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BRC-1 acts in the inter-sister pathway of meiotic double-strand break repair

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The breast and ovarian cancer susceptibility protein BRCA1 is evolutionarily conserved and functions in DNA double-strand break (DSB) repair through homologous recombination, but its role in meiosis is poorly understood. By using genetic analysis, we investigated the role of the *Caenorhabditis elegans* BRCA1 orthologue (*brc-1*) during meiotic prophase. The null mutant in the *brc-1* gene is viable, fertile and shows the wild-type complement of six bivalents in most diakinetically nuclei, which is indicative of successful crossover recombination. However, *brc-1* mutants show an abnormal increase in apoptosis and RAD-51 foci at pachytene that are abolished by loss of *spo-11* function, suggesting a defect in meiosis rather than during premeiotic DNA replication. In genetic backgrounds in which chiasma formation is abrogated, such as *him-14/MSH4* and *syp-2*, loss of *brc-1* leads to chromosome fragmentation suggesting that *brc-1* is dispensable for crossing over but essential for DSB repair through inter-sister recombination.

Keywords: *Caenorhabditis elegans*; meiosis; DNA repair

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INTRODUCTION

DNA interruptions, including double-strand breaks (DSBs), are caused by endogenous (DNA synthesis, recombination) and exogenous (genotoxic agents) factors. In all organisms, imprecise repair of such discontinuities leads to an assortment of genetic alterations, such as point mutations, deletions and rearrangements. There are two main DSB repair pathways—homologous recombination (HR) and non-homologous end joining (NHEJ)

(West, 2003; Wyman & Kanaar, 2006). Homologous recombination is an accurate repair pathway that uses an intact sister/homologue as a template, whereas NHEJ involves direct religation of the DSB and is prone to errors. Failure to repair DSBs accurately is associated with several cancer-predisposition syndromes, such as Fanconi anaemia (FANCD2), Bloom syndrome (BLM) or breast and ovarian cancer susceptibility (BRCA1, BRCA2/FANCD1), which are affected for DSB repair by homologous recombination (Venkitaraman, 2002; D'Andrea, 2003; Wu & Hickson, 2003; Mankouri & Hickson, 2004). As many of the genes in the homologous recombination pathway, including breast and ovarian cancer susceptibility genes *BRCA1* and *BRCA2/FANCD1* (Martin *et al*, 2005; Petalcorin *et al*, 2006), have been conserved during metazoan evolution, their roles and interactions can be investigated in model systems that are suited for the study of DSB repair and meiosis.

The U-shaped gonad of *Caenorhabditis elegans* contains germ cells, the physical location of which correlates with their stage in meiotic progression, which facilitates the study of the effect of gene depletions at various stages of meiotic prophase (for reviews, see Couteau *et al*, 2004; Colaiacovo, 2006; Garcia-Muse & Boulton, 2007). During meiotic prophase, DSBs are induced by the conserved meiotic protein SPO-11 (Keeney *et al*, 1997; Dernburg *et al*, 1998). The repair of at least one meiotic DSB to generate a crossover between each pair of homologous chromosomes is essential for accurate chromosome segregation at the first meiotic division (Brenner, 1974; Hawley, 1988; Hillers & Villeneuve, 2003). Failure to generate the obligate crossover results in chromosome non-disjunction and aneuploidy in the next generation. Mutants affecting homologous recombination repair of meiotic DSBs show increased embryonic lethality owing to aneuploidy, high incidence of males owing to X-chromosome non-disjunction, anomalies in the number and shape of chromosomes at diakinesis, and altered levels and distribution of RAD-51 foci at the pachytene stage.

A battery of mitotically silent genes, including *him-14/MSH4*, *msh-5*, *syp-1*, *syp-2* and *him-3*, have been described in *C. elegans* that are involved in directing recombination between homologous chromosomes during meiosis (Zalevsky *et al*, 1999; Zetka *et al*, 1999; Kelly *et al*, 2000; MacQueen *et al*, 2002; Colaiacovo *et al*,

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2003). Null mutants in these genes show 12 well-structured univalents at diakinesis instead of the 6 bivalents in wild-type (representing homologous chromosomes held together by chiasmata). In these mutants, meiotic DSBs are believed to be repaired using non-crossover pathways, but the factors that promote this type of homologous recombination repair are not known (Colaiacovo *et al*, 2003).

