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Greatwall kinase: a nuclear protein required for proper chromosome condensation and mitotic progression in *Drosophila*

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Mutations in the *Drosophila* gene *greatwall* cause improper chromosome condensation and delay cell cycle progression in larval neuroblasts. Chromosomes are highly undercondensed, particularly in the euchromatin, but nevertheless contain phosphorylated histone H3, condensin, and topoisomerase II. Cells take much longer to transit the period of chromosome condensation from late G_2 through nuclear envelope breakdown. Mutant cells are also subsequently delayed at metaphase, due to spindle checkpoint activity. These mutant phenotypes are not caused by spindle aberrations, by global defects in chromosome replication, or by activation of a caffeine-sensitive checkpoint. The Greatwall proteins in insects and vertebrates are located in the nucleus and belong to the AGC family of serine/threonine protein kinases; the kinase domain of Greatwall is interrupted by a long stretch of unrelated amino acids.

Introduction

Many molecules participate in the condensation of chromosomes during mitosis. The highly conserved condensin complex clearly plays a key role, promoting chromosome condensation by directing the supercoiling of DNA (for review see Hirano, 2002). Condensin is unlikely to determine all levels of compaction because disruption of the complex only partially interferes with mitotic chromosome condensation (Steffensen et al., 2001; Hagstrom et al., 2002). Other molecules and events have been proposed to mediate additional aspects of condensation, including topoisomerase II and the phosphorylation of histone H3 by aurora B kinase. However, the importance of such factors in chromosome condensation remains controversial.

Several preconditions must be fulfilled to allow chromosomes to condense. First, DNA replication must be completed; for example, *Drosophila* mutants in DNA replication factor genes show defects in chromosome condensation (Krause et al., 2001; Pflumm and Botchan, 2001). Second, several cell cycle regulators must become activated. The first visible stages of condensation during late G_2 or early prophase are mediated by cyclin A-CDK, but not by cyclin B-CDK. Cyclin A-CDK activity increases throughout G_2, and injection of cyclin A-CDK into G_2 cells rapidly induces chromosome condensation (Furuno et al., 1999). In contrast, cyclin B-CDK is inactive and stays in the cytoplasm during these early condensation stages (Rieder and Cole, 1998; Pines and Rieder, 2001). Later aspects of chromosome condensation do require cyclin B-CDK. Late in prophase, the nuclear accumulation and activation of cyclin B-CDK1 triggers nuclear envelope breakdown (NEB), and also mediates subsequent chromosome condensation. In particular, cyclin B-CDK1 phosphorylates and activates the 13S condensin complex (Kimura et al., 1998; Sutani et al., 1999).

Here, we describe a novel *Drosophila* gene, *greatwall* (*gwl*), that is required for proper chromosome condensation. The Greatwall protein is conserved in insects and vertebrates,

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Key words: *Drosophila*; cell cycle; kinase; chromosome condensation; nuclear protein

Abbreviations used in this paper: dsRNA, double-stranded RNA; *gwl*, *greatwall*; NEB, nuclear envelope breakdown; RNAi, RNA interference.

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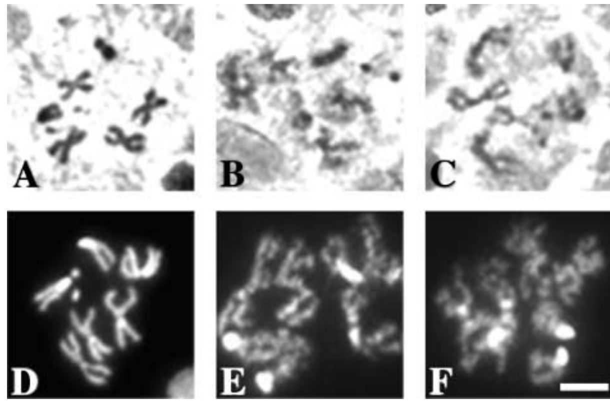

Figure 1. **Chromosome condensation defects in *gwl* mutant brain cells.** Wild-type (A and D), gwl^{716} mutant (B and E), and gwl^{2970} mutant (C and F) brains were treated with colchicine, and the chromosomes were stained with either orcein (A–C) or Hoechst 33258 (D–F). Bar, 5 μm.

