph1* females is more severe than that of embryos from null *grp* females, suggesting that enhancement of phosphorylation of GRP (Chk1) substrates is not the sole function of MCPH1. Furthermore, we found both the DNA checkpoint in larval stages and its developmentally regulated use at the MBT to be intact in *mcph1* mutants, suggesting a requisite role for MCPH1 in the DNA checkpoint evolved in higher organisms.

Studies of human cells suggest a role for MCPH1 in regulation of chromosome condensation. Microcephalic patients homozygous for a severely truncating mutation in MCPH1 show increased frequency of G2-like cells displaying premature chromosome condensation (PCC) with an intact nuclear envelope (Alderton et al., 2006; Trimborn et al., 2004). Depletion of Condensin II subunits by RNAi in *MCPH1*-deficient cells leads to reduction in the frequency of PCC, suggesting that MCPH1 is a negative regulator of chromosome condensation (Trimborn et al., 2006). Alderton et al. (Alderton et al., 2006) observed a decreased level of inhibitory phosphates on CdK1 that correlated with PCC in *MCPH1*-deficient cells. The authors proposed that MCPH1 maintains CdK1 phosphorylation in an ATR-independent manner because PCC is not seen in cells of patients with Seckel syndrome, which is caused by mutation of *ATR*; residual ATR present in these cells, however, may be sufficient to prevent PCC (O’Driscoll et al., 2003). Furthermore, in several experimental systems, ATR and Chk1 have been implicated in an S-M checkpoint that prevents premature mitotic entry with
unreplicated DNA (reviewed by Petermann and Caldecott, 2006).

We have shown that embryos from grp (Chk1) females occasionally undergo mcph1-like arrest in early syncytial cycles, prior to the time at which inhibitory phosphorylation of Cdk1 is thought to control mitotic entry. Thus, decreased signaling through the DNA checkpoint resulting in less Cdk1 phosphorylation is unlikely to explain this mcph1-like arrest. In contrast to studies of MCPH1-deficient human cells, we detect no decrease in pY15-Cdk1 levels in mcph1 embryos allowed to progress beyond their normal arrest point by mutation of mnk (Chk2). Based on these data and the PCC phenotype associated with loss of MCPH1 in humans, we propose a model in which MEI-41/GRP cooperate with MCPH1 in syncytial embryos in a Cdk1-independent manner to delay chromosome condensation until DNA replication is complete (Fig. 9). In the absence of mcph1, we hypothesize that embryos condense chromosomes before finishing S phase, resulting in DNA defects (bridging chromatin), Chk2 activation, and mitotic arrest. We were precluded from directly monitoring chromosome condensation in mnk mcph1 embryos expressing Histone-GFP as previously described (e.g. Brodsky et al., 2000) because we were unable to establish fly stocks carrying this transgene in the mnk background. Live imaging of mcph1 embryos was not technically feasible because they arrest prior to cortical stages, and yolk proteins obscure more interior nuclei in early embryos. grp embryos have been reported to initiate chromosome condensation with normal kinetics (Yu et al., 2000), although a subtle PCC phenotype might be difficult to detect.

Support for our model that MCPH1 allows completion of S phase by delaying chromosome condensation comes from the observation that inhibition of DNA replication in syncytial embryos (via injection of aphidicolin or HU) results in phenotypes similar to those observed in mcph1 embryos, including chromatin bridging, which is presumably a direct consequence of progressing through mitosis with unreplicated chromosomes (Raff and Glover, 1988), and Chk2 activation (Takada et al., 2003). Alternatively, mcph1 might be required during S phase for timely completion of DNA synthesis; in this case, mcph1 embryos would initiate chromosome condensation with normal kinetics prior to completing replication. Coordination of S-phase completion and mitotic entry may be particularly critical in the rapid cell cycles of the early embryo that lack gap phases and may explain why loss of Drosophila mcph1 is most apparent at this developmental stage. Interestingly, even in the absence of exogenous genotoxic stress, MCPH1-deficient human cells also exhibit a high frequency of chromosomal aberrations (Rai et al., 2006), which may be a consequence of PCC.