Studies in mammalian cells have established a role for BRCA1 and its heterodimeric partner BARD1 in DSB repair by homologous recombination (Scully *et al*, 1997; Moynahan *et al*, 1999). A role for BRCA1 in meiosis is also suggested from knockout mice that are infertile as a result of pachytene arrest (Xu *et al*, 2003). Recent studies have shown that BRCA1 has a specialized meiotic role in the XY body that is maintained in a transcriptionally silent state through meiotic sex chromosome inactivation (MSCI; Turner *et al*, 2004). BRCA1 is required for the localization of ATR and subsequent phosphorylation of H2AX at the XY body, which is important for the establishment of MSCI (Turner *et al*, 2004). It is clear that BRCA1 functions in both mitotic and meiotic cells, but its contribution to meiotic DSB repair is less well understood. Here, we investigate the contribution of BRCA1 of *C. elegans* (*brc-1*) during meiotic recombination by exploiting specific aspects of *C. elegans* meiosis: meiotic DSBs induced by SPO-11 are not a prerequisite for the assembly of the synaptonemal complex (Dernburg *et al*, 1998), and axis morphogenesis and synaptonemal complex formation are not required for loading RAD-51 onto DSBs (Colaiacovo *et al*, 2003). Our data indicate that BRC-1 contributes to meiotic DSB repair by a non-crossover pathway.

RESULTS

Apoptosis and RAD-51 foci increase in the *brc-1* mutant

Previous studies have shown that the *brc-1* mutant is viable and fertile, but shows a weak meiotic phenotype: the incidence of males (Him phenotype), reflecting the frequency of X-chromosome non-disjunction in *C. elegans* meiosis, is elevated in the *brc-1* mutant (~2% versus ~0.1% in wild type; Boulton *et al*, 2004). However, the levels of embryonic lethality (0.38%) are not significantly increased in the absence of *brc-1*, indicating that the segregation of autosomes during meiosis occurs as normal. Most of the diakinetic nuclei in *brc-1* mutants show the wild-type complement of six 4,6-diamidino-2-phenylindole (DAPI)-stained bivalents at diakinesis, representing homologous chromosomes joined by chiasmata (Fig 1). However, approximately 12% of oocyte nuclei show seven DAPI-stained bodies, which might account for the Him phenotype. Thus, *brc-1* is largely dispensable for crossover recombination on the autosomes.

A possible meiotic defect is also suggested by findings that *brc-1* mutants show elevated basal levels of apoptosis of pachytene nuclei (Boulton *et al*, 2004). To determine whether this apoptotic phenotype is dependent on meiotic DSB formation, we generated a *brc-1;spo-11* double mutant. Eliminating meiotic DSB formation with the *spo-11* mutation (Dernburg *et al*, 1998) suppressed the apoptotic phenotype of *brc-1* mutants (Table 1). To examine the consequence of eliminating apoptosis in the absence of *brc-1*, we combined *brc-1* with a mutation in *ced-3*, which is essential for apoptosis (Yuan *et al*, 1993). A fourfold elevation in embryonic lethality was observed in the *brc-1;ced-3* double mutant when compared with the single mutants alone (Table 1), suggesting that in the absence of BRC-1, apoptosis is required to eliminate

compromised meiotic cells that are incompatible with producing viable offspring.

To analyse further a potential meiotic DSB repair defect in *brc-1* mutants, we examined RAD-51 foci, which mark homologous recombination events (Alpi *et al*, 2003; Colaiacovo *et al*, 2003). The loading of RAD-51 at meiotic DSBs in the early stages of meiotic prophase occurs as normal in *brc-1* mutants. However, *brc-1* mutants show abnormally high levels of RAD-51 foci in early pachytene nuclei, a phenotype that is suppressed by the *spo-11* mutation (Fig 2). A similar elevation in RAD-51 foci is also seen in the meiotic nuclei of *brd-1* mutants, which lack the heterodimeric partner protein of BRC-1 (Fig 2; Boulton *et al*, 2004; Polanowska *et al*, 2006). These data indicate a role for *brc-1* in the efficient repair of a subset of SPO-11-dependent meiotic DSBs.

