Australin: a chromosomal passenger protein required specifically for Drosophila melanogaster male meiosis

Shan Gao, Maria Grazia Giansanti, Graham J. Buttrick, Sharada Ramasubramanyan, Adam Auton, Maurizio Gatti, and James G. Wakefield

1Department of Zoology, University of Oxford, OX1 3PS Oxford, England, UK
2Istituto Pasteur-Fondazione Cenci Bolognetti, Dipartimento di Genetica e Biologia Molecolare and 3Istituto di Biologia e Patologia Molecolari del Consiglio Nazionale della Ricerca, Università di Roma “La Sapienza,” 00185 Rome, Italy
5Department of Statistics, University of Oxford, OX1 3QY Oxford, England, UK

Correspondence to J. Wakefield: james.wakefield@zoo.ox.ac.uk

Abbreviations used in this paper: Aust, Australin; Borr, Borealin-related; CPC, chromosomal passenger complex; dsRNA, double-stranded RNA; INCENP, inner centromere protein; MBP, myelin basic protein; MT, microtubule; Pav, Pavarotti; UTR, untranslated region.

The online version of this paper contains supplemental material.

Introduction

The accurate segregation of chromosomes to opposite sides of the cell and the physical cleavage of the cell into two are fundamental aspects of cell division. Both processes are orchestrated by microtubules (MTs), which are dynamic polymers of α- and β-tubulin. Upon entry into mitosis or meiosis, the MTs form a bipolar spindle apparatus capable of interacting with specialized proteinaceous sites on condensed chromosomes termed kinetochores. Once they have all been aligned at the center of the spindle and a protective spindle assembly checkpoint has been satisfied, the chromosomes are segregated to the poles of the cell (Musacchio and Salmon, 2007). Immediately afterward, a subpopulation of MTs organizes between the separating chromosomes to form the central spindle. This polarized structure is then able to direct the accumulation of proteins important in the formation of the actomyosin contractile ring that mediates cytokinesis (Glotzer, 2003).

The chromosomal passenger complex (CPC) regulates many processes during cell division including chromosome condensation, spindle formation and stability, monitoring the interaction between kinetochores and MTs, central spindle assembly, and cytokinesis (Kallio et al., 2002; Murata-Hori et al., 2002; Vader et al., 2006). The CPC contains four core subunits: the kinase aurora B (Shindo et al., 1998; Adams et al., 2001; Giet and Glover, 2001; Kallio et al., 2002; Shannon and Salmon, 2002), the MT-binding protein inner centromere protein (INCENP; Cooke et al., 1987; Mackay et al., 1993), survivin, which additionally has a well-defined role in regulating apoptosis (Ambrosini et al., 1997; Li et al., 1998), and Borealin/DASRA/CSC-1 (Romano et al., 2003; Gassmann et al., 2004; Sampedro et al., 2004; Hanson et al., 2005; Klein et al., 2006). Although different subcomplexes have been shown to exist in cells, disruption of INCENP, survivin, or Borealin leads to mislocalization of the entire CPC (Gassmann et al., 2004; Lens et al., 2006). The CPC components show a dynamic, cell cycle–dependent localization,
Furthermore, Aust is absolutely required for central spindle assembly and the localization of proteins involved in cytokinesis. This study therefore not only provides an example of gene specialization during gametogenesis but also sheds further light on the meiotic functions of the CPC.

**Results**

**aust, a gene required for male fertility and meiotic chromosome segregation and cytokinesis**

The *aust* mutation was isolated by a cytological screen of the “Zuker’s collection,” which includes >2,000 male sterile mutants (Wakimoto et al., 2004). The *aust* mutant allele caused defects in meiotic chromosome segregation and cytokinesis both as a homozygote and a hemizygote over *Df(2L)30A-C*, a deficiency that failed to complement the *aust* mutation (see Materials and methods and Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200708072/DC1). Onion stage spermatids from wild-type flies display one phase-dense mitochondrial derivative (Nebenkern) and one phase-light nucleus, each of a similar size (Fig. 1, A and C). In contrast, screening >1,000 cells from *aust*/ *Df(2L)30A-C* hemizygotes failed to identify a single cell with wild-type morphology. Instead, spermatids showed a single large Nebenkern surrounded by two, three, four, or more nuclei of varying size (Fig. 1 B). By far the most predominant spermatids were those containing four nuclei of irregular size and a single Nebenkern (observed in >75% of cells; Fig. 1, D–J; and Table I). This phenotype is diagnostic of failures in both chromosome segregation and cytokinesis (Gonzalez et al., 1989; Fuller, 1993; Wakefield et al., 2001). As such, male meiosis has proved an excellent system in which to screen for genes required for karyokinesis and cytokinesis (Gonzalez et al., 1989; Fuller 1993; Giansanti et al., 2001, 2004).
chromosome segregation and cytokinesis during the two male meiotic divisions.

*aust* is a paralogue of *borr*
Using complementation analysis to a series of deficiency chromosomes and individual mutants, we mapped the *aust* locus to one of six genes (see Materials and methods and Fig. S1). To define which of these corresponded to the *aust* locus, we both assessed the expression of each gene in wild-type or *aust* flies using RTPCR and sequenced each gene from genomic DNA extracted from wild-type or *aust* flies. For five of the six genes, the expression and the DNA sequence were identical between wild-type and *aust* flies (Fig. 2A). However, *aust* flies failed to express CG17009, and sequencing of the gene from *aust* individuals identified a point mutation in the splice donor site at the exon–intron boundary (Fig. 2, A and B). Sequencing of genomic DNA isolated from a second *aust* allele identified during the course of our studies, *aust*2, also identified a single point mutation in the CG17009-coding region, resulting in an Ile–Ser amino acid substitution toward the C terminus of the protein (Fig. 2B and not depicted). Together, these results confirm CG17009 as the *aust* locus.

