Cyclin G is involved in meiotic recombination repair in *Drosophila melanogaster*

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Summary

Cyclin G (CycG) belongs to the atypical cyclins, which have diverse cellular functions. The two mammalian CycG genes, *CycG1* and *CycG2*, regulate the cell cycle in response to cell stress. Detailed analyses of the role of the single *Drosophila cycG* gene have been hampered by the lack of a mutant. We generated a null mutant in the *Drosophila cycG* gene that is female sterile and produces ventralised eggs. This phenotype is typical of the downregulation of epidermal growth factor receptor (EGFR) signalling during oogenesis. Ventralised eggs are also observed in mutants (for example, mutants of the spindle class) that are defective in meiotic DNA double-strand break repair. Double-strand breaks (DSBs) induce a meiotic checkpoint by activating Mei-41 kinase (the *Drosophila* ATR homologue), thereby indirectly causing dorsoventral patterning defects. We provide evidence for the role of CycG in meiotic checkpoint control. The increased incidence of DSBs in *cycG* mutant germaria may reflect inefficient DSB repair. Therefore, the downregulation of Mei-W68 (an endonuclease that induces meiotic DSBs), Mei-41, or *Drosophila melanogaster* Chk2 (a downstream kinase that initiates the meiotic checkpoint) rescues the *cycG* mutant eggshell phenotype. In *vivo*, CycG associates with Rad9 and BRCA2. These two proteins are components of the 9-1-1 complex, which is involved in sensing DSBs and in activating meiotic checkpoint control. Therefore, we propose that CycG has a role in an early step of meiotic recombination repair, thereby affecting EGFR-mediated patterning processes during oogenesis.

Key words: 9-1-1 complex, BRCA2, Cyclin G, Double-strand break repair, DSB, meiotic checkpoint

Introduction

Meiosis is characterised by the recombination of homologous chromosomes, thereby guaranteeing the regular segregation of homologous chromosomes and, incidentally, increasing the genetic diversity in the offspring. The process is initiated by the controlled induction of double-strand DNA breaks (DSBs), followed by homologous recombination repair. Early in meiosis DSBs are induced by the type II DNA topoisomerase Mei-W68, the homologue of Spol1 in budding yeast (McKim and Hayashi-Hagihara, 1998). DNA double-strand breaks induce a well-conserved repair machinery: they are sensed by the 9-1-1 complex which consists of Rad9, Rad1, Hus1 and BRCA2, and results in the recruitment of repair enzymes at these sites (Kloostad et al., 2008; Peretz et al., 2009; overview in Pardo et al., 2009). DNA repair genes fall phenotypically into the ‘spindle’ class of genes, including the Rad51 and Rad54 homologues, i.e. *spindle A* (*span-A*) and *okra* (*okr*). The respective proteins catalyse DNA-strand exchange as part of the recombination repair process (Ghabrial et al., 1998; Morris and Lehmann, 1999; Staeva-Vieira et al., 2003). Disruption of this well-conserved DSB repair machinery initiates a meiotic checkpoint, mediated by the activities of the Mei-41 and DmChk2 kinases, the *Drosophila* ATR and Chk2/Mnk homologues that act in DNA damage signalling (Abdu et al., 2002; overview in Shiloh, 2006).

The eponymous phenotype of the *spindle* class of genes is a ventralised eggshell, characterised by fused or absent dorsal appendages because of a patterning defect. Dorsoventral patterning of the egg is governed by the epidermal growth factor receptor (EGFR)-signalling pathway. The epidermal growth factor receptor is activated in the dorsal follicle cells by the ligand Gurken (Grk) which is localised to the anterodorsal side of the oocyte. If Grk is absent or mislocalised then the dorsalising signal is missing, and the egg cannot develop dorsal appendages (Schüpbach, 1987; overview in Morris and Lehmann, 1999). If the meiotic checkpoint is active, i.e. from persistent or increased numbers of DSBs, the germline helicase Vasa, which acts as a translation initiation factor of *grk* mRNA, is modified so that the efficient translation of *grk* mRNA is suppressed and the correct establishment of the dorsoventral axis is impaired (Abdu et al., 2002; Ghabrial and Schüpbach, 1999; overview in Morris and Lehmann, 1999). Thus the ventralised eggshell phenotype of the *spindle* class mutants is a side effect of meiotic checkpoint activation. Likewise, axis specification defects are observed in mutants of the *Drosophila* small repeat-associated siRNA (rasiRNA) and Piwi-interacting RNA (piRNAs) pathways, which mediate silencing of transposons and retrotransposons, thereby ensuring genome stability during germline development (Klattenhoff et al., 2007; Klattenhoff et al., 2009). Mutations in pathway members result in an increase of DSBs independent of Mei-W68; a meiotic checkpoint results presumably as a direct consequence (Klattenhoff et al., 2009).