BRC-1 acts in DSB repair in the absence of crossovers

In *C. elegans*, one crossover per homologue pair is necessary and sufficient to generate interhomologue connections irrespective of the length of the chromosome (Hawley, 1988; Edgley & Riddle, 1993; Hillers & Villeneuve, 2003), therefore, it follows that additional DSBs generated by SPO-11 must be repaired by non-crossover pathways. The bias towards the homologue as a homologous recombination template is regulated by pro-crossover factors such as the HIM-14/MSH4–MSH-5 complex and the synaptonemal complex. Mutants in either of these complexes are achiasmata and show 12 intact univalents at diakinesis (Zalevsky *et al*, 1999; MacQueen *et al*, 2002). To analyse the potential role of *brc-1* in non-crossover DSB repair pathways, we examined the effects of combining the *brc-1* mutant with (i) the *him-14/MSH4* mutant, in which the synaptonemal complex is present, but crossing over is abolished, and (ii) the *syp-2* mutant, in which both synaptonemal complex formation and crossing over are absent (Fig 1). Combining the *brc-1* mutation with either *him-14/MSH4* or *syp-2* mutants resulted in a statistically significant increase in DAPI-stained bodies at diakinesis, which is indicative of chromosome fragmentation (Fig 1; supplementary Table A online). Unlike the *brc-1;him-14/MSH4* double mutant, approximately 25% of the observed *brc-1;syp-2* nuclei at diakinesis resolve in misshapen, poorly condensed chromatin (Fig 1). This phenotype is similar to that observed in *brc-2*- and *rad-51*-deficient strains, which are compromised for repair of all meiotic DSBs through the homologous recombination pathway (Rinaldo *et al*, 2002). These data indicate that in the absence of crossing over, BRC-1 contributes to meiotic DSB repair by using the sister chromatid as a template.

It is possible that the fragmentation observed in *brc-1;him-14/msh-4* and *brc-1;syp-2* double mutants might result from premature loss of chromosome cohesion or from a DSB repair defect. To distinguish between these two possibilities, *brc-1;him-14/MSH4* and *brc-1;syp-2* diakinetic nuclei were immunostained for the meiosis-specific cohesin REC-8 (Pasierbek *et al*, 2001). In both double mutants, DAPI-stained bodies retained REC-8, indicating that chromosome fragmentation arises as a result of a repair defect rather than the premature loss of cohesion (supplementary Fig A online).

If a non-crossover pathway of homologous recombination is impaired in the absence of *brc-1*, this defect might be compensated by an elevation in the frequency of crossovers. Therefore, we assayed the frequency of recombination in the *brc-1* mutant in two different chromosomal intervals: *dpy-5 unc-29* on