Stringent BLAST searches failed to identify any putative homologues of CG17009 in humans, *Xenopus laevis* or *Cae- norhabditis elegans* (unpublished data). However, we noticed that CG17009 possessed significant homology to its neighboring gene, *D. melanogaster* borr, located ~1 kb distal to CG17009 (Fig. 2B and Fig. S1; Eggert et al., 2004; Hanson et al., 2005). Closer examination confirmed that CG17009 is a paralogue of *borr*. A local alignment revealed that of the 315 residues in Bor, 99 (31%) are conserved in the CG17009 product (Fig. 2C), with the strongest conservation occurring at the N and C terminals of the proteins. Interestingly, a region of ~140 amino acids, roughly one third of protein, in the intermediate sequence of Bor is not present in the CG17009 product, which is suggestive of at least one indel event in the common history of the genes (Fig. 2, B and C). Because of its homology to Borealin (named after the aurora borealis, or northern lights) we have named CG17009 *australin* (after the aurora australis, or southern lights).

To further investigate the relationship between *aust* and *borr*, we collected sequences of both genes from within the genus *Drosophila* (http://flybase.bio.indiana.edu/). Phylogenetic analysis suggests that the divergence of the two genes predates the divergence of the *Drosophila* species (Fig. 2D). Of the two genes, *borr* appears to show a slightly higher degree of conservation between species (Fig. 2D), which suggests a greater level of evolutionary constraint. We also examined the ratio of non-synonymous to synonymous substitution rates (dN/dS) and confirmed that the *aust* gene has a marginally higher substitution rate (dN/dS = 0.11) than *borr* (dN/dS = 0.09; Yang and Nielsen, 2000). Importantly, however, the dN/dS ratios are comparable, which indicates that both genes are under strong selective constraint and are therefore likely to be functional.

**Aust biochemically interacts with DmINCENP, Borr, and itself**
Borealin is a conserved component of the CPC, which is composed of four core subunits: aurora B, INCENP, survivin, and Borealin itself (Vagnarelli and Earnshaw, 2004; Vader et al., 2006). Work in human cells has shown that Borealin directly associates with itself and with INCENP (Gassmann et al., 2004; Klein et al., 2006). Given the homology between Aust and Borr, we wondered whether both proteins share similar biochemical properties. To test this, we purified bacterially expressed myelin basic protein (MBP), MBP-Borr, and MBP-Aust, immobilized them on amylose beads, and added the beads to extracts of mammalian tissue culture cells expressing either HA-Borr or HA-Aust. We found that both MBP-Borr and MBP-Aust but not MBP alone coprecipitated with their respective HA fusion proteins and with each other (Fig. 3A). Furthermore, both MBP-Aust and MBP-Borr were capable of coprecipitating FLAG-DmINCENP. However, in agreement with previously published work in other systems, neither protein directly coprecipitated HA–aurora B (Fig. 3B; Gassmann et al., 2004).

It has also been recently shown that human Borealin is able to directly bind to DNA in an in vitro assay (Klein et al., 2006). Similarly, we found that both MBP-Borr and MBP-Aust but not MBP alone were able to interact with DNA (Fig. 3C). We conclude that Borr and Aust share key properties with the human Borealin protein.

**Aust is a testis-specific chromosomal passenger protein**
The presence of two Borealin-like proteins in *D. melanogaster* with apparently identical biochemical properties suggested to us

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Table I. **Defects associated with *aust* onion stage spermatids**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of spermatids</th>
<th>Type of spermatids</th>
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<tr>
<td></td>
<td>N 2×N 4×N &gt;4×N</td>
<td>N 2×N 3×N 4×N 5×N</td>
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<td></td>
<td>Nu Nu 2Nu 2Nu 4Nu 4Nu</td>
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Data represent percentage of spermatid type. 100% of wild-type spermatids possess a single nucleus and a single Nebenkern of regular size. In contrast, no spermatids in *aust* mutants exhibit regular morphology. Over 75% of onion stage spermatids possess a single large Nebenkern surrounded by four nuclei of irregular size. N refers to the size of the Nebenkern observed. N indicates a Nebenkern of regular size, 2×N indicates a Nebenkern twice the normal size, and 4×N indicates a Nebenkern four times the size of those seen in wild-type spermatids. Nu refers to the number of nuclei present. A and B indicate regular and irregular nuclear sizes, respectively.
that Borr and Aust might function similarly within cells but at different developmental stages. To assess this, we both examined the expression pattern of the genes throughout development and produced antibodies against the two proteins to investigate the localization of Borr and Aust in different cell types.

RT-PCR analysis on cDNA from a variety of tissues showed that although Borr was expressed in all tissues analyzed, Aust expression was confined to the testes (Fig. 4 A). In agreement with the ubiquitous expression pattern of Borr, affinity-purified anti-Borr antibodies showed a typical chromosomal passenger protein distribution in both syncytial blastoderm embryos and larval brains (Fig. 4, B and C). During interphase, Borr was present in the nucleus, redistributing to discrete chromosomal sites early in prophase. By metaphase, the antibodies recognized specific......