We studied the role of the atypical *cycin G* (*cycG*) in *Drosophila* development. CycG had been identified as a cofactor of the chromatin factor Corto that is involved in the transcriptional regulation of the homeotic gene *Abdominal B* (Salvaing et al., 2008a; Salvaing et al., 2008b). More recent data,
relying mainly on gain of function and RNAi based studies, point to a role of CycG in the regulation of cell proliferation and growth (Faradji et al., 2011). In order to validate and extend our current knowledge of the functions of CycG, we generated a null mutant in the Drosophila cycG gene by homologous recombination. Unexpectedly, this cycG mutant is viable; however, the females are sterile. Eggs from cycG mutant mothers (herein called cycG mutant eggs) show a typical ventralised phenotype, often with fused dorsal appendages. The increased incidence of DSBs, karyosome defects and altered Vasa mobility in cycG mutant germlia suggest a role of CycG in meiotic checkpoint regulation. In accordance, the cycG eggshell defects were rescued by the downregulation of Mei-W68, Mei-41 and DmChk2 activity. Finally we find that CycG protein defects were rescued by the downregulation of Mei-W68, Mei-41 and DmChk2 activity. Finally we find that CycG protein associates with BRCA2 and Rad9, both members of the 9-1-1 complex in Drosophila. Our data suggest an early role of CycG in mediating of meiotic recombination repair, thereby ensuring proper development of the oocyte.

**Results**

**Generation and verification of a cycG null mutant**

In order to generate a cycG mutant, we deleted the cycG locus precisely by homologous recombination using the ends-in technique (Rong et al., 2002). Flanking the I-SceI restriction site, two point mutations were introduced that caused frame shift mutations in the two resultant cycG gene copies (Fig. 1A). This strategy led to the generation of the cycGHR7 mutant allele, which was molecularly verified (supplementary material Fig. S1). Southern blot analysis confirmed the predicted genotype; however, an additional 350 bp deletion was discovered in the distal of the two gene copies (Fig. 1A; supplementary material Fig. S1). CycG is ubiquitously expressed during embryonic and larval development (Salvaging et al., 2008a). However, during oogenesis cycG mRNA was strongly enriched in the presumptive oocyte already from vitellarial stage 1 on, and was expressed at low levels in region 2 pro-oocytes (Fig. 1B,B’). No cycG mRNA was seen in cycGHR7 mutant oocytes, indicating that it is a null mutant (Fig. 1B,C). Moreover, no CycG protein was detected by western blot analysis in extracts derived from cycGHR7 mutant animals (Fig. 1D). Unfortunately our polyclonal anti-CycG antibodies did not allow for detection of CycG protein in ovaries by immunohistochemistry because of high background labelling.

In order to assure the integrity of the neighbouring medea gene, we tested the level of medea mRNA in the cycGHR7 mutant and found no obvious differences compared to a wild-type control (Fig. 1E).

**Females lacking cycG are sterile and lay ventralised eggs**

Unexpectedly, the homozygous cycGHR7 mutant is viable, but females are sterile and produce ventralised eggs that are characterised by fused respiratory appendages (compare Fig. 2A,A’ and Fig. 2B-C’). In order to verify that the mutant phenotype results from a loss of CycG and not from some secondary mutations introduced during the homologous recombination, we induced CycG overexpression throughout development in a homozygous cycGHR7 mutant background. We found that the eggshell defects were nearly completely rescued in these combinations (Fig. 2D), showing that the ventralised phenotype does result from a loss of the cycG gene.

The ventralised eggshell phenotype is reminiscent of the weak phenotype of loss-of-function mutations in EGFR signalling (Schüpbach, 1987). The epidermal growth factor receptor is activated in the somatic follicle cells by a localised Gurken (Grk) signal derived from the oocyte. The apparent downregulation of EGFR-signalling activity observed in the cycGHR7 mutant (Nagel...
et al., 2012) could result from a defect at any level of the pathway, either on the signalling or on the receiving side. Thus we asked whether CycG functions in the follicle cells or in the oocyte by generating homozygous cycGHR7 mutant germline clones using the female-sterile ovoD1 technique (Chou and Perrimon, 1992). Eggs from the cycGHR7 germline clones developed ventralised eggshells similar to those observed from cycGHR7 homozygous eggs (Fig. 2C,C'), showing that CycG activity is required in the germline. This is fully consistent with cycG mRNA accumulation in oocytes (Fig. 1B,B') but not in follicle cells.

Since CycG has been implicated in the transcriptional regulation of Abdominal B (Salvaing et al., 2008a), we wondered, whether it might affect transcription of grk mRNA as well. We did not observe conspicuous changes in the accumulation of grk mRNA in cycG mutant egg chambers, although the amount of Grk protein is reduced (Fig. 3).