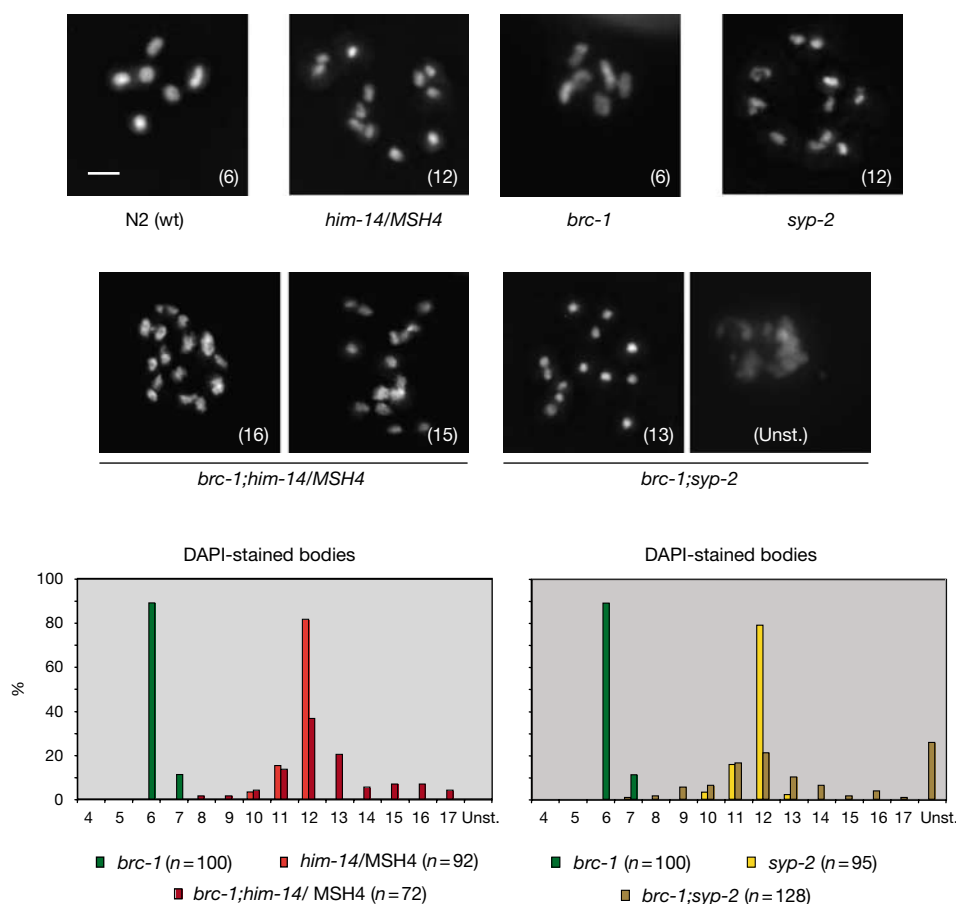


Fig 1 | Increased number of DAPI-stained structures at diakinesis in *brc-1;him-14/MSH4* and in *brc-1;syp-2* double mutants. Representative images of DAPI-stained oocyte nuclei at diakinesis in the indicated genotypes double mutants. The number in parenthesis within each image indicates the number of DAPI-stained bodies that were detectable through the z stack of the nucleus. Histograms represent quantification of the DAPI-stained bodies. The number (*n*) of observed nuclei is indicated next to each genotype. The y axis represents the percentage of nuclei in each class and the x axis indicates the number of DAPI-stained bodies. The difference in DAPI-stained bodies between *brc-1;him-14/MSH4* and *him-14/MSH4*, and between *brc-1;syp-2* and *syp-2* are statistically significant, (see supplementary Table A online). Scale bar, 2 μ m. DAPI, 4,6-diamidino-2-phenylindole; Unst, nuclei with unstructured chromatin; wt, wild type.

chromosome I, which spans a region with a low level of recombination (about 1 cM/Mb), and *unc-60 dpy-11* on chromosome V, where the frequency of recombination (3.7 cM/Mb). In both intervals tested, the frequency of recombination (3.52% and 19.64%, respectively) was comparable with that observed in the wild type and with the map units reported previously (Edgley & Riddle, 1993). Therefore, *brc-1* does not significantly influence either recombination frequency or crossover distribution.

DSBs can be repaired by homologous recombination or by NHEJ. NHEJ is generally believed to act in somatic cells before S phase, but might contribute to meiotic DSB repair when homologous recombination is compromised. To assess the latter, we compared the meiotic outcome of *him-14/MSH4;lig-4* (*C. elegans* DNA ligase-4; Martin et al, 2005) and *brc-1;him-14/MSH4* double mutants. In contrast to *brc-1;him-14/MSH4*, chromosome fragmentation was not observed in *him-14/MSH4;lig-4* double mutants. Rather, the diakinetin nuclei in *him-14/MSH4;lig-4* showed a pattern similar to that of *him-14/MSH4*

single mutants (supplementary Fig B online). These data indicate that *brc-1* and *lig-4* function in different DSB repair pathways. Furthermore, NHEJ has little or no role in meiotic DSB repair in wild-type *C. elegans*.

Collectively, our results are consistent with a role for BRC-1 in a non-crossover homologous recombination pathway of meiotic DSB repair through inter-sister recombination.

DISCUSSION

Recent evidence indicates that germ cells switch between specific DSB repair pathways depending on their temporal and spatial position in the *C. elegans* germ line (Hayashi et al, 2007). In the case of meiotic DSB repair, a subset of DSBs per nucleus are selected for repair through crossover recombination, which ultimately produce chiasmata between homologous chromosomes. As the number of SPO-11-induced DSBs created at the onset of meiotic prophase exceed the number of final crossovers, these extra breaks must be repaired using alternative pathways. It is assumed that in the presence of an intact synaptonemal

Table 1 | Germline apoptosis and embryonic lethality in the *brc-1* mutant

	Apoptosis			Lethality	
	No. of gonad arms analysed	Apoptotic nuclei	Average apoptotic nuclei/arm	No. of embryos scored	Embryonic lethality (%)
Wild type	85	337	3.96	1,977	<0.05
<i>brc-1</i> *	73	563	7.71	1,048	0.38
<i>spo-11</i>	82	267	3.26	ND	ND [†]
<i>brc-1;spo-11</i>	65	238	3.66	ND	ND [†]
<i>cep-1/p53</i>	89	273	3.07	1,032	0.19
<i>brc-1;cep-1/p53</i>	94	337	3.58	1,339	0.22
<i>ced-3</i>	64	6	0.09	1,240	1.94
<i>brc-1;ced-3</i>	83	7	0.08	1,369	9.72