Figure 2.  CG17009 encodes aust, a paralogue of borr.  (A) RT-PCR of aust candidate genes using cDNA from wild-type or aust testes.  (B) Representation of CG17009 and its neighboring gene CG4454 (borr).  Regions corresponding to the proximal and distal exons of borr show homology with the two CG17009 exons (broken lines).  Sequencing from two independent aust alleles identified point mutations in CG17009.  (C) Alignment of Aust and Borr protein sequences using ClustalW (Thompson et al., 1994).  Arrows denote amino acids.  Black asterisks, conserved residues; red asterisks, point mutations present in the aust alleles.  (D) Phylogenetic tree of aust and borr for Drosophila species using the XP_309424 gene from Anopheles gambiae as the outgroup.  Significance was assessed via bootstrapping with 1,000 replicates.  Bootstrap values are shown.
To verify that Aust does, indeed, accumulate on kinetochores during metaphase, we costained cells for both Aust and the chromosomal passenger protein DmINCENP. We found that both antigens co-localized to discrete foci on the condensed chromosomes (Fig. 5 B). Again, similarly to DmINCENP, Aust remained on kinetochores throughout anaphase of meiosis I (Fig. 5 A; Resnick et al., 2006). Together, these results show Aust to be a chromosomal passenger protein and suggest that it replaces Borr specifically for the two male meiotic divisions.

Aust is required for localization of the CPC during male meiosis

Borealin has been shown to be required for the localization of other CPC components both in *D. melanogaster* and other organisms (Gassmann et al., 2004; Sampath et al., 2004; Hanson et al., 2005). Thus, we analyzed the localization of other chromosomal passenger proteins in *aust* spermatocytes. To ensure that the effects on meiosis observed were specifically caused by a loss of Aust function, all analyses were performed using *aust*/ Df(2R)30A-C hemizygotes (henceforth *aust* mutants).

In contrast, although anti-Aust antibodies failed to localize in mitotic tissue (unpublished data), they stained condensing chromosomes just before male meiosis I. Throughout both meiotic divisions, Aust showed a distribution typical of that of a chromosomal passenger protein. In addition to the central spindle midzone accumulation in anaphase and telophase and in good agreement with previous work on DmINCENP (Resnick et al., 2006), anti-Aust antibodies also stained the region of the equatorial cell cortex during anaphase (Fig. 5 A; unpublished data). This dynamic localization pattern was absent in *aust* spermatocytes, confirming the specificity of the anti-Aust antibody (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200708072/DC1).

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Thus, Aust function is required for the recruitment of other CPC components to the kinetochore during male meiosis.

**Aust is required during prometaphase I to maintain sister chromatid cohesion**

The defects observed in *aust* spermatids are the result of problems in chromosome segregation and cytokinesis (Fig. 1 and Table I). To analyze how these defects occur, we stained *aust* mutant testes preparations for DNA and α-tubulin and analyzed the first meiotic division (Fig. 7 A). Several distinct phenotypes in chromatin organization could be discerned. First, the number of chromosomal masses during prometaphase I appeared to vary between cells. Second, chromosomes often failed to align correctly on the metaphase plate. Third, chromosomes were often segregated inappropriately during anaphase. The defects in alignment and segregation can be explained by the well-characterized role of the CPC in rectifying inappropriate kinetochore–MT attachments (Hauf et al., 2003; Lens and Medema, 2003; Cimini et al., 2006; Knowlton et al., 2006) and by the lack of a robust checkpoint in *D. melanogaster* male meiosis, which allows spindles to proceed into anaphase in the presence of misattached chromosomes (Rebollo and Gonzalez, 2000).

However, it was not initially clear why *aust* spermatoocytes should contain abnormal masses of chromatin before metaphase I. To analyze how these defects occur, we stained *aust* mutant testes preparations for DNA and α-tubulin and analyzed the first meiotic division (Fig. 7 A). Several distinct phenotypes in chromatin organization could be discerned. First, the number of chromosomal masses during prometaphase I appeared to vary between cells. Second, chromosomes often failed to align correctly on the metaphase plate. Third, chromosomes were often segregated inappropriately during anaphase. The defects in alignment and segregation can be explained by the well-characterized role of the CPC in rectifying inappropriate kinetochore–MT attachments (Hauf et al., 2003; Lens and Medema, 2003; Cimini et al., 2006; Knowlton et al., 2006) and by the lack of a robust checkpoint in *D. melanogaster* male meiosis, which allows spindles to proceed into anaphase in the presence of misattached chromosomes (Rebollo and Gonzalez, 2000).
We also compared the localization of the centrosomal protein centrin to wild-type and aust mutant spermatocytes expressing β-tubulin–GFP. We did not observe any MT bundling or interdigitation, either at the cell cortex or internally. Instead, centrosome-nucleated MTs probe the cytosol and contact the cell cortex in the vicinity of the cell equator, where they begin bundling (Fig. 8 B and Videos 1–3, available at http://www.jcb.org/cgi/content/full/jcb.200708072/DC1). This is accompanied by an interdigitation of MTs in the center of the cell, leading to a well-organized central spindle. Furrow ingression then begins and the interzonal bundle of MTs becomes pinched in the middle (Fig. 8 B and Video 1; Inoue et al., 2004). In aust cells, although centrosome-nucleated MTs reached the cell equator during anaphase, we did not observe any MT bundling or interdigitation, either at the cell cortex or internally. Instead, centrosome-nucleated MTs continued to grow from opposite poles toward and past the center of the cell (Fig. 8 B and Videos 2 and 3).