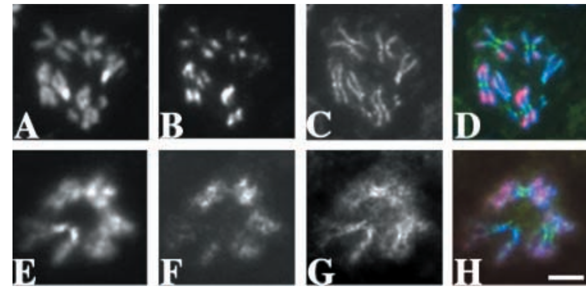

Figure 2. **Chromosomes from *gwl* mutants contain the condensin component Barren and phosphohistone H3.** Wild-type (A–D) and gwl^{2970}mutant (E–H) neuroblasts stained for DNA (A and E), phosphohistone H3 (B and F), and Barren (C and G). (D and H) Merge of signals for DNA (blue), phosphohistone H3 (red), and Barren (green). Bar, 5 μm.

and contains a kinase domain bifurcated by a long stretch of unrelated amino acids. Greatwall kinase is found in the nuclei of interphase cells, but is distributed throughout mitotic cells. Mitotic chromosomes in *gwl* mutants are notably undercondensed in the euchromatin. Surprisingly, condensin, phosphorylated histone H3, and topoisomerase II are recruited to these abnormally condensed chromosomes. Mutant cells have major delays in cell cycle progression. The period of chromosome condensation before NEB is much longer than normal. After NEB, mutant cells display a delay in metaphase that is dependent on the spindle checkpoint.

Results and discussion

Neuroblasts in *gwl* mutants have undercondensed chromosomes that nonetheless bind phosphohistone H3 and condensin

We isolated a mutation in *Drosophila* that causes neuroblasts in third instar larval brains to display irregular chromosome condensation and a high mitotic index indicative of mitotic arrest (see Materials and methods). We named the gene identified by this mutation *greatwall* because the mutant phenotype suggests a role in protecting chromosome structure. Later, we found that four uncharacterized mutants (Kitamoto et al., 2000) were allelic to the original *gwl* mutation and caused identical mitotic defects.

The most obvious phenotype associated with *gwl* mutations is chromosome undercondensation. This is apparent in mutant larval brains whose chromosomes were stained with either orcein or Hoechst 33258 (Fig. 1), and is most visible in brains treated with colchicine, which arrests cells under conditions that promote chromosome condensation. In these chromosomes, the euchromatin is highly undercondensed, whereas the heterochromatin remains more compacted. Many aberrantly condensed chromosomes were nonetheless clearly composed of two sister chromatids.

Surprisingly, *gwl* mutant chromosomes still reacted with antibodies directed against phosphohistone H3 and the condensin component Barren (the fly homologue of XCAP-H; Fig. 2). The signal intensities on *gwl* chromosomes were roughly similar to those seen in wild type, but Barren and phosphohistone H3 were more diffuse on the undercondensed mutant chromosomes.

Progression through mitosis is delayed or arrested in *gwl* mutants

Cytological examination of fixed brains from larvae homozygous or hemizygous for all five *gwl* alleles revealed defective mitotic progression. The mitotic index was ~2.5-fold higher than controls, and the proportion of mitotic cells that were in anaphase or telophase was only 10–15% of that in wild type (Table S1, available at http://www.jcb.org/cgi/content/full/jcb.200310059/DC1). Thus, *gwl* mutant cells delay or arrest before anaphase onset. Almost all of the few residual anaphases/telophases in *gwl* brains were aberrant, with lagging chromosomes or chromosome bridges. The spindles in *gwl* mutant cells were nevertheless morphologically normal at all mitotic stages (Fig. S1).

More detailed observations showed that *gwl* cells are defective in two aspects of cell cycle progression (Table S1 and Table S2). First, the fraction of cells in late G_2/prophase was elevated, indicating that NEB is delayed. Second, as evidenced by the very low frequency of anaphases, mutant cells in which NEB has occurred are subsequently delayed in entering anaphase.

To better understand the *gwl* phenotype, we examined the division of live neuroblasts in time-lapse microscopy using newly described methods that maintain culture viability for >12 h and that provide excellent four-dimensional resolution (Fleming and Rieder, 2003). To allow simultaneous visualization of chromosome condensation, we generated mutant and control flies expressing GFP-tagged histone H2AvD (Clarkson and Saint, 1999), and filmed cultured neuroblasts with both epifluorescence and differential interference contrast microscopy.