**brc-1* is statistically different from wild type, *brc-1;spo-11* and *brc-1;cep-1/p53* (Student's *t*-test; *P*-value <0.0001). [†]*spo-11* and *brc-1;spo-11* embryonic lethality has not been scored as is more than 99% due to progeny aneuploidy. ND, not determined.

complex and the HIM-14/MSH4-MSH5 complex, excess DSBs are repaired as inter-homologue non-crossovers. Evidence suggests that the bias towards inter-homologue repair is released before the end of pachytene, to ensure that any remaining DSBs are repaired before the first meiotic division. This is based on the observation that mutants that lack the synaptonemal complex, for example, *syp-2* mutants, show persistent meiotic DSBs that are ultimately repaired late in meiotic prophase in a *rad-51*- and *rec-8*-dependent manner, giving rising to 12 intact univalents (Colaiacono et al, 2003).

The data presented here support a role for BRC-1 in meiotic DSB repair through inter-sister homologous recombination. This is suggested by the fact that BRC-1 is dispensable for crossover formation, but *brc-1* mutants show abnormally high levels of apoptosis and meiotic RAD-51 foci, which are both SPO-11 dependent. In the absence of the synaptonemal complex (*syp-2* mutant) where inter-homologue recombination is eliminated, repair of meiotic DSBs can only proceed by inter-sister recombination. The observation that *syp-2* mutants show 12 intact univalents, yet *brc-1; syp-2* double mutants show a large number of diakinetid nuclei with uncondensed, misshapen chromatin, similar to that previously observed in the HR-deficient mutants *rad-51* and *brc-2* (Chin & Villeneuve, 2001; Rinaldo et al, 2002; Colaiacono et al, 2003; Martin et al, 2005), strongly suggests that BRC-1 is required for meiotic DSB repair by inter-sister recombination. Given the subtle meiotic phenotype of the *brc-1* single mutant, our data suggest that once the single obligate crossover per homologue pair is generated in *C. elegans*, most remaining meiotic DSBs are repaired by inter-homologue non-crossover recombination, rather than by inter-sister recombination. A specific role for BRC-1 in DSB repair using the sister chromatid as a template is consistent with the known function of BRCA1 in mitotic cells, where the synaptonemal complex is absent and crossover-promoting genes are not expressed.

Similar to its human counterpart, BRC-1 is recruited to sites of DSBs (Scully et al, 1997; Polanowska et al, 2006), but how BRC-1/BRCA1 promotes DSB repair in mitotic or meiotic cells remains unclear. BRCA1 deficiency in both *C. elegans* and humans is

associated with a DSB repair defect (Moynahan et al, 1999; Bhattacharyya et al, 2000; Boulton et al, 2004). However, one important difference is that BRC-1 is dispensable for RAD-51 recruitment to sites of DSBs (Polanowska et al, 2006). This difference, coupled with our present finding that BRC-1 is required for inter-sister recombination, raises the possibility that BRCA1 might have additional functions in DSB repair independent of its role in recruiting Rad51 to DSBs. As BRC-1/BRCA1 have E3-ubiquitin ligase activity, which is stimulated by DSBs, identification of substrates for this activity might provide important insights into the control of DSB repair (Morris & Solomon, 2004; Polanowska et al, 2006). So far, CtIP and H2AX are the only definitive BRCA1 substrates identified in mitotic cells, whereas substrates in meiotic cells have yet to be found (Yu et al, 2006; Zhao et al, 2007). Recent studies have also revealed connections between BRCA1 and the Fanconi anaemia proteins during the DNA-damage response (Wang, 2007). It will be important to assess possible interactions between BRC-1 and CeFANCD2 during *C. elegans* meiosis (Collis et al, 2006). These studies might provide mechanistic insight into the function of BRCA1 and FANCD2 that could have important implications for the understanding of genome stability and tumorigenesis.

METHODS

DAPI analysis and immunostaining. DAPI-staining, immunostaining and analysis of meiotic nuclei were carried out as described by Colaiacono et al (2003). Quantitative analysis of RAD-51 foci was carried out on z series of images acquired using a Leica DM5000 fluorescence microscope, Leica DC 350 FX camera under the control of Leica LAS AF 6000 software. Optical sections were collected at 0.25 µm increments. Quantitative analysis of DAPI-staining bodies in diakinetid nuclei was carried out on z series of images (optical sections 0.50 µm increments). The numbers of nuclei scored for genotype are indicated in Fig 1 next to the coloured bar codes.

Apoptosis assay. Germline apoptosis was visualized with SYTO12 (Invitrogen, Molecular Probes, San Giuliano Milanese, Italy) as described by Gumienny et al (1999).