An evolutionary role for mcph1 in expansion of brain size along primate lineages has emerged in recent years (reviewed by Woods et al., 2005). In brains of Drosophila mcph1 males, we find low-penetrance defects in MB structure. Both MCPH1 isoforms are expressed in larval brains, and all mcph1 mutations described here affect both isoforms, so it is unclear whether MB formation requires one or both isoforms. The lack of MB defects in mcph1 females is puzzling because both isoforms are found in male and female larval brains (data not shown); other sex-specific factors are probably involved. Larval brains of mcph1 males show no obvious aneuploidy (data not shown) or spindle orientation defects (Andrew Jackson, personal communication), so the cellular basis for these defects remains to be determined. It will be interesting to test in future studies whether mei-41 and grp, which cooperate with mcph1 to regulate early embryogenesis, are similarly required in Drosophila males for brain development.

In conclusion, we have demonstrated an essential role for Drosophila MCPH1 in maintaining genomic integrity in the early embryo. Our data suggest that MCPH1 cooperates with MEI-41/GRP in a Cdk1-independent manner to promote genomic integrity in embryos, possibly by controlling timing of chromosome condensation.

**Fig. 9.** Proposed model of Drosophila MCPH1 function. Asterisks represent key points at which human MCPH1 reportedly functions. Our data suggest that MCPH1 cooperates with MEI-41/GRP in a Cdk1-independent manner to promote genomic integrity in embryos, possibly by controlling timing of chromosome condensation.

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**Materials and Methods**

**Drosophila stocks**

Flies were maintained at 25°C using standard techniques (Greenspan, 2004). Wild-type stocks used were y w or Oregon-R. Ziker alleles of mcph1 are cn bw and balanced over CyO. Ziker stock designations have been shortened and superscripted to indicate that they are alleles of mcph1 (e.g. ZII-1861 becomes mcph1<sup>ZII-1861</sup>). Deficiency strains, P-element lines for mapping, mutants for complementation
testing (grp, aurora2, wee1ES1, nanos-Gal4:VP16 stock, and mei-41 mutants were from Bloomington Stock Center. mcph1 P-element insertions were from Bloomington Stock Center (EY11307), Kyoto Stock Center (NP6229-5-1), or a gift from Steven Hou (l(2)SH0220). teja259, man5060 and gen620 stocks were gifts from Mike Brodsky, Bill Theurkauf and Tin Tin Su, respectively.

Identification of new alleles of cell-cycle regulators

A combination of female meiotic recombination, deficiency mapping and direct complementation testing of candidates was used to identify mutants from our screen. Complementation testing with known cell-cycle regulators was performed by assessing fertility of females carrying a Zucker chromosome in trans to a known mutation. We used the following alleles: wee1ES1 (Price et al., 2000), grp (Fogarty et al., 1997), teja259 (Okunemus et al., 2004) and awol (Glover et al., 1995).

Quantification of embryonic hatch rates

For hatch rate assays, embryos (0–4 hours) were collected on grape plates, counted and aged 40 hours at 25°C. The number of hatched embryos was determined by subtracting the number of unhatched ( intact) embryos from the total number collected. Hatch rate is the ratio of hatched to total embryos expressed as a percentage.

Genetic and molecular mapping of awol

The awol gene was localized by a combination of mapping strategies. We first screened a collection of deficiencies on the second chromosome for non-complementation of the female sterility of awol259. We found that females carrying awol259 in trans to Df(2R)BSC39 produced embryos with the awol phenotype; similar results were obtained for awol259 and awol256. Thus, awol lies between the breakpoints of Df(2R)BSC39 in the polytene interval 4SC–E1, a region that contains ~35 genes for eye color and body pattern, and X-linked genes for eye color and body pattern (Chen et al., 1998) relative to the following insertion lines: Mto19505, Erg600250, KO4952, ok22007 and CG8327P260. We thereby narrowed awol to a region of five genes (including mcph1) that lie distal to Erg600250 and proximal to KG04952. The awol stock used (cn ZII-1861 bw/CyO) has visible flanking markers en and bw. The source of transposase was Delta2-3 Sb. Multiple independent recombinant chromosomes were recovered for each P-element line tested. Genomic DNA was extracted from whole flies homozygous for awol mutations essentially as previously described (Ballinger and Benzer, 1989). mcph1 coding regions were PCR-amplified from genomic DNA and sequenced.