This analysis suggests that Aust has an essential role very early in central spindle formation. The first known event in cleavage plane specification in D. melanogaster male meiosis is the accumulation of anillin to the equatorial cell cortex (Giansanti et al., 1999, 2004). We therefore investigated whether anillin was correctly localized in aust cells. Although anillin was readily visible as an equatorial ring in wild-type spermatocytes, it failed to localize in aust cells during anaphase or telophase (Fig. 9A).

**Figure 5. Localization of Aust in primary spermatocytes.** (A) Primary spermatocytes fixed and stained with antibodies to α-tubulin (green), Aust (red), and with DAPI (blue). Upon entry into meiosis I, Aust accumulates on condensing chromosomes, concentrating at specific chromosomal locations by metaphase. In anaphase, Aust remains associated with chromosomes but additionally accumulates at the cell equator. By telophase, Aust staining is apparent at the central spindle midzone. (B) Metaphase I spermatocytes stained for DmINCENP (green), Aust (red), and DNA (blue). DmINCENP and Aust colocalize at kinetochores. Bars, 10 μm.
or telophase in *aust* spermatocytes (Fig. 9 C; Fig. S3 B). Thus, Aust is essential for both central spindle formation and anillin accumulation at the equatorial cortex.

Aust can functionally replace Borr in *D. melanogaster* S2 cells

Given the similarity between Borr and Aust in their localization, their biochemical properties and their ability to recruit the CPC to kinetochores in their respective tissues, we decided to investigate whether the two proteins are functionally equivalent.

Overexpression of Borr using testis-specific promoters did not result in accumulation of Borr to specific subcellular structures during male meiosis and did not rescue the male sterility observed in *aust* flies (unpublished data). Therefore, to investigate the functional similarity between the two proteins, we asked whether Aust can replace Borr during mitosis. Borr localizes as a passenger in S2 cells (Fig. 10 A; Eggert et al., 2004), and RNAi against *borr* in S2 tissue culture cells has been shown to result in a robust binucleate phenotype (Eggert et al., 2004). Double-stranded RNA (dsRNA) from the untranslated regions (UTRs) of *borr* (see Materials and methods) caused a similar phenotype, including defects in mitotic spindle formation, chromosome alignment, chromosome segregation, and cytokinesis (Fig. 10 B). Staining with anti-Borr antibodies confirmed the loss of Borr protein in cells showing such phenotypes (Fig. 10 B). However, RNAi against Borr combined with the simultaneous introduction of *aust* transgenes (FLAG/Myc-*aust* or RFP-*aust*) rescued the phenotypes observed (Fig. 10, C and D). In addition, the protein products of both *aust* transgenes localized similarly to endogenous Borr (Fig. 10 D and not depicted).

We quantified this rescue by comparing the proportion of polyploid binucleate cells in cultures treated with either *borr* dsRNA or *borr* dsRNA in the presence of FLAG/Myc- or RFP-Borr with those treated with *borr* dsRNA in the presence of FLAG/Myc- or RFP-Aust (Fig. 10, E-G). RNAi against the UTR of *borr* resulted in ~80% polyploid binucleate cells after 5 d (Fig. 10, E-G). In contrast, the simultaneous addition of either FLAG/Myc- or RFP-Aust reduced this proportion to ~27%. This is similar to the proportion of polyploid binucleate cells seen in cultures cotransfected with FLAG/Myc- or RFP-Borr (23%; Fig. 10 E). Thus, Aust is functionally equivalent to Borr when expressed in S2 cells.

Discussion

In this study, we show that *D. melanogaster* has a second borealin-like gene, which we term *aust*, that is expressed and functions specifically during male meiosis. These proteins share identical biochemical properties. In addition, Aust is able to functionally replace Borr during mitosis in S2 tissue culture cells. Aust is required for sister chromatid cohesion during prometaphase and metaphase, chromosome alignment at metaphase, and chromosome segregation. Furthermore, loss of Aust leads to a complete absence of the central spindle and a concomitant failure of cytokinesis during male meiosis, which suggests that the CPC functions early on in anaphase to regulate the initial events leading to cytokinesis.

Similar results were obtained for actin (Fig. 9 B). We also investigated the accumulation of the MT-bundling protein Fascetto, the *D. melanogaster* homologue of PRC-1/Ase1 (Verni et al., 2004), and the kinesin-like protein PAV-KLP (Adams et al., 1998). Again, we found no accumulation of either protein during anaphase
conclusions about their ancestral lineages. The most that can be said from our analyses with any certainty is that the duplication that resulted in \textit{borr} and \textit{aust} was independent of the event that resulted in \textit{DASRA A} and \textit{DASRA B} in \textit{X. laevis} (unpublished data).

Strikingly, \textit{Aust} lacks \textit{/H11601} and yet is able to replace the longer \textit{Borr} protein in tissue culture cells, localizing as a passenger and rescuing the spindle organization, chromosome alignment and segregation, and central spindle and cytokinesis phenotypes present in the absence of \textit{Borr}. Whether this functional redundancy works reciprocally, however, remains unclear. We attempted to overexpress \textit{Borr} in \textit{aust} mutants using a testes-specific promoter but were not able to observe localization to any specific structures during meiosis or to successfully rescue the male sterility (unpublished data).

