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Differential requirements of a mitotic acetyltransferase in somatic and germ line cells

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Abstract

During mitosis different types of cells can have differential requirements for chromosome segregation. We isolated two new alleles of the *separation anxiety* gene (*san*). *san* was previously described in both *Drosophila* and in humans to be required for centromeric sister chromatid cohesion (Hou et al., 2007; Williams et al., 2003). Our work confirms and expands the observation that *san* is required *in vivo* for normal mitosis of different types of somatic cells. In addition, we suggest that *san* is also important for the correct resolution of chromosomes, implying a more general function of this acetyltransferase. Surprisingly, during oogenesis we cannot detect mitotic defects in germ line cells mutant for *san*. We hypothesize the female germ line stem cells have differential requirements for mitotic sister chromatid cohesion.

Keywords

Drosophila; germ line; oogenesis; mitosis; sister chromatid cohesion; *separation anxiety*

Introduction

Drosophila embryonic development starts with thirteen nuclear divisions without cytokinesis (Foe et al., 1993). The nuclei migrate outward to the egg periphery during nuclear division 8 and 9, with most nuclei arriving at the surface of the embryo during interphase 10. After four additional nuclear divisions, the cortical nuclei arrest mitosis during interphase 14. Once arrested, the nuclei become synchronously encased by polarized invaginations of the plasma membrane and a monolayer of epithelial cells is formed *de novo*.

Sister chromatid cohesion is crucial for chromosome alignment during metaphase (Losada, 2007; Nasmyth and Haering, 2005). The evolutionarily conserved multisubunit cohesin complex is required for sister chromatid cohesion. This complex contains four core subunits: Smc1, Smc3, Scc3, and Scc1/Mcd1/Rad21 (Losada, 2007). In vertebrates, Scc3 has two isoforms: SA1 and SA2 (Losada et al., 2000; Sumara et al., 2000). Another protein, Pds5, is

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weakly associated with the cohesin complex and may regulate the dynamic interaction of cohesin with chromatin (Hartman et al., 2000; Panizza et al., 2000)

In yeast cohesion is established in multiple steps: before S-phase the Scc2 and Scc4 proteins regulate the chromosomal loading of cohesin at centromeres and at regularly spaced intergenic regions along chromosome arms (Ciosk et al., 2000; Glynn et al., 2004; Tomonaga et al., 2000). During ensuing DNA replication, sister chromatid cohesion is established (Uhlmann and Nasmyth, 1998). The Eco1/Ctf7 protein is the main regulator of this event (Skibbens et al., 1999; Toth et al., 1999). Yeast mutants lacking the acetyltransferase Eco1/Ctf7 (or Eso1 in fission yeast) exhibit defective cohesion despite cohesins continuing to localize to the chromosomes (Skibbens et al., 1999; Tanaka et al., 2000; Toth et al., 1999). Recent studies support the idea that this protein makes a direct connection with the replication fork when establishing cohesion (Lengronne et al., 2006; Moldovan et al., 2006). The cohesin links will then remain until anaphase when they are removed through proteolytic cleavage of Scc1 (Uhlmann et al., 1999).

Meiotic and mitotic sister chromatid cohesion are distinct. Meiotic cells contain specific cohesin subunits, including the α-kleisin Rec8 (Nasmyth and Haering, 2005). Rec8 is crucial for meiotic cohesion and for synaptonemal complex (SC) formation in all organisms studied (Klein et al., 1999; Molnar et al., 1995; Pasierbek et al., 2001). In *Drosophila*, meiotic cohesion depends on the protein Orientation Disruptor (Ord) (Bickel et al., 1996; Bickel et al., 1997; Miyazaki and Orr-Weaver, 1992). In *ord* mutants, sister chromatids segregate randomly through both meiotic divisions (Bickel et al., 1997; Webber et al., 2004). Ord is enriched at the centromeres of meiotic chromosomes in both males and females (Bickel et al., 1997; Webber et al., 2004). Smc1 and Smc3 subunits colocalize with Ord at centromeres of ovarian germ line cells and in flies lacking Ord activity, cohesin SMCs fail to accumulate at oocyte centromeres (Khetani and Bickel, 2007).

In *Drosophila*, the *separation anxiety* (*san*) gene encodes an acetyltransferase known to be required for mitotic sister chromatid cohesion in neuroblasts and S2 cells (Williams et al., 2003). *san* function was associated with sister chromatid cohesion since mutant cells showed loss of the cohesin Scc1 specifically at the centromeres. Recently, *san* was shown to have a homologue in humans (Arnesen et al., 2006). RNAi experiments depleting *SAN* in HeLa cells caused defects in sister chromatid cohesion and cohesin SMC1 was no longer detected at the centromeres (Hou et al., 2007).