**Cyclin G acts upstream of Mei-41 in meiotic checkpoint control**

Females defective in genes required for meiotic double-strand break (DSB) repair lay eggs with dorsoventral patterning defects, similar to grk and EGFR-signalling mutants (Ghabrial et al., 1998; González-Reyes et al., 1997; Staeva-Vieira et al., 2003). Obstruction of repair induces a meiotic checkpoint via the activation of the Mei-41 kinase which acts through the downstream DmChk2 kinase, thereby affecting the translation of grk mRNA (Abdu et al., 2002; Ghabrial and Schüpbach, 1999; overview in Shiloh, 2006). DNA double-strand break formation is catalysed during early meiosis by the Mei-W68 protein (McKim and Hayashi-Hagihara, 1998). If the ventralised eggshell phenotype in the cycGHR7 mutant is really provoked by the induction of a meiotic DSB checkpoint, this phenotype should no longer be detectable in the absence of DSB formation. We therefore generated double mutants for mei-W68 and cycG and analysed the eggshell phenotype of the progeny of these mutant females. As expected, eliminating meiotic DSBs resulted in a nearly complete suppression of the dorsal appendage defects in about 94% of cycG mutant eggs (Fig. 4). Moreover, if the egg phenotype associated with the cycGHR7 mutation is a result of meiotic checkpoint induction, lack of the executing Mei-41 kinase should rescue the cycGHR7 mutant phenotype, which was indeed the case (Fig. 4). A similar effect was observed by reducing the copy number of DmChk2, the downstream kinase of Mei-41 (Fig. 4). Moreover, feeding caffeine to cycGHR7 mutant females resulted in a higher frequency of normal eggs (Fig. 4), corroborating the above results since caffeine inhibits the activity of DmChk2 (Sarkaria et al., 1999). From these results we conclude that CycG is involved in meiotic checkpoint regulation upstream of the Mei-41 kinase.

**The cyclin G mutant is defective in meiotic DSB repair and induces the meiotic recombination checkpoint**

To assess the degree of DSB repair in cycGHR7 mutant germaria, we made use of an antibody that detects a phosphorylated form of DmChk2 kinase should rescue the cycGHR7 mutant phenotype, which was indeed the case (Fig. 4). A similar effect was observed by reducing the copy number of DmChk2, the downstream kinase of Mei-41 (Fig. 4). Moreover, feeding caffeine to cycGHR7 mutant females resulted in a higher frequency of normal eggs (Fig. 4), corroborating the above results since caffeine inhibits the activity of DmChk2 (Sarkaria et al., 1999). From these results we conclude that CycG is involved in meiotic checkpoint regulation upstream of the Mei-41 kinase.

**Fig. 2. Cyclin G affects dorsoventrally patterning of the oocyte.** (A–C') Dark field micrographs of deposited eggs shown in the dorsal view (A–C) and lateral view (A'–C'). The anterior is to the left (A,A'). Wild type eggs (the control) show an egg chamber with normal dorsal appendage morphology. (B–C') Homozygous cycGHR7 mutant eggs (B,B') and eggs derived from cycGHR7 mutant maternal germinal clones (C,C') are characterised by fused dorsal appendages with variable penetrance, typical of ventralised eggshells. Scale bars: 100 μm. (D) Phenotypic classification of eggs laid by cycGHR7 homozygous females (left), and in the background of ubiquitous CycG overexpression (the genotype is da-Gal4 cycGHR7/UASp cycG cycGHR7) (right). The frequency of eggshell patterning defects is shown by percentage with the wild type shown in black; intermediate, in grey; and completely fused appendages, in white. n, the number of analysed eggs.

**Fig. 3. Grk protein levels are reduced in cycG mutant ovaries.** (A–B') Grk mRNA distribution is shown in wild-type ovaries (A,B) and cycGHR7 homozygous mutant ovaries (A',B'). During early oocyte development, grk expression can be detected in the germarium (arrow in A and A'). The grk mRNA later becomes localised to the posterior, and then to the anterior-dorsal side of the oocyte by stage 10 (B,B'). (C,C') Grk protein distribution was detected with antibody staining in wild-type follicles (C) and cycGHR7 mutant follicles (C'). The false colours reflect the intensity of the staining. The pictures were taken with the same setting to allow for a direct comparison. Note low expression levels of Grk protein in the cycGHR7 mutant. Scale bars: 50 μm.
the histone H2A variant (γ-H2AV) which accumulates at the sites of DNA breaks and serves as a marker for meiotic DSBs (Jang et al., 2003; Mehrotra and McKim, 2006). In wild-type females γ-H2AV foci can be observed in nurse cells and pro-oocytes within region 2a and 2b of the germarium but no longer in region 3, suggesting that DSBs catalysed within region 2 are repaired by the time germline cells reach region 3 (Fig. 5A) (Jang et al., 2003; Mehrotra and McKim, 2006). Meiotic DNA repair mutants, e.g. spn-A, are characterised by delayed repair of DSBs seen by the persistence of γ-H2AV staining: on average 20 foci can be counted in spn-A mutant pro-oocytes within region 3, which were immunostained with anti-Orb antibodies to detect the oocyte (Fig. 5A) (Mehrotra and McKim, 2006). CycGHR7 mutant germaria displayed a mean of 17.1 γ-H2AV foci in the region 3 pro-oocytes consistent with a defect in DSB repair, which is abolished in mei-W68; cycG double mutants, where DSB formation is impaired (Fig. 5A).