Generation of mcph1 excision line

P-element insertions have been identified in the 5′-UTR of mcph1 (CG8327P260). Generation of mcph1 excision line EY11307 - UT R of mcph1 (NP6229-5-1) and within its largest intron (l(2)SH0220 and EY11307) (Grumbling and Strelets, 2006). l(2)SH0220 (Ob et al., 2003) outside of the mcph1 genomic region (data not shown). We found that EY11307 homozygous and EY11307/mcph1Z1861 has no visible flanking markers en and bw. The source of transposase was Delta2-3 Sb. Multiple independent recombinant chromosomes were recovered for each P-element line tested. Genomic DNA was extracted from whole flies homozygous for awol mutations essentially as previously described (Ballinger and Benzer, 1989). mcph1 coding regions were PCR-amplified from genomic DNA and sequenced.

Embryo fixation, staining and microscopy

Embryos (1–2 hours unless otherwise indicated) were collected for staining using standard techniques (Rothwell and Sullivan, 2000). For mouse anti-α-tubulin (DM1α, 1:500, Sigma) or rabbit anti-Centromerin (1:10,000, a gift from W. Theurkauf) staining, embryos were dechorionated in 50% bleach, fixed, and dehydrated in a series of ethyl alcohol (50% and 70%) and 1:1 propylene glycol (50% and 70%). Embryos were then fixed for 20 minutes in a mixture of 3.7% formaldehyde in PBS and heptane (1:1). The aqueous layer containing formaldehyde was removed and embryos dehydrated as described above. Embryos were incubated in primary antibodies at 4°C overnight except for anti-MCPH1 (4°C for three days). Secondary antibodies were conjugated to Cy3 (Jackson Immunoresearch). Embryos were stained with propidium iodide (Sigma) and cleared as previously described (Fenger et al., 2000). A Nikon Eclipse 80i microscope equipped with a CoolSNAP ES camera (Photometrics) and Plan-Apo (20×, 100×) or Ultraview 40× objectives was used: for confocal images, we used a Zeiss LSM510 microscope equipped with a Plan-Neofluar 100× objective.

Embryo squashes and quantification of DNA bridging

Methanol-fixed embryos (40–80 minutes) were placed in 2-μl drops of 45% acetic acid on coverslips for 1–2 minutes. Slides were lowered onto coverslips, inverted and allowed to dry. Coverslips were reapplied and blotted dry. Slides were then immersed in ethanol at −20°C for 10 minutes and air-dried. Vectashield mounting medium with DAPI (Vector Labs) and new coverslips were added to slides. Fluorescence microscopy (100× objective) was used to visualize DNA. Late anaphase and telophase figures (cycle-5 to -7) were examined. The presence of one or more linkages between DNA mass segregating to opposite poles was scored as a bridging defect.

Live embryo imaging

For analysis of cell-cycle timing, embryos (0–1.5 hours) were dechorionated in 50% bleach, glued (octane extract of tape) to glass-bottomed culture dishes (MatsTek Co.) and covered with half-strength embryo medium. DIC images of dividing embryos at 21.5–22.5°C were captured (20-second intervals) using a Nikon Eclipse TE2000-E inverted microscope with a CoolSNAP HQ CCD camera (Photometrics), Plan-Apo 20× objective, and IPLab image acquisition software (BD Biosciences). Interphase length was determined by counting frame numbers from nuclear envelope formation to breakdown. Mitosis length was determined by counting frame numbers from nuclear envelope break down to reformation. Cycle number was determined by nuclear size and density.

mcph1 cDNA clones and transgen es

cDNA clones encoding MCPH1-B (LD43341) and MCPH1-A (LP15451) were from the Genome Science Center and covered with hair oil (Sigma). Dic images of dividing embryos at 21.5–22.5°C were captured (20-second intervals) using a Nikon Eclipse TE2000-E inverted microscope with a CoolSNAP HQ CCD camera (Photometrics), Plan-Apo 20× objective, and IPLab image acquisition software (BD Biosciences). Interphase length was determined by counting frame numbers from nuclear envelope formation to breakdown. Mitosis length was determined by counting frame numbers from nuclear envelope break down to reformation. Cycle number was determined by nuclear size and density.