In this study we isolated two new loss-of-function alleles of *san* in a forward genetic screen for maternal mutants defective in blastoderm cellularization. We confirm and expand the observation that during mitosis *san* is required *in vivo* and in different types of somatic cells for chromosome segregation. In addition, our work suggests that *san* is also important for chromosome resolution. This implies a more general function of this acetyltransferase and a possible interplay between cohesion and chromosome condensation/resolution. Surprisingly, during oogenesis we cannot detect mitotic defects in germ line cells mutant for *san*. We hypothesize the female germ line stem cells have differential requirements for mitotic sister chromatid cohesion.

Results

atado is required maternally for the correct segregation of chromosomes during syncytial blastoderm

To identify new genes involved in *Drosophila* blastoderm cellularization and germ-band extension, we took advantage of a previously reported maternal screen (Barbosa et al., 2007). This screen used the FLP-FRT/ovo^{D} system (Chou and Perrimon, 1992) to generate germ line

mutant clones. The screen was carried out in the right-arm of the second chromosome (2R) and 137 independent mutant lines within the "germ cells only" class of mutants were isolated on the basis of an extremely abnormal soma but where the germ cells were formed normally at the posterior pole of the embryo. The secondary screen involved isolation of mutants defective in cuticle production. Absence of cuticle is a good marker for defects in apicobasal polarization of epithelial cells. Secondary screening allowed the isolation of 47 of the initial 137 lines. Complementation studies of these mutants identified 9 complementation groups by zygotic lethality or sterility. Complementation group 2 contained two alleles, which we will initially refer to as *atado*^{1} and *atado*^{2}. These two alleles will later be renamed *san*^{3} and *san*^{4}, respectively.

To characterize the role of the *atado* gene during *Drosophila* early embryonic development, we examined germ line clone embryos of both alleles of *atado*. Both mutant alleles had similar maternal phenotypes, with 87% (n=39) of *atado*^{2} embryos exhibiting nuclear division abnormalities during syncytial blastoderm (2.5%, n=40, in wild-type embryos) (Supplementary Fig. 1). During syncytial blastoderm development wild-type nuclei divide synchronously and are evenly distributed throughout the embryo (Fig. 1A, D, G). We observed that the *atado* embryos frequently showed nuclei division asynchrony (data not shown) and irregular distribution of the nuclei (Fig. 1B). Yet, the most striking phenotype in *atado* embryos was chromosome segregation defects during mitosis (Fig. 1). Anaphase, whilst being mainly bipolar in *atado* embryos, appeared significantly more disorganized in *atado* than in wild-type embryos (Fig. 1D, E). *atado* embryos showed a high frequency of chromosome lagging (Fig 1F) and formation of chromosome bridges (Fig. 1H, I). We also observed interphase nuclei fused together or attached by chromosome bridges (Fig. 1C, Supplementary Fig. 1). Due to these chromatin bridges we refer to our mutant as *atado*, "tied-up" in Portuguese.

Chromosome lagging in *atado* embryos may be explained by kinetochore abnormalities, but we failed to detect any obvious defects in the localization of Centromere identifier (Cid) during metaphase or anaphase (Supplementary Fig. 2A, B). Chromosome lagging could also be explained by centrosome/mitotic spindle defects. Yet, we did not detect any obvious defects in the localization of Centrosomin (Cnn) (Supplementary Fig. 2C–F), and the mitotic spindle was bipolar and correctly attached to chromosomes and centrosomes (Supplementary Fig. 2E, F).

atado is necessary zygotically for neuroblasts mitosis and imaginal discs development

atado mutations were zygotically lethal. To better characterize the zygotic lethality of *atado* alleles, we followed the development of transheterozygote *atado*^{1}/*atado*^{2} mutants. All isolated transheterozygote *atado* larvae reached the third instar larval stage, pupated and died at the pupa stage (n=14). All isolated heterozygous larvae (*atado*/+) were viable (n=16). Therefore, the maternal contribution of *atado* was sufficient for development to larval stage, but not for pupa and metamorphosis to the adult.

We expected the lethality to be associated with mitotic defects, and hence we analyzed the brains and imaginal discs of transheterozygote third instar larvae. We observed that *atado* mutant larvae had smaller brains and extremely small imaginal discs (Supplementary Fig. 3, data not shown). This is the typical zygotic phenotype of several cell cycle mutants (Gatti and Baker, 1989; Krause et al., 2001). *atado* neuroblasts showed chromosome congression defects during metaphase (Fig. 2A, B) and abnormal segregation of chromosomes during anaphase (Fig. 2D, E). To further confirm that *atado* is important for cell proliferation, we induced *atado* mutant clones in imaginal discs of an otherwise heterozygote larva (one copy GFP). Imaginal disc clones mutant for *atado*^{2} (marked by the absence of GFP) were absent or significantly smaller than the twin-spot wild-type clones (marked by two copies of GFP) (Fig. 2G, H). In contrast, control clones also marked by absence of GFP had a similar size to the

twin-spot clones (Fig. 2I). This suggested that *atado* is required for normal development of larvae imaginal discs.