After recombination is completed and the synaptonemal complex is disassembled, the meiotic chromosomes detach from the nuclear envelope and compact into a karyosome. This process is completed by stage 6 of oogenesis (Lancaster et al., 2010). The karyosome forms a spherical body that is located in the centre of the oocyte nucleus, the germinal vesicle (Fig. 5B). Activation of the meiotic checkpoint causes defects in the karyosome: it appears less condensed, often threadlike or even fragmented and is nearly always located at the nuclear periphery (Ghabrial et al., 1998; Ghabrial and Schüpbach, 1999; González-Reyes et al., 1997). Oocytes of spindle class mutants like spn-A show these karyosome defects with high frequency (Fig. 5B) (Ghabrial et al., 1998; González-Reyes et al., 1997). The cycGHR7 mutant egg chambers also displayed karyosome defects that were, however, considerably weaker: in 53% of the egg chambers we observed a mislocalisation of the DNA which was attached to the nuclear membrane instead of the central location, along with a mildly distorted shape of the germinal vesicle (Fig. 5B); the other 47% were normal.

One consequence of meiotic checkpoint activation in Drosophila is the inefficient translation of grk mRNA, which is required for dorsoventral egg patterning. A target of the executing kinase DmChk2 is Vasa, an eIF4A-like translation initiation factor involved in Grk translation, which is modified post-translationally upon checkpoint activation (Abdu et al., 2002; Ghabrial and Schüpbach, 1999; Johnstone and Lasko, 2004). In order to determine whether Vasa was likewise modified in the absence of CycG, western blot analysis from cycGHR7 ovarian lysates was performed. In comparison to wild-type extracts, Vasa protein mobility was retarded in the cycGHR7 extracts (Fig. 5C). Moreover, the altered Vasa mobility was restored to a more wild-type situation in extracts from cycG and mei-W68 or DmChk2 doubly mutant ovaries (Fig. 5C). Taken together, these results show that a meiotic checkpoint is induced in Drosophila oocytes in the absence of cycG and that hence CycG plays an important role in the meiotic checkpoint control of Drosophila.
components. We therefore performed co-immunoprecipitations with factors involved in DSB sensing and signal transduction, i.e. the Rad9 component of the 9-1-1 complex and BRCA2, respectively, and with factors involved in DNA-strand exchange and resolution, i.e. Spn-A (Rad51) and Okr (Rad54). Because no functional antibodies are available for most of the proteins involved in meiotic checkpoint control and DSB repair we made use of epitope-tagged constructs under UASp-control which were induced specifically within the germline.

We first attempted to co-precipitate CycG with either Spn-A or Okr however, we failed (supplementary material Fig. S2). Because Spn-A protein amounts were low, we can neither confirm nor exclude an association from this failure. However, no protein interaction was found between CycG and Okr, indicating that CycG is not required for DNA-strand resolution.

In order to assay possible interactions between CycG and Rad51 proteins, we switched to genetic interactions. Unfortunately the close proximity of the CycG and Spn-A loci prevented recombination of the two genes. Instead we used the spn-B locus which encodes a Rad51-like protein (Ghabrial et al., 1998). We recombined spn-B mutant alleles with cycGHR7 and analysed the frequency of ventralised eggs from doubly mutant females. We noted an enhancement of the ventralised phenotype: in contrast to the single mutants, the double mutants gave extremely ventralised eggs at a high frequency, lacking dorsal appendages completely (Fig. 4). Since the spn-B allelic combination we used is nearly null, CycG and Spn-B presumably act in parallel.