It is remains possible, therefore, that \textit{Borr} is unable to functionally compensate for a lack of \textit{Aust}. However, given that endogenous \textit{Borr} becomes suddenly undetectable at the onset of meiosis (Fig. 4 F), it is also possible that both the endogenous and transgenic \textit{Borr} proteins are degraded before meiosis. We are currently following alternative strategies to address the functional relationship between these two proteins.

\textit{Aust} does not contribute to male meiotic spindle formation but is required for chromosome alignment and segregation

Many studies on the CPC have relied on investigating partial loss of function through assessing either RNAi or weak allelic

\textit{D. melanogaster} possess two distinct borealin-like genes with similar functions

The human CPC is composed of four subunits: aurora B, INCENP, survivin, and Borealin (Vagnarelli and Earnshaw, 2004; Vader et al., 2006), and homologues of all four have previously been described in \textit{D. melanogaster} (Jones et al., 2000; Adams et al., 2001; Giet and Glover, 2001; Eggert et al., 2004). It was therefore initially surprising to find that \textit{D. melanogaster} possess a second borealin-like gene. \textit{Aust} has diverged from \textit{borr} to such an extent that initial BLAST searches using the human Borealin protein as bait failed to identify \textit{aust} as a homologue (Gassmann et al., 2004). However, the sequence similarity between \textit{borr} and \textit{aust} and their neighboring chromosomal locations strongly suggest that these genes are true paralogues, originating from a single ancestral gene. Our phylogenetic analysis shows the presence of \textit{Borr} and \textit{Aust} homologues in all \textit{Drosophila} species for which there are sequence data, which suggests that the duplication event occurred before the diversification of the \textit{Drosophilidae}. Interestingly, other species, including \textit{X. laevis} and \textit{Danio rerio}, have two borealin-like genes (Gassmann et al., 2004). In \textit{X. laevis}, the two paralogues, \textit{DASRA A} and \textit{DASRA B}, are both expressed in oocytes (Sampath et al., 2004), and it is tempting to speculate that, like the products of the two \textit{D. melanogaster} genes, one might function during meiosis and the other during mitosis. We attempted to carry out a thorough phylogenetic analysis of the Borealin proteins from many different species. However, because of the divergence of borealin-like genes, it is difficult to make any firm
induced MT nucleation has been observed in D. melanogaster male meiosis (Bonaccorsi et al., 1998; Rebollo et al., 2004), D. melanogaster spermatocytes can form functional bipolar spindles in the complete absence of chromosomes, relying only on centrosomally nucleated MTs (Bucciarelli et al., 2003). The limited role of chromatin-induced MTs during spermatocyte spindle assembly therefore offers an explanation as to why, in the absence of Aust, spindle formation proceeds normally.

Despite the normal spindle morphology of aust spermatoocytes, chromosome alignment on the metaphase plate is severely compromised. Given the known role of the CPC in monitoring and correcting erroneous MT–kinetochore attachments, this is to be expected. Additionally, however, it appears that sister chromatid cohesion is perturbed. Analysis of chromosome morphology in aust spermatocytes during prometaphase I clearly showed the presence of single chromatids, whereas the number of CID-positive spots during metaphase I often exceeded the expected

Figure 8. Time-lapse imaging of MT organization in aust spermatocytes. Testes from wild-type and aust mutants expressing α-tubulin–GFP were dissected and spermatocytes undergoing meiosis I were imaged every 60 s. (A) Stills of metaphase/early anaphase spindles. aust cells show normal meiotic spindle morphology. (B) Selected frames from cells undergoing anaphase and telophase. In wild-type cells (see Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200708072/DC1), a robust central spindle is formed early in anaphase and constricts as cells progress through telophase. In aust mutants (see Videos 2 and 3), spindles elongate during anaphase but no central spindle is formed. Bars, 10 μm.

Figure 9. Localization of cytokinesis proteins in aust spermatocytes. Wild-type and aust mutant spermatocytes were fixed and stained for α-tubulin (green), DNA (blue), and either anillin (A), actin (B), or Fascetto (C, red). Although each protein localizes to the cleavage furrow in wild-type cells, anillin, actin, and Fascetto are not recruited in aust mutants. Bar, 10 μm.

phenotypes (Adams et al., 2001; Chen et al., 2003; Lens et al., 2006; Resnick et al., 2006). As Aust is required only for male meiosis, we were able to use the potentially null aust1 allele to assess the direct effects of fully inactivating the Borealin-dependent functions of the CPC. We found that in the absence of Aust, the recruitment of centrosomal components to meiotic spindle poles is normal and spindle formation is unaffected. This appears to be at odds with previous work that has clearly shown that X. laevis DASRA, and the CPC in general, is required for chromatin-induced MT stabilization and spindle formation (Sampath et al., 2004; Kelly et al., 2007). However, this observed difference is probably caused by the way in which the meiotic spindles in X. laevis oocytes and D. melanogaster spermatocytes are built. In the X. laevis in vitro system, robust bipolar spindles form around condensing chromatin in the absence of centrosomes in both Ran-dependent and -independent pathways (Sampath et al., 2004; Kelly et al., 2007). In contrast, although chromatin-induced MT nucleation has been observed in D. melanogaster male meiosis (Bonaccorsi et al., 1998; Rebollo et al., 2004), D. melanogaster spermatocytes can form functional bipolar spindles in the complete absence of chromosomes, relying only on centrosomally nucleated MTs (Bucciarelli et al., 2003). The limited role of chromatin-induced MTs during spermatocyte spindle assembly therefore offers an explanation as to why, in the absence of Aust, spindle formation proceeds normally.