atado is allelic to *separation anxiety* (*san*), a gene required for mitotic sister chromatid cohesion

To identify the gene responsible for the *atado* mutant phenotypes, we mapped both *atado* alleles using the Bloomington 2R deficiency kit (See Materials and Methods), defining a cytological interval comprising 47 genes. By a candidate gene approach we concluded that *atado* was most likely allelic to the gene *separation anxiety* (*san*) since both *atado* alleles failed to complement san^2 (Fig. 3A), a lethal P-element of the *san* gene (Williams et al., 2003). Furthermore, san^2 germ line clones produced mutant embryos phenotypically indistinguishable from $atado^1$ and $atado^2$ (Supplementary Fig. 4). The *san* gene was previously predicted to encode an acetyltransferase, which transfers acetyl groups to the N-terminus of other proteins (Williams et al., 2003). San protein contains 184 amino acids and analysis of San primary sequence revealed an acetyltransferase domain composed by two acetyltransferase subdomains from amino acids 74–94 and 117–129, respectively (Fig. 3B) (Williams et al., 2003). Sequencing both alleles of *atado* confirmed that *atado* was allelic to *san*, as both alleles contained distinct nonsense mutations within *san* open reading frame (ORF). These two nonsense mutations were predicted to cause severe truncations of the San protein (Fig. 3B). We failed to detect San from embryonic total protein extracts (Fig. 3C). The San antibody is polyclonal and it was raised against most of San protein (Williams et al., 2003). It was nevertheless possible that it did not recognize the truncate proteins encoded by the *atado* alleles. We expressed in bacteria the smallest truncated protein, which was predicted to be encoded by $atado^2/san^4$. The San antibody used in this work was able to recognize the recombinant protein in total extracts from bacteria (Supplementary Fig. 5). We concluded that the isolated *atado* alleles are loss-of-function alleles of *san*. We therefore renamed $atado^1$ and $atado^2$, respectively to san^3 and san^4 alleles.

san is required for chromosome resolution

Syncytial blastoderm embryos mutant for *san* showed dramatic chromosome segregation defects during anaphase (Fig. 1). We observed that during metaphase the defects in chromosome congression and alignment were comparatively mild (Fig. 4A–C and Supplementary Fig. 4). This is in contrast to the dramatic defects in chromosome congression and alignment recently reported in Scc1-depleted embryos (Pauli et al., 2008). Additionally, we only detected a minor separation of the sister chromatid kinetochores (due to the loss of centromeric cohesion) in *san* mutant embryos arrested in metaphase (Fig. 4D, E). Given this evidence we decided to investigate if San had additional functions that could explain the observed phenotypes.

Cohesion defects can explain the lagging chromosomes and the high frequency of chromosome bridges observed in *san* mutant embryos, but we noticed that a large proportion of these bridges did not involve centromeric regions of the chromosome (negative for Cid staining). Of the scored bridges between nuclei in late-mitosis/interphase, 55.6%± 18.2 were negative for Cid staining, 32%±11.5 were positive for Cid staining, and in 12.4%±10 of the cases the result was inconclusive (n= 104 bridges) (Supplementary Fig. 6). Bridges involving distal chromosome regions were previously described in mutants defective in chromosome condensation and/or resolution (Bhat et al., 1996; Steffensen et al., 2001). Consistent with the hypothesis that *san* could be involved in chromosome resolution/condensation; we observed that during mitosis a subset of nuclei showed a dramatic decrease in the levels of Barren (Fig. 4F–J) (number of cortical nuclei with a detectable reduction of Barren localization, wild-type: 0±0 nuclei, n=780, 11 embryos, san^4: 19.7%±12.9 nuclei, n=980, 11 embryos; Student's t test, 95% confidence interval, $p<3\times10^{-5}$). Embryos mutant for *barren* are defective in chromosome segregation with

the formation of chromosome bridges (Bhat et al., 1996). Although the reduction of Barren localization suggests defects in chromosome resolution and/or condensation, we did not detect reduced levels of this condensin specifically at chromosome bridges (data not shown). Consistent with chromosome resolution defects, during interphase we observed a subset of chromosome bridges showing reduced levels of Topoisomerase II (Fig. 4K, L). TopoII is important for DNA decatenation and chromosome resolution (Holm et al., 1989; Uemura et al., 1987). Chromosomal localization of TopoII is condensin-dependent (Coelho et al., 2003). We did not detect any change in the levels of TopoII in embryo total extracts (data not shown).