Next we addressed molecular interactions between CycG, Rad9 and BRCA2. We were able to co-precipitate CycG with an HA-tagged version of BRCA2 as well as with Flag-tagged Rad9 from ovarian extracts (Fig. 6). Rad9 is an integral component of the 9-1-1 complex that associates with BRCA2 (Klovstad et al., 2008). Hence, CycG is likely to function via the 9-1-1/BRCA2 complex. Consistent with this deduction, the mild eggshell patterning defect observed in brca2 mutants was not altered in
the *brca2; cycG* doubly mutant combination, suggesting that *brca2* acts epistatic over *cycG* (Fig. 4). Similar results were obtained before with *brca2; spn-A* or *hus1 spn-B* doubly mutant combinations (Abdu et al., 2007; Klattenhoff et al., 2008). They can be best explained by a direct involvement of the 9-1-1/*BRCA2* complex in meiotic checkpoint induction (Abdu et al., 2007; Klattenhoff et al., 2008). In conclusion, CycG may act upstream of the 9-1-1 complex, i.e. as a regulatory factor of one or several of its components, or in parallel with regard to meiotic checkpoint activation.

**Discussion**

In this study, we generated a null mutant of *cycG* by homologous recombination. Intriguingly, flies lacking *cycG* are viable, but female-sterile, indicating that this gene has essential functions during oogenesis. Egg chambers mutant for *cycG* exhibited dorsalventral patterning defects and failed to accumulate a wild-type level of Grk protein during oogenesis. CycG has been found to associate with the chromatin factor Corto and to control the transcription of the *Abdominal B* gene (Salvaing et al., 2008a; Salvaing et al., 2008b). Here, however, *grk* mRNA levels were unaltered in the *cycG* mutant, indicating that CycG does not act as a transcriptional regulator of *grk*. Instead, our results show that the ventralised egg phenotype of the *cycG* mutant is a consequence of compromised DSB repair, resulting in the activation of a meiotic checkpoint, since it was rescued by the downregulation of the DSB-inducing Mei-W68 endonuclease. Thus, meiotic DNA breaks are a prerequisite for the defects observed in the *cycG*-mutant germline. This is in contrast to mutants of the rasiRNA or priRNA pathways, where *mei-W68* mutations failed to suppress the egg phenotypes, which phenotypically matched those of the *cycG* mutant. For those other pathways, DNA breaks were found to accumulate during oogenesis because of defects in transposon silencing mechanisms rather than in meiotic DSB repair (Klattenhoff et al., 2007; Klattenhoff et al., 2009). Since mutants of *mei-41* and *DmChk2* kinases, which trigger the meiotic checkpoint, reversed the eggshell defects of the *cycGHR7* mutant, CycG presumably acts upstream of Mei-41 in the regulation of the meiotic checkpoint. This is consistent with the expression of *cycG* mRNA in region 2 of the germarium (Fig. 1B’). Moreover, the physical association of CycG protein with components of the 9-1-1/*BRCA2* complex in ovaries suggests an involvement of CycG in early steps of meiotic recombination repair. Interestingly, the overexpression of *Drosophila* CycG renders larval cells sensitive to irradiation-induced apoptosis (Faradji et al., 2011). This observation may hint at a role of CycG in DSB repair in somatic cells as well.

*CycG, a mediator of meiotic recombination repair*

A genome-wide interaction database combining genetic and molecular data sets (DroID) allows predictions and starting points for further biochemical and genetic analyses (Murali et al., 2011; Yu et al., 2008). Interestingly, the DroID dataset reports a direct molecular interaction of CycG with Spn-A in a yeast two-hybrid screen (Stanyon et al., 2004). *Spn-A* encodes the *Drosophila* Rad51 homologue required for the repair of meiotic DSBs (Staeva-Vieira et al., 2003). Rad51 is recruited to the damaged DNA, where it forms a nucleoprotein complex with the resected DNA and initiates strand invasion in the homologous recombination pathway (overview in Forget and Kowalczykowski, 2010). This step requires the association of several proteins with Rad51/Spn-A, including BRCA2 in *Drosophila* (Brough et al., 2008). Our data show an association of CycG with Rad9 and BRCA2, both components of the 9-1-1/*BRCA2* complex that is important for DSB-sensing and checkpoint activation (Klattenhoff et al., 2008, Peretz et al., 2009; overview in Pardo et al., 2009). Genetic interaction analyses showed that *brca2* is epistatic to *cycG* since the eggshell phenotype of *brca2; cycG* double mutants is similar to that of the *brca2* single mutant. A similar epistatic relationship was observed in doubly mutant combinations of different *spindle* class mutants and either *brca2* or *hus1* and can be reconciled by the requirement for the 9-1-1/*BRCA2* complex for meiotic checkpoint activation (Abdu et al., 2007; Klattenhoff et al., 2008; Peretz et al., 2009). In contrast, meiotic checkpoint activation is independent of CycG because it does occur in the *cycGHR7* mutant. Based on the phenotypic enhancement observed in the *cycG spn-B* double mutants, CycG may act as a mediator of repair enzyme function, possibly in the course of BRCA2 mediated Spn-A loading onto single-strand DNA. This is in line with the reported interaction of CycG with Spn-A (Murali et al., 2011; Stanyon et al., 2004) and also with the lack of interaction of CycG with the Rad54 homologue Okr that is expected to act downstream of Spn-A (overview in Forget and Kowalczykowski, 2010).