Despite the normal spindle morphology of aust spermatoocytes, chromosome alignment on the metaphase plate is severely compromised. Given the known role of the CPC in monitoring and correcting erroneous MT–kinetochore attachments, this is to be expected. Additionally, however, it appears that sister chromatid cohesion is perturbed. Analysis of chromosome morphology in aust spermatocytes during prometaphase I clearly showed the presence of single chromatids, whereas the number of CID-positive spots during metaphase I often exceeded the expected
In contrast, our live analysis of MT dynamics in *aust* spermato- cytes has conclusively demonstrated that the central spindle does not form. The inability of *aust* cells to accumulate anillin in a tight band at the equatorial cortex provides strong evidence that *Aust* and the *D. melanogaster* CPC are essential in the very early steps of meiotic cytokinesis. This is in agreement with a recent study in yeast that showed the localization of Boi-1 and Boi-2, the yeast homologues of anillin, to the cleavage site is dependent on Ipl-1, the yeast homologue of aurora B (Norden et al., 2006). Previous studies in *D. melanogaster* have shown that anillin accumulates at the cell equator in anaphase before actomyosin ring assembly (Giansanti et al., 1999). The analysis of 25 mutants defective in spermatocyte cytokinesis revealed that anillin localization is affected only by mutations in the *pebble* and *diaphanous* genes, which encode a Rho GTP exchange factor and a Formin homology protein, respectively (Giansanti et al., 2001, 2004; unpublished data). Thus, anillin accumulation at the equatorial cortex is one of the earliest events of spermatocyte cytokinesis.

Studies in *D. melanogaster* and mammalian cells have shown that anillin interacts with actin, nonmuscle myosin II, and the septins, and it has been suggested that anillin may mediate the interactions between the contractile ring and the equatorial membrane (Field and Alberts, 1995; Somma et al., 2002; Echard et al., 2004; Field et al., 2005; Straight et al., 2005). We have found that *Aust* localizes to both the equatorial membrane and eight, which suggests precocious sister kinetochore separation (Fig. 6 A). Although DmINCENP has been shown to be required to protect sister chromatid cohesion at the kinetochores through regulating the accumulation of Mei-S332 (Resnick et al., 2006), this cannot account for the presence of individual chromatids earlier in meiosis. Mei-S332 is required only from the onset of anaphase I, and null mei-S332 mutants do not show precocious separation of sisters before this point (Lee et al., 2005). Thus, *Aust*, and probably the CPC, must affect chromosome cohesion not only through Mei-S332 but also via an as yet undefined, Mei-S332–independent, mechanism.

**Aust is absolutely required for central spindle formation**

The existence of a compromised spindle assembly checkpoint during *D. melanogaster* male meiosis (Rebollo and Gonzalez, 2000) allowed us to perform a detailed analysis of central spindle formation and cytokinesis in *aust* mutants. RNAi experiments for *D. melanogaster* aurora B and DmINCENP and analysis of male meiosis in a *dmINCENP* hypomorphic allele have shown that reduced levels of the CPC result in a lower density of central spindle MTs and defects in cytokinesis in a proportion of cells (Adams et al., 2001; Giet and Glover, 2001; Resnick et al., 2006). Consistent with these results, RNAi studies in tissue culture cells and phenotypic analysis of *barr* mutants have shown that Borr is required for cytokinesis (Eggett et al., 2004; Hanson et al., 2005). In contrast, our live analysis of MT dynamics in *aust* spermatocytes has conclusively demonstrated that the central spindle does not form.

The inability of *aust* cells to accumulate anillin in a tight band at the equatorial cortex provides strong evidence that *Aust* and the *D. melanogaster* CPC are essential in the very early steps of meiotic cytokinesis. This is in agreement with a recent study in yeast that showed the localization of Boi-1 and Boi-2, the yeast homologues of anillin, to the cleavage site is dependent on Ipl-1, the yeast homologue of aurora B (Norden et al., 2006). Previous studies in *D. melanogaster* have shown that anillin accumulates at the cell equator in anaphase before actomyosin ring assembly (Giansanti et al., 1999). The analysis of 25 mutants defective in spermatocyte cytokinesis revealed that anillin localization is affected only by mutations in the *pebble* and *diaphanous* genes, which encode a Rho GTP exchange factor and a Formin homology protein, respectively (Giansanti et al., 2001, 2004; unpublished data). Thus, anillin accumulation at the equatorial cortex is one of the earliest events of spermatocyte cytokinesis. Studies in *D. melanogaster* and mammalian cells have shown that anillin interacts with actin, nonmuscle myosin II, and the septins, and it has been suggested that anillin may mediate the interactions between the contractile ring and the equatorial membrane (Field and Alberts, 1995; Somma et al., 2002; Echard et al., 2004; Field et al., 2005; Straight et al., 2005). We have found that *Aust* localizes to both the equatorial membrane and
the central spindle MTs. A similar localization has been observed for the Pav component of the centralspindlin complex (Minestrini et al., 2003). In addition, we observed that the Pav kinesin fails to localize at the equatorial membrane in aust mutants. Together, our observations suggest that Aust acts very early during spermatocyte cytokinesis, mediating both anillin and centralspindlin recruitment at the cleavage site. This would prevent bundling of the spindle MTs that contact the equatorial cortex and inhibit initiation of both central spindle and contractile ring assembly.

Why have a CPC specifically for male meiosis?