During oogenesis *san* is not required for germ line mitosis

Our data implied *san* as being important for mitosis of distinct types of somatic cells. This is consistent with previous published work that showed that *san* is required *in vivo* in neuroblasts, *ex vivo* in S2 cells and human HeLa cells, for mitotic sister chromatid cohesion (Hou et al., 2007; Williams et al., 2003). At this point it is important to emphasize that the maternal screen from which we isolated both san^3 and san^4 alleles was designed to exclude mutants with mitotic defects during oogenesis as egg laying had to be normal. To investigate if egg laying from females with a germ line mutant for *san* was normal, we induced germ line clones using the FLP-FRT/ovo^D system (Chou and Perrimon, 1992). We compared the number of eggs laid by *san* and control females (with an identical FRT chromosome), and concluded that egg laying between these females was identical for more than 15 days after pupa eclosion (Supplementary Fig. 7) (See Materials and Methods). Since clones were induced at larvae stages by heat-shock, this suggested that *san* and control germ line stem cells divided continuously and at similar rates for almost 20 days after clone induction.

To test the hypothesis that *san* mitotic function is not required during oogenesis, we generated females with genetically mosaic ovaries using the FLP-FRT-mediated mitotic recombination and a nuclear GFP clone marker. Absence of GFP (green) indicates that the cells are homozygous for *san* mutations. After the induction of clones at larvae stages, we observed a consistently high frequency of *san* clones in the adult ovaries (data not shown). This suggested that the proliferating larvae primordial germ cells mutant for *san* could efficiently compete for the adult germ line stem cell niche. Confirming that *san* is not required during oogenesis for germ line mitosis, egg chambers mutant for *san* from females with 7/8 or 15 days old after pupa eclosion showed a normal determination of the oocyte (Fig. 5B–D), a normal condensed karyosome (Fig. 5E - asterisks indicates a condensed karyosome), a normal number of nurse cells (15 nurse cells, n=16), a normal fusome (Fig. 5F), and a normal eggshell without fused dorsal appendages (*spindle* phenotype) (data not shown). Similar results were obtained with a previously isolated loss-of-function allele of *san* (san^2) (Supplementary Fig. 8A–E).

Since germ line clones mutant for *san* did not show mitotic defects during oogenesis, we investigated if San is required within the somatic follicle cells. Each *Drosophila* egg chamber contains 16 germ line cells surrounded by a follicle cell epithelium of somatic origin. Follicle cell clones were induced by heat-shock at larvae stages (as described for germ line clones - See Material and Methods). Whereas we were able to isolate large clones (negative for GFP) using a control chromosome (Supplementary Fig. 8G–L), in the case of *san* mutants (FRT san^2 and FRT san^4), the isolated clones were both significantly smaller and less frequent (Supplementary Fig. 8A–F); suggesting a requirement of *san* function within follicle cells.

We were able to recover *san* clones within the follicle cells if they were induced in adult flies and if the ovaries were dissected two/three days after induction (data not shown). This was most likely due to San protein perdurance within the follicle cells.

We also tested whether San was expressed in wild-type ovaries. Western blots of total protein extracts showed that San was expressed in the ovaries (Fig. 5H), with no obvious differences

in the expression levels detected between younger (3 days) and older females (8 days). Additionally, San immunostaining of mosaic ovaries confirmed San expression within the ovaries germ line, as we detected a specific cytoplasmic staining between the control (presence of GFP) and mutant egg chambers (absence of GFP) (Fig. 5G, I).

Previous studies with *san* homologue in HeLa cells showed that the inactivation of *san* expression by RNAi induced an abnormal expression of cohesin SMC1 (Hou et al., 2007). To check if integrity of the cohesin complex during oogenesis was affected in *san* mutants, mosaic ovaries were stained for Smc1. Consistent with the absence of defects during oogenesis, we could not detect any change in Smc1 localization in germaria whose germ-line was mutant for *san* (Fig 5J–O), with bright foci and a diffuse staining present as was previously described for wild-type germarium (Khetani and Bickel, 2007). We failed to detect specific immunostaining for Scc1 in wild-type germarium (data not shown). We also did not detect specific immunostaining for Scc1 and Smc1 in the syncytial blastoderm embryo (data not shown).

Discussion

We isolated two new alleles of the *san* gene: san^3 and san^4. *san* was previously described to be required for centromeric sister chromatid cohesion in *Drosophila* (neuroblasts and *ex vivo* in S2 cells) and in humans (*ex vivo* in HeLa cells) (Hou et al., 2007; Williams et al., 2003).

Our work confirms and extends the observation that *san* is required *in vivo* for mitosis of different types of somatic cells. It also suggests that in addition to the previously described role in sister chromatid cohesion, *san