Possible molecular mechanisms of *Drosophila* CycG activity in the meiotic checkpoint control

The two mammalian CycG genes, CycG1 and CycG2, have been involved in cell cycle regulation and stress response, although many details of the molecular mechanisms of CycG activity remain elusive. Studies on a targeted mouse mutant revealed a role of CycG1 in the DNA damage response, since mutants were sensitive to irradiation, and stressed cells arrested at the G2/M phase of the cell cycle (Klattenhoff et al., 2001). In contrast overexpression of CycG2 promoted a G1/S phase arrest (Bennin et al., 2002). Of note, an association of mammalian CycG was only established in mitotic, not yet in meiotic cell cycle control. Mammalian CycG1 has been found to associate with GAK (CycG associated kinase) and cyclin dependent kinase Cdk5 on the one hand, and with B’ subunits of protein phosphatase 2A (PP2A) on the other hand (Kanaoka et al., 1997; Okamoto et al., 2002). The latter also interact with CycG2 (Bennin et al., 2002). Similarly *Drosophila* CycG was identified as a partner of several cyclin dependent kinases and also of Widborst (Wdb), the *Drosophila* B56-2 homologue of the B’ subunit of PP2A in large scale yeast two-hybrid screens (Giot et al., 2003; Stanyon et al., 2004). In mammals the molecular interaction of CycG with PP2A increased the activity of PP2A, which has led to the idea that CycG may serve as a specificity factor recruiting PP2A to its substrates (overview in Chen, 2002). Recent work has highlighted a possible underlying mechanism: It was shown that CycG1 served to recruit GAK to the B’γ subunit of PP2A, which by a direct phosphorylation of the B’γ subunit enhanced holoenzyme assembly and activity of PP2A (Naito et al., 2012). Moreover, phosphorylated PP2A concentrated at DSB foci induced by irradiation and colocalised there with phosphorylated H2AX (γ-H2AX) and with phosphorylated Chk2 (Naito et al., 2012), suggesting that this association resulted in a specific dephosphorylation of Chk2 after DNA damage in somatic cells. However, the GAK-target site in B’γ is found only in two of the five mammalian B’ members, and in
none of the three known Drosophila B subunits, rendering an analogous regulatory mechanism in the fly unlikely. In the context of meiotic homologous repair, PP2A is known
to dephosphorylate activated checkpoint kinases ATM and ATR as well as γ-H2AX, thereby abrogating the checkpoint and
allowing a cell to resume the cell cycle (Chowdhury et al., 2005; Petersen et al., 2006; overview in Shiloh, 2006). Assuming
Drosophila CycG enhances dephosphorylation of these checkpoint proteins via its interaction with PP2A, the checkpoint
signals would stay in force in the cycG mutant for a prolonged time despite a normal repair process. As γ-H2AX is
known to amplify the signal during the DSB response (Pardo et al., 2009), failure of its removal might suffice to activate
the checkpoint to generate the observed phenotypes. However, in Drosophila γ-H2AV is constantly replaced with H2AV by
histone exchange, requiring constant phosphorylation of H2AV by ATM and ATR kinases at DSB sites until repair is completed (Joyce et al., 2011).

Alternatively, Drosophila CycG may act as a co-factor of cyclin dependent protein kinases during meiotic recombination repair. DNA damage repair involves a plethora of phosphorylation steps of several players (Summers et al., 2011), so a regulatory role for CycG can easily be envisaged. Importantly, we identified CycG in a complex with BRCA2 in ovaries. One of the key functions of BRCA2 in mammals is the loading of the Rad51 recombinase onto single-strand DNA during somatic recombinational repair (overview in Forget and Koszalczykowski, 2010). This activity of BRCA2 is cell cycle regulated: it is thought that phosphorylation of BRCA2 by Cdks releases Rad51 from BRCA2/Rad51 complexes, enabling the formation of nucleoprotein filaments required for strand invasion (Esashi et al., 2005; overview in Summers et al., 2011). Whereas the physical interaction of Drosophila BRCA2 with the Rad51 homologue Spn-A has been shown in cell culture (Brough et al., 2008), no data exist so far for BRCA2 mediated Spn-A loading onto single-strand DNA or a possible dependence on Cdk mediated phosphorylation in Drosophila.