Live imaging of *gwl* neuroblasts revealed delays in several mitotic phases (Fig. 3 and Table S3). Prophase in mutant neuroblasts lasted >10 times longer than in controls. After NEB, mutant cells were somewhat slower than controls (~2 times) to reach metaphase, and 20% of the mutant cells never achieved a stable metaphase. The duration of metaphase was also longer in *gwl* mutants than in controls (>4 times), and this does not take into account the 17% (2/12)

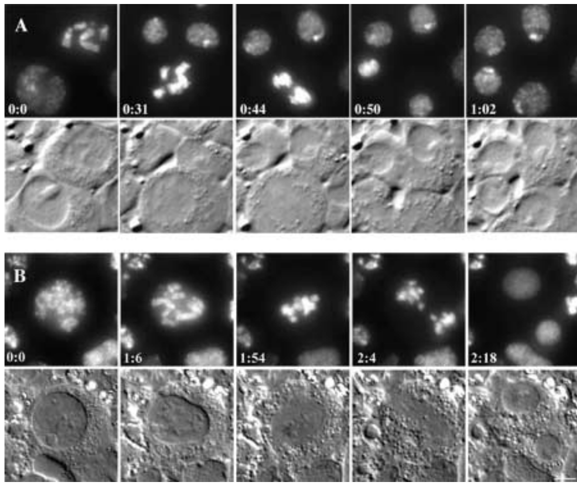

Figure 3. **Division of cultured neuroblasts in time-lapse microscopy.** Time in h and min (h:min). (A) Wild-type neuroblasts. (B) *gwl*^{716} mutant neuroblast. First and third rows, histone H2Av-GFP signal; second and fourth rows, concurrent DIC images. In the particular mutant neuroblast shown, chromosome condensation takes place at least in part, and the cells enter a defective anaphase with lagging chromatids. However, the time required to complete various stages of mitosis is much longer than in wild type. Bar, 5 μm.

of mutant cells that reached metaphase but failed to enter anaphase during the recording period. Thus, our results with both fixed and live material present a consistent view of the mutant's difficulties in mitotic progression.

To determine whether the delay in NEB might reflect mitotic spindle defects, we filmed mutant and control cells in the presence of colchicine (Table S3 and Fig. S2). Chromosome condensation took much longer in drug-treated *gwl* mutant cells than in similarly treated controls, and 30% of the mutant cells failed to condense their chromosomes fully or initiate NEB during periods exceeding 4 h. Thus, Greatwall is needed for proper chromosome condensation and timely NEB even in the absence of a mitotic spindle.

Mutations in *gwl* do not block chromosome replication or activate a caffeine-sensitive G_2 checkpoint

The phenotypes described thus far might possibly be secondary consequences of DNA replication problems; similar defects are associated with mutations in many *Drosophila* genes encoding DNA replication proteins (Pflumm and Botchan, 2001). We used several techniques to show that DNA replication is substantially complete in G_2 mutant cells before mitosis. First, as seen in Fig. 1, many *gwl* mutant chromosomes appear as two complete sister chromatids late in mitosis. Second, we analyzed BrdU incorporation in whole brains (Fig. S3). Mutant brains, even from animals with the null *gwl*^{2970} allele, showed strong BrdU incorporation. In contrast, BrdU incorporation is virtually abolished in the brains of DNA replication mutants (Krause et al., 2001; Pflumm and Botchan, 2001). Third, we quantified the amount of DNA in individual cells by integrating Hoechst 33258 fluorescence signals in cells also stained for phosphohistone H3 to discriminate mitotic stages. In both wild-type and *gwl* brains, many interphase cells and all mitotic cells had roughly twice the DNA of the interphase cells with the lowest DNA content (unpublished data). Together, these results reveal that DNA replication in *gwl* mutants is globally normal, but we cannot exclude that subtle S phase defects might occur.

An alternative explanation for the mutant phenotype is that absence of Greatwall might activate a G_2/early prophase checkpoint that reverses the early stages of chromosome condensation and delays NEB (Pines and Rieder, 2001; Mikhailov and Rieder, 2002). Many checkpoints of this type monitor DNA damage and are mediated by the ATM and ATR kinases; when activated, these checkpoints inhibit cyclin B-CDK via the inhibition of Cdc25 phosphatase (for review see Abraham, 2001). Thus, we filmed live *gwl* neuroblasts treated with levels of caffeine that disrupt these checkpoints (De Marco and Polani, 1981). If such a checkpoint was activated in *gwl* mutants, caffeine should shorten the period of chromosome condensation before NEB. In fact, we saw exactly the opposite: many caffeine-treated mutant prophase cells never underwent NEB (unpublished data).

The spindle checkpoint delays anaphase onset in *gwl* mutants

As already described in this paper, *gwl* mutant cells are also delayed in a metaphase-like state after NEB. This arrest almost certainly results from spindle checkpoint activity caused by improper metaphase chromosome condensation. First, because sister chromatids remain connected in *gwl* cells after NEB, the cells have not entered anaphase (unpublished data). Second, all *gwl* cells staining for phosphohistone H3 have elevated cyclin B levels, as expected if the checkpoint is active (Fig. S4, A–H). Third, the checkpoint protein Bub1 accumulates on *gwl* mutant kinetochores, as is true in wild-type cells before anaphase onset (Fig. S4, I–T). That the metaphase arrest in *gwl* mutants is due to the spindle checkpoint is consistent with published reports showing that improper chromosome condensation or DNA damage can disrupt the centromere/kinetochore and delay satisfaction of the checkpoint (Pflumm and Botchan, 2001; Garber and Rine, 2002; Mikhailov et al., 2002).

gwl encodes an evolutionarily conserved, putative protein kinase

Fig. S5 details our assignment of the gene CG7719 (protein kinase-like 91C) as *gwl.* All five *gwl* alleles contain missense or nonsense mutations in CG7719. The four missense alleles affect conserved amino acids in the serine/threonine protein kinase catalytic domain of the encoded protein, whereas the null allele *gwl*^{2970} has a nonsense mutation that truncates the protein. We verified this assignment by depleting the CG7719 protein from *Drosophila* tissue culture cells using RNA interference (RNAi). Chromosomes from cells treated with CG7719 double-stranded RNA (dsRNA) were highly undercondensed (Fig. 4), reminiscent of the chromosomes in *gwl* mutant brains. This undercondensation is strikingly similar to that observed when fly tissue culture cells were treated with dsRNA for two condensin complex components (Gluon [XCAP-C] and Barren [XCAP-H]; Fig. 4 E–H). Greatwall-depleted cells

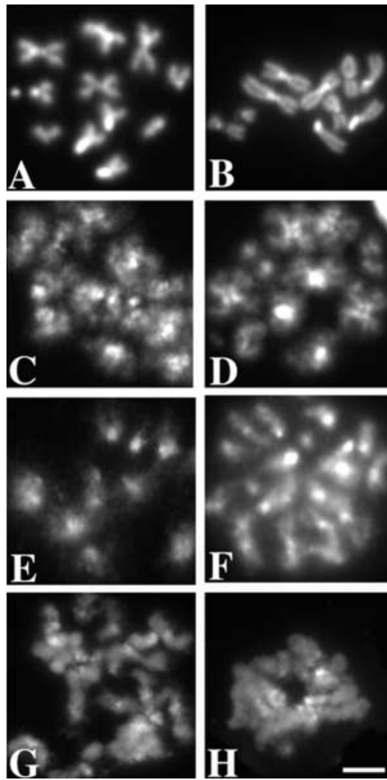

Figure 4. **Depletion of Greatwall by RNAi.** Treatment of *Drosophila* tissue culture cells with *gwl* dsRNA causes aberrant chromosome morphology reminiscent of that produced by depletion of condensin subunits. (A and B) Karyotypes of control colchicine-treated tissue culture cells in the absence of dsRNA. (C and D) RNAi with *gwl* dsRNA. (E) RNAi with dsRNA for Gluon (XCAP-C). (F) RNAi with dsRNA for Barren (XCAP-H). (G and H) Simultaneous RNAi with Greatwall and either Gluon (G) or Barren (H) dsRNAs. Bar, 5 μm.

also showed a 2–3-fold increase in the mitotic index and a threefold decrease in the percentage of anaphase/telophase cells (unpublished data), consistent with the mitotic delays in *gwl* brains. Western blotting with antibody to the CG7719 product established that the dsRNA-treated cells are indeed Greatwall deficient (Fig. S6). Of interest, immunostaining of dsRNA-treated cells showed that topoisomerase II localizes normally to chromosomes in cells lacking Greatwall and cohesin components (Fig. S7).