Polyconal antibodies against MCPH1

Mabese-binding protein (MBP) fused to MCPH1-B protein (residues 1-352) was used to produce antibodies. Intron MCPH1-B sequence was PCRamplified from LD43341 and subcloned into pMal (New England Biolabs). MAB-N-MCPH1-B was made in bacterial cells, purified using amylose beads, and injected into guinea pigs for antibody production (Covance). Anti-MCPH1 antibodies were affinity purified using standard techniques.

Protein extracts and immunoblot s

Protein extracts were made by homogenizing either embryos (1–2 hours old unless otherwise indicated) or dissected tissues in urea sample buffer as described previously (Tang et al., 1998). Proteins were transferred to nitrocellulose for immunoblotting using standard techniques. MCPH1-A and -B (unlabeled proteins) were made by Genpeptide transcription-translation of LP15451 and LD3341, respectively. MCPH1-B coding region was PCR-amplified from LD3341, subcloned into pMAL (New England Biolabs), MAB-N-MCPH1-B was made in bacterial cells, purified using amylose beads, and injected into guinea pigs for antibody production (Covance). Anti-MCPH1 antibodies were affinity purified using standard techniques.

DNA damage response assays

We used a Mark I cesium-137 irradiator as a source of irradiation (IR). To test the G2-M checkpoint post-IR, we used the method of Brodsky et al. (Brodsky et al., 2000) except that fluorescently coupled secondary antibodies were used. To test the intra-S phase checkpoint post-IR, we used the method of Jaklevic and Su (Jaklevic and Su, 2004) except that larvae were exposed to 40 Gray (4000 Rad). To test sensitivity to irradiation, third instar larvae were untreated or exposed to 10 Gray (1000 Rad), transferred to food and raised to adulthood and eclosed as adults. Mutant chromosomes were balanced over cyt, arm-gfp (Sullivan et al., 2000) and homozygotes identified by lack of GFP signal. Numbers of pupae formed and empty pupal cases (due to eclosion) were scored up to 10 days post-IR. Percentage eclosion (measure of survival) is the number of empty pupal cases expressed as a percentage of total pupae. All irradiated larvae formed pupae in these experiments. To test hydrosophy (HU) sensitivity, heterozygous adults (ten males and ten virgin females) were added to vials. After embryo collection (48 hours), adults were removed and 500 ml of 20 μM HU in water was added to food 24 hours later. Adult progeny were scored after 2 weeks. HU sensitivity is indicated by preferential loss of a specific genotypic class.

Adult brain immunostaining

Adult brains were fixed, immunostained and examined by confocal microscopy as previously described (Krashes et al., 2007) using mouse anti-Fasciclin II antibodies (ID4, 1/4, Developmental Studies Hybridoma Bank).
The maternal-effect mutant collection was kindly provided by Charles Zuker. We thank Edmund Koundakjian, David Cowan and Robert Hardy for establishing the collection and the labs of Barbara Wakimoto, Dan Lindsay and Mike McKeown for identifying the female flies used in this subset. We gratefully acknowledge Terry Orr-Weaver and in whose lab the screen was performed (with support from NIH grant GM39341 and NSF grant MCB0132237 to T.O.-W) and her lab members for participation in the screen. We thank Irina Kaverina and Saeo Takada for expert advice on live analysis of embryos by DIC microscopy. Erin Loggins, Audrey Frist and Joshua Tarkoff provided technical assistance. Curtis Thorne helped map awol. Bill Theurkauf, Tin Tin Su and Mike Brodsky provided antibodies and fly stocks. We thank Bill Theurkauf and Tin Tin Su for helpful discussions and Andrew Jackson for sharing unpublished data. Daniela Drummond-Barbosa, Andrea Page-McCaw, Terry Orr-Weaver and members of the Lee lab provided critical comments on the manuscript. This work was supported by a Basil O’Connor Starter Scholar Research Award (Grant 5-FY05-29) and Grant 1-FY07-456 from the March of Dimes Foundation and NIH grant GM70444 to L.A.L.

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