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Materials and Methods

Generation and verification of the cycG mutant

Homologous recombination was performed on the experimental species Drosophila melanogaster (Meigen) according to the ‘ends-in’ method as described in Rong et al. (Rong et al., 2002). A 6.1 kb Bso UI hindIII fragment that covers the coding region of cycG was isolated from a λ-pherage derived from a Kr°phage library (Maier et al., 1992) using the cycG cDNA as a probe; it was then cloned into pTV2 (Rong et al., 2002). The I-Scel site was inserted with primers at the BamHI site after two frame shift mutations had been inserted on either side by filling in the neighbouring Neo I (upstream) and Ngo MIV sites (downstream). P-element mediated transformation resulted in a starting line that contained the construct on the X-chromosome. From 500 mosaic females, 12 red-eyed flies were obtained. Two lines (Salvaing et al., 2008a) were used. The secondary antibodies used were coupled mouse anti-HA (Sigma-Aldrich; St. Louis, MO), rabbit anti-Flag (Rockland; do). Detection mouse anti-GFP (sc-9996, Santa Cruz Biotechnology; Santa Cruz, CA), anti-CycG (Salvaing et al., 2008a) or rat anti-HA bound to Protein A-Agarose (Santa Cruz, CA) or Protein G-Agarose (Rockland, Rockland, CA) were used. The primary antibodies used were coupled with alkaline phosphatase (Jackson Immunoresearch, Dianova; Hamburg, Germany). The figures were assembled using Corel Photo Paint and Corel Draw software (Ottawa, ON, Canada).

Genetic interactions

Genetic interaction tests were performed with bra256E/Cyo, bra256E/Cyo (Klovstad et al., 2008), Df(2L)BSC285/Cyo (BL 23158) and Df(2L)BSC60/SM6a (BL 29030) [both of these deficiencies uncover the DmDmc2 locus, DmDmc2 deficiency uncovers the mec-41 gene, mec-41/+/FMTa (BL 4169) and Df(2L)BSC772/FMT (BL 26869) [this deficiency uncovers the mei-41 gene, spn-A/TM3Sb (BL 3322), spn-B/TM3Sb and spn-B/TM3Sb (Ghabrial et al., 1998). Germine clones were generated using the dominant female sterile protocol developed by Chou and Perrimon (Chou and Perrimon, 1992) with the hsFLP, (neo)FRT82B ov205b (BL 2149) and FRT82B cycG205 flies. Standard genetic procedures were used to generate doubly mutant combinations. Oregon-1 served as wild-type control.

Documentation of eggshell morphology

Eggs were mounted in Hoyer’s medium (Van der Meer, 1977) for microscopic examination by a Zeiss Axioskop (Carl Zeiss AG; Oberkochen, Germany) coupled to a Canon 450D camera (Canon; Tokyo, Japan). Alternatively, pictures of uncoated eggs were taken with a table-top scanning electron microscope (Neoscope JCM-5000, Nikon; Tokyo, Japan).

Caffeine feeding assays

The protocol of Klattenhoff et al. (Klattenhoff et al., 2009) was followed for the caffeine treatment: cycG156 homozygous and wild type control flies were fed with yeast paste containing 2% w/v caffeine (Sigma-Aldrich; St. Louis, MO). Embryos from these animals were collected on apple juice plates and their phenotypes were analysed.

In situ hybridisation and histochemistry

In situ hybridisations on salivary gland chromosomes and ovaries were performed with digoxigenin-labelled cDNA probes according to standard protocols (Tautz and Pfeifle, 1989). For the histochemical analyses, ovaries of 3–5 day-old females were dissected and fixed for 30 min in 4% paraformaldehyde in phosphate buffered saline (PBS) plus heptane. For the anti-Gurken staining ovaries were preincubated with 4% normal goat serum in PBX (PBS with 0.3% Triton X-100). After several washes in PBS the mouse anti-Gurken antibody (1:50, 1D12 DSHB, developed by T. Schüpbach) was incubated overnight at 4°C. The anti-H2AV staining was performed according to the buffer A protocol (McKim et al., 2009) by using rabbit anti-γ-H2AV (1:500; Mehrtra and McKim, 2006). Mouse anti-Orb antibodies (1:5; 4H8 and 6H4 DSHB, developed by P. Schiedl) were used to label individual cysts in the developing germania and YOYO3 iodide (Molecular Probes/Life Technologies; Grand Island, NY) to stain the DNA. Secondary antibodies coupled to either DTAF, Cy3 or Cy5 were from Jackson Research (Dianova; Hamburg, Germany). Karyosome morphology was analysed in fixed ovaries, using propidium iodide as nuclear marker and mouse anti-Lamin C (1:50, LC28.26 DSHB, developed by P. Fisher) to visualise the nuclear lamina. Fluorescently labelled tissue was mounted in Vectashield (Vector Laboratories; Burlingame, CA) and analysed with a Bio-Rad MRC1024 confocal microscope coupled to a Zeiss Axiohot, using LaserSharp2000TM software (Carl Zeiss AG; Oberkochen, Germany). The figures were assembled using Corel Photo Paint and Corel Draw software (Ottawa, ON, Canada).