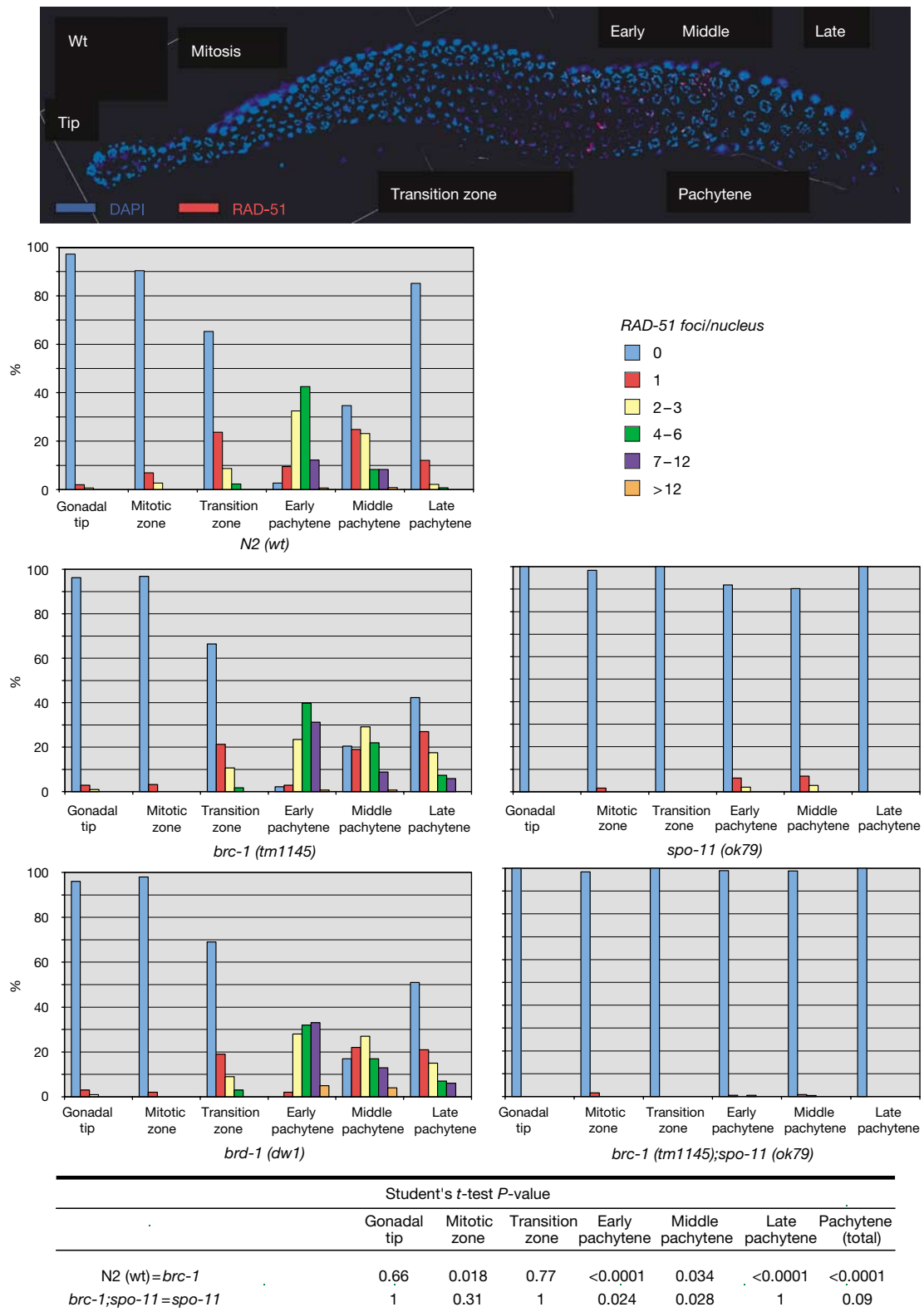


Fig 2 | *brc-1* mutants show *spo-11*-dependent elevation of RAD-51 foci. Representative image of a wild-type germline immunostained with anti-RAD-51 (red) and DNA counterstained with DAPI (blue). Histograms represent quantification of RAD-51 foci in germlines of animals of the indicated genotypes. The *y* axis represents the percentage of nuclei with the indicated number of foci. The *x* axis represents the position (zone) along the germline. Statistical analysis was carried out using the Student's *t*-test. DAPI, 4,6-diamidino-2-phenylindole; wt, wild type.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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