Together, our data point toward the presence in D. melanogaster of a male meiosis–specific CPC, where specificity is provided by the Borealin-like Aust subunit. Interestingly, it appears that other organisms have also evolved a male meiosis–specific CPC. A distinct mammalian aurora kinase, aurora C, is expressed only during spermatogenesis (Dieterich et al., 2007; Kimmins et al., 2007). Both knockout mice and humans carrying a single nucleotide deletion in the aurora C gene display polyploid spermatid nuclei and compromised fertility. Importantly, the infertile individuals exhibit a normal somatic karyotype, which suggests that male meiosis and not mitosis is specifically perturbed (Dieterich et al., 2007). Thus, the presence of a male meiotic CPC may be a general feature of higher eukaryotes.

If we accede that Aust is a component of a male meiotic CPC, it poses the question, why have a CPC specifically for male meiosis in D. melanogaster? As Borr (and not Aust) is expressed during female meiosis, it seems likely that the Aust-containing CPC is not required for processes common to male and female meiosis such as cosegregation of sister kinetochores and preservation of sister chromatid cohesion during meiosis I. The Aust-containing CPC is likely therefore to be involved in a process that specifically characterizes male meiosis. Although in female meiosis, homologous chromosomes recombine and are held together by the synaptonemal complex and chiasmata, this is not the case in male meiosis (McKim, 2005). In D. melanogaster spermatocytes, homologous chromosome pairing is instead mediated by specialized proteins, including SNM, MNM, and TEF (Tomkiel et al., 2001; Thomas et al., 2005). If the CPC were required for recruitment or maintenance of these proteins to specific chromosomal locations, this could provide a reason for the presence of a specialized CPC for male meiosis. Although our preliminary results have shown that SNM is recruited and maintained at the chromosomal sites with normal dynamics in aust mutants (unpublished data), it is possible that Aust and the meiotic CPC regulate the function of other pairing proteins.

One further difference between male meiosis and either female meiosis or other cell divisions that occur in D. melanogaster is the sheer size and robustness of the central spindle. D. melanogaster primary spermatocytes are much larger than normal somatic cells and have a comparably larger central spindle that is essential for cytokinesis (Cenci et al., 1994; Giansanti et al., 2001). If Aust were to contribute to the formation and stabilization of the central spindle with much greater efficiency than Borr, e.g., by interacting with other specialized proteins, it may explain why Borr is replaced by Aust upon entry into meiosis. However, we presently have no evidence that this is the case. The reason for the existence of two developmentally distinct Borealin-like proteins in D. melanogaster therefore remains a matter for further study.

Materials and methods

Fly strains and isolation of aust mutations

The aust1 mutation was identified by a cytological screen of the Zuer’s collection of male sterile mutants (Wakimoto et al., 2004). The aust2 mutation was isolated from the same collection by complementation analysis. The second chromosome lines of the Zuer’s collection carry a maternally derived chromosome marked with cm and bw balanced over CyO; CySc2. The aust1 mutation was mapped by recombination using multiply marked chromosomes with al dp br c px sp and al dp br Bl c px sp and tested for failure to complement the deletions from the chromosome 2 deficiency kit (provided by the Bloomington Stock Center). Two nonoverlapping deficiencies resulted in male sterility in combination with aust1, and aust2 (Fig. S1). Analysis of aust1/Df(2L)B75C and aust2/Df(2L)B75C uncovered aust1, the other led to male sterility because of a spermatid differentiation phenotype that did not affect meiosis. We conclude that there are two independent male sterile mutations on the original aust1 chromosome and that the aust1 locus is located within region uncovered by deficiency Df(2L)30A-C region.

The flies expressing Bubulin-EGFP were kindly provided by M. Savoini and D. Glover (University of Cambridge, Cambridge, UK; Inoue et al., 2004). All flies were reared according to standard procedures and maintained at 25°C.

Cloning of the aust locus

To further define the region containing the aust1 locus, we obtained a group of three smaller deficiencies overlapping Df(2L)30A-C, Df(2L)N22-5, and Df(2L)B75C (gift from D. Kalderon, Columbia University, New York, NY). Although both Df(2L)N22-5 and Df(2L)162B75C uncovered the sterility and meiotic phenotype, Df(2L)162B75C complemented the aust1 mutation (Fig. S1). We were able to define the distal breakpoints of Df(2L)B75C and Df(2L)30A-C using complementation analysis to known lethal mutations in the region. This showed the breakpoint of Df(2L)30A-C to be between two genes, tao and cks (cks mutants were a gift from A. Swan, University of Windsor, Ontario, Canada). We then molecularly defined the distal breakpoint of Df(2L)162B75C using single embryo PCR (Fig. S1). Genomic DNA was extracted from 20 individual embryos and screened for PCR for the presence or absence of CG3759 identified by mutations that failed to complement Df(2L)162B75C, a control gene from a different location in the genome. The extracted DNA from embryos in which these genes were absent (i.e., homologous Df(2L)162B75C embryos) was subjected to further PCR using primers specific for other genes in the region between CG3759 and tao. This localized the breakpoint to between CG13110 and CG13111, a region containing eight putative genes (Fig. S1). Of these, two (cks and Gr50A) were identified by mutant alleles that complemented the aust1 locus. Comparative RT-PCR of the remaining six loci from both wild-type (siblings stocks from other chromosome 2 mutants of the Zuer’s collection) and aust1 cDNA identified a single locus, CG17009, in which expression levels were altered (see Results).