Immunoprecipitations and immunoblotting

Induction of pUA3p CycG, pUA3p Flag-Rad9b and pUA3p HA-bra2 (Klovstad et al., 2008) or pUA3p sm-A-GFP (a kind gift from T. Schüpbach); was attained by using the maternal Gal4 driver P(Gal4::VP16-UTR-CG6325MVD1) (BL 4937). 100 ovary pairs of the respective genotypes were homogenised in RIPA I buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, protease and phosphatase inhibitor cocktail (Roche; Basel, Switzerland) and incubated for 15 min on ice. After precipitation with immobilized protein A-Agarose beads (Roche; Basel, Switzerland) the cleared lysate was incubated for several hours at 4°C in RIPA II (10 mM Tris pH 7.5 including protease and phosphatase inhibitor cocktail (Roche; Basel, Switzerland). For negative control a pig anti-CycG (Salvaing et al., 2008a) or rat anti-HA bound to Protein A-Agarose beads (Roche; Basel, Switzerland). After several washes with RIPA II buffer at 4°C samples were run on a 10% SDS-PAGE followed by western blotting. For detection mouse anti-gp (sc-9996, Santa Cruz Biotechnology; Santa Cruz, CA), mouse anti-HA (Sigma-Aldrich; St. Louis, MO), rabbit anti-Flag (Rockland; Gibertsville, PA), rabbit anti-Rad54 (Alexiadis et al., 2004) or rat anti-CycG (Salvaing et al., 2008a) were used. The secondary antibodies used were coupled with alkaline phosphatase (Jackson Immunoresearch, Dianova; Hamburg, Germany).

For the preparation of ovarian extracts, 40 ovaries of each genotype were dissected in ice-cold PBS and homogenised in RIPA I buffer on ice. After centrifugation, a loading buffer was added to the supernatant and the probes were loaded onto an SDS-PAGE followed by western blotting. Rat-anti Vasa (1:1000, DSHB; developed by A. C. Spradling and D. Williams) was used for the detection of Vasa. Extracts from larval tissues were probed with guinea pig anti-CycG.
(1:1000) or rat anti-CycG (1:500) (Saiainga, 2008a) and rabbit anti-Actin (1:250) Sigma-Aldrich; St. Louis, MO) as a loading control.

**Semi-quantitative RT-PCR analysis**
The mRNA from wild-type and cycG204 mutant male adults and unsexed third instar larvae was isolated using the Poly ATtract® System 1000 (Promega; Madison, WI). Reverse transcription was performed with the ProtocScript® M-MulV Taq RT-PCR kit (New England Biolabs; Ipswich, MA) at 42°C. The PCR was performed using the following primer sets: Tubb (internal control), Tubb656229UFP (5'-GAA CCT ACC ATG ATC ACG C) and Tubb656507LP (5'-GAA GCC AAG CAG GCA GCA) primers, and Medea transcripts: U Med (5'-GGT CGC TCA CGC AAA CCG A) and L Med (5'-AGG CGC AGG ATG CAT AAC CG).

**Acknowledgments**

**References**


Fig. S1. Molecular verification of the cycGHR7 mutant. (A) Verification of the homologous recombination event by in situ hybridisation to salivary gland chromosomes using a white probe. Note insertion of a white gene at the tip of 3R in addition to the endogenous locus (arrows). (B) Enlargement of the 3R tip; the insertion is within the 100C region (arrow), the predicted locus of cyclin G. (C) Southern blot analysis of wild type (WT) and homozygous cycGHR7 (HR7) DNA digested with Bam HI and Eco RI; on the right, a restriction map depicts the relevant region and the probe used for hybridisation. Note the size reduction of the lower band in the mutant which results from a ~ 0.35 kb deletion within the left gene copy resulting from the insertion event. Size standard is given in kilo base pairs (kb).
Fig. S2. Neither Spn-A nor Okr was detected in precipitates of CycG. Western blots of immunoprecipitations (IP) on ovarian lysates are shown; the input column shows 20% of the extract used for the IP. IPs without antibodies served as mock control. (A) UASp-*cycG* was expressed in the germline and IP was performed with anti-CycG. Okr was detected with Rad-54 specific antibodies (anti-Rad-54). (B) Females expressing UASp *cycG* and UASp *Spn-A*-GFP in the germline were used for the IP, which was performed with anti-CycG. Spn-A was detected with anti-GFP; note the low levels in the input control that indicate very low expression of Spn-A-GFP.