Greatwall belongs to the AGC family, a diverse group of serine/threonine kinases that phosphorylates targets surrounded by basic amino acids (Hanks and Hunter, 1995; Morrison et al., 2000). The kinase domain of Greatwall is split (Fig. 5), with ~500 amino acids separating subdomains VII and VIII (Hanks and Quinn, 1991). Other insects and vertebrates, including humans, have proteins very closely related to Greatwall; the kinase domains of the fly and human Greatwall proteins share 59% overall amino acid sequence identity (Fig. 5). The homologies between Greatwall proteins extend beyond the kinase domain into the flanking blue and green regions shown in Fig. 5. The insertions of several hundred amino acids between subdomains VII and VIII are less conserved, but we can still detect limited homology between the insect and vertebrate insertions. The Greatwall proteins all form a single homology group: they

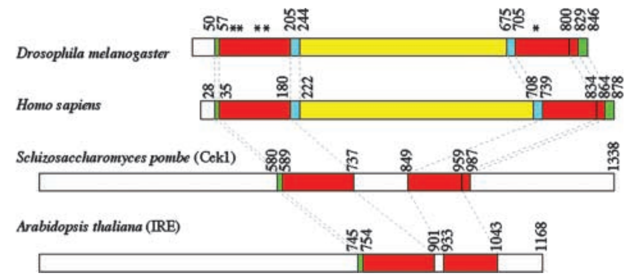

Figure 5. **Structure of Greatwall proteins.** Conserved domains of the *D. melanogaster* and human Greatwall proteins, compared with the related kinases Rim15p/Cek1p and IRE. The kinase domain in all these proteins (red) is split by the insertion of unrelated amino acids between kinase subdomains VII and VIII. Green and blue areas are less well-conserved regions found in all Greatwall kinases, but not in other kinases (except the short green stretch preceding the kinase domains in Rim15p/Cek1p and IRE). A small degree of homology is apparent between the inserts in insect and vertebrate Greatwall proteins (yellow). Asterisks indicate the lesions associated with mutant alleles: gwl^{716} = Gly66Ser; gwl^{180} = Gly69Glu; gwl^{2970} = Gln102Ter; gwl^{1028} = Ser127Leu; gwl^{873} = Ala728Val.

are the closest relatives found in all pairwise searches between these species. Greatwall is more distantly related to several other proteins that also contain an interruption between kinase subdomains VII and VIII, including IRE and IREH1 in *Arabidopsis*, CEK1 in *Schizosaccharomyces pombe*, and RIM15 in *Saccharomyces cerevisiae*.

The Greatwall protein localizes to the nucleus

To determine Greatwall's intracellular localization, we used anti-Greatwall antibodies as immunofluorescence probes (see Fig. S6 for the characterization of these antibodies). Greatwall accumulated in a punctate pattern in the nuclei of all interphase and prophase cells (Fig. S8). The distribution of the protein within nuclei does not match that of the DNA, but some Greatwall protein might still be chromosome associated. Greatwall was relatively evenly distributed throughout mitotic cells, with no obvious accumulation over the chromosomes or the spindle. The nuclear localization of Greatwall was verified by overexpressing GFP-tagged Greatwall in tissue culture cells (unpublished data).

Possible functions of the Greatwall kinase

Mitotic progression in *gwl* mutants is much slower than normal, with delays during both late G_2/prophase and the metaphase–anaphase transition. Mutations in *gwl* also cause improper chromosome condensation. In live recordings, chromosomes remain poorly condensed throughout a prolonged prophase, eventually condensing rapidly just before NEB (Fig. 3). However, because the undercondensed chromosomes seen in fixed, colchicine-treated brains (Fig. 1) are from arrested metaphase-like cells, we conclude that the condensation before NEB is incomplete and does not proceed further during prometaphase/metaphase. Incomplete chromosome condensation could disrupt centromere structure, preventing satisfaction of the spindle checkpoint and thus delaying anaphase onset. The aberrations seen in the few residual anaphases in mutants could be caused by the tangling of incompletely condensed chromosomes.

What is the primary defect in *gwl* mutants? Our results argue strongly that the mutant phenotypes are not secondary consequences of DNA damage or incomplete chromosome replication. Thus, we imagine that Greatwall is directly involved either in chromosome condensation or in the basic cell cycle machinery. Though problems in chromosome condensation during G_2/M could trigger an ATM/ATR-independent checkpoint that delays the commitment to mitosis (Goldstone et al., 2001; Pearce and Humphrey, 2001; Rui and Tse-Dinh, 2003), we do not favor this hypothesis. Greatwall is unlikely to play a structural role in chromosome condensation, as it does not appear to associate with chromosomes (Fig. S8), and because cells contain <5,000 Greatwall molecules as estimated by Western blots (unpublished data). Furthermore, chromosome undercondensation in *gwl* mutants reflects neither a lack of histone H3 phosphorylation nor a failure of condensin or topoisomerase II to associate with chromosomes (Fig. 2 and Fig. S7).

Instead, we believe that Greatwall helps activate cell cycle regulators that prepare interphase cells for entry into mitosis (Pines and Rieder, 2001). Our preliminary efforts to investigate Greatwall in *Xenopus* egg extracts suggest that Greatwall is needed to establish high cyclin B-CDK activity during mitotic entry. This hypothesis explains many aspects of the *gwl* phenotype, given that cyclin B-CDK phosphorylates the 13S condensin complex during mitosis (Kimura et al., 1998; Sutani et al., 1999).

In summary, though the importance of Greatwall to chromosome condensation and mitotic progression is clear, the biochemical function of this novel kinase remains to be determined. We are currently trying to identify potential substrates for Greatwall phosphorylation, and other proteins with which Greatwall might associate.

Materials and methods

Fly stocks

1,538 lines containing EMS-induced mutations on the third chromosome causing late larval/pupal lethality were provided by C. Zuker and E. Koundakjian (University of California, San Diego, San Diego, CA). Homozygous mutant larvae in each line were screened for mitotic defects; one line contained the gwl^{1028} mutation. Four other alleles (gwl^{180}, gwl^{716}, gwl^{874}, and gwl^{2970}) were the gift of T. Kitamoto (Beckman Research Institute of the City of Hope, Duarte, CA; Kitamoto et al., 2000). The deletions *Df(3R)Cha9*, *Df(3R)Dl-FX2*, and $Df(3R)Cha^{M5}$ were from the Bloomington *Drosophila* Stock Center. Oregon-R was the wild-type strain. Mutations and deficiencies were balanced over *TM6B* with *Humeral* (*Hu*) and *Tubby* (*Tb*), or over *TM6C* with *Stubble* (*Sb*) and *Tb*. For filming, gwl^{716} was crossed with a stock containing a GFP-tagged histone H2AvD transgene on the third chromosome (supplied by R. Saint, Australian National University, Canberra, Australia; Clarkson and Saint, 1999). Desired recombinants were rebalanced over *TM6C*.

Cytology and RNAi

Mitosis in larval brains was examined as described previously (Bentley et al., 2002). Mouse anti-lamin antibody was the gift of P. Fisher (State University of New York at Stony Brook, Stony Brook, NY; Stuurman et al., 1995), rabbit anti-Bub1 was provided by C. Sunkel (Universidade do Porto, Porto, Portugal; Basu et al., 1999), and rabbit anti-Barren was from H. Bellen (Baylor College of Medicine, Houston, TX; Bhat et al., 1996). To generate anti-Greatwall antibodies, sequences from a *gwl* cDNA clone (LD35132) encoding aa 371–846 were inserted into the pMAL-C2 vector (New England Biolabs, Inc.). Rabbit sera against the resultant Greatwall-maltose binding protein fusion made in *Escherichia coli* were affinity purified as in Bentley et al. (2002).

Observations of living Greatwall-H2AvD-GFP neuroblasts were performed according to Fleming and Rieder (2003). In some cases, isolated brains were exposed to 50 μM colchicine at RT for 1.5 h before dissection. For caffeine treatment, brains were incubated with 100 μM caffeine in neuroblast culture medium throughout the filming.

We followed the protocols of Somma et al. (2002) to deplete Greatwall, Barren, and Gluon from *Drosophila* Kc cells by RNAi and to examine the phenotypic consequences. Rabbit polyclonal anti-topoisomerase II antibody was the gift of D. Arndt-Jovin (Max Planck Institute for Biophysical Chemistry, Göttingen, Germany; Gemkow et al., 2001).

Online supplemental material

The supplemental material contains eight figures and three tables that document the Greatwall phenotype, the specificity of the anti-Greatwall antibody, and the intracellular localization of the Greatwall protein. The online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200310059/DC1.

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