Phylogenetic analysis of aust and borr homologues

The phylogenetic analysis of aust and borr from different Drosophila species was generated using MEGA 3 (Kumar et al., 2004). The minimum evolution method was used with the modified Nei-Gojobori method with Jukes-Cantor correction and a transition/transversion ratio (Zhang et al., 2001). The full-length cDNA of CG17009 (aust) was amplified by PCR using primers that include BamHI and HindIII restriction sites. The MBP-tagged proteins were purified as described previously (Gergely et al., 2000). To express genes in mammalian cell culture, full-length INCENP and aurora B were amplified and cloned into pGEM-T vector and fully sequenced before subcloning into either HA-tagged pCDF3 or FLAG-tagged pCDF3 using Ndel and NheI restriction sites. The expression of transgenes in mammalian cells was performed as described previously (Perez-Quintero et al., 2004).
In vitro pull-down and DNA-binding assays
HEK293T cells expressing HA-dBorr, HA-Aust, HA-dmINCENP, or FLAG-aust were lysed in lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, and protease inhibitors [Roche]) before overnight incubation at 4°C with either 10 μg of MBP, 5 μg of MBP-Borr, or MBP-Aust immobilized on amylase beads. Beads were washed three times in the lysis buffer and boiled in 30 μl of SDS–PAGE buffer (Bio-Rad Laboratories) for 10 min. Proteins were analyzed by Western blotting using anti-HA and anti-FLAG antibodies (Sigma-Aldrich). The in vitro DNA-binding assay was performed using DNA–cellulose beads (Sigma-Aldrich) as described previously (Klein et al., 2006).

RNAi and rescue experiments
Tagged Borr and Aust rescue constructs were made using Gateway technology (Invitrogen). Full-length Borr and Aust coding regions were inserted pENTR-D/Topo and recombined into pAWR (RFP) and pAFMW (6x FLAG/3x Myc) vectors to be expressed in S2 cells under the control of the actin promoter. Regions of the 5′ and 3′ UTR of borr were amplified using the following primers: forward, 5′-TCATTCCTCTCTCTATAGCCGGACACCTGGACGCGCTTGTATTTGAGCCTGTTAATATTCCG-3′; reverse, 5′-TCATTCCTCTCTCTCTGAGAGGAGGATCCGGGACACCTGGACGCGCTTGTATTTGAGCCTGTTAATATTCCG-3′. Cells were incubated with the transfection mix for 1 h at 25°C before the addition of FBS to a final concentration of 10% and left for 5 days before fixation and staining (Invitrogen) according to the manufacturer’s instructions. Cells were washed twice using serum-free medium before addition of 10 μM of pAFMW-Aust and pAFMW-Borr. Cells were then frozen in liquid nitrogen and, after removal of the coverslip, imaged. Four different high-resolution images of MTs and DNA in cells treated with dsRNA plus pAFMW-Borr cells were created using Metamorph software and show each fluorescent image on May 24, 2017 Downloaded from

- **Temperature, and mildly squashed.** Slides were then frozen in liquid nitrogen and, after removal of the coverslip, imaged in 0.1% PBST and 3% BSA for 30 min before incubation with the appropriate primary and secondary antibodies. The following antibodies were used: mouse anti-a-tubulin (DM1A clone, Sigma-Aldrich) at 1:500, rat anti-a-tubulin (Jackson ImmunoResearch Laboratories) at 1:500, mouse anti-a-tubulin (Sigma-Aldrich) at 1:500, purified rabbit anti-Bor-Arr at 1:100, purified mouse anti-Aust at 1:10, rabbit anti-dmINCENP (gift of M. Carmena, University of Edinburgh, UK) at 1:250, rabbit anti-a-tubulin B (gift of D. Glover, University of Cambridge) at 1:200, chicken anti-CID (gift of G. Karpen, The University of California, Berkeley, CA) at 1:250, rabbit anti-Asp (gift of J. Raff, University of Cambridge) at 1:500, rabbit anti-anillin (gift from C. Field, Harvard University, Cambridge, MA), and rabbit anti-Fascetto (Verni et al., 2004). Appropriate Alexa 488, Cy3, and Cy5 secondary antibodies were obtained from Invitrogen or Jackson ImmunoResearch Laboratories. DNA was visualized with 0.5 μg/ml DAPI.

Preparations were examined under oil at 25°C with a microscope (Eclipse TE2000-U; Nikon) with a Plan APO VC 60× 1.4 NA objective (Nikon) and a 1.5x zoom integrated zoom using a camera (c8484-056; Hamamatsu). Pictures were captured using IPLab software (BD Biosciences), converted to TIFF files, pseudocolored, and merged in Photoshop CS2 (Adobe). Levels of individual channels were adjusted where applicable to maximize pixel intensity.

Online supplemental material
Fig. S1 details the strategy used to identify the genomic location of the aust locus. Fig. S2 shows the staining of the anti-Aust antibody in aust mutant primary spermatocytes. Fig. S3 shows the localization of a-tubulin, Asp, and pav kinesin in wild-type and aust mutant spermatocytes. Fig. S4 shows high resolution images of MTs and DNA in cells treated with bor dRNA and those treated simultaneously with bor dRNA and pAFMW-aust. Video 1 shows wild-type spermatocytes expressing a-tubulin–GFP undergoing anaphase and telophase of meiosis I. Videos 2 and 3 show aust spermatocytes expressing a-tubulin–GFP undergoing anaphase and telophase of meiosis I. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200708072/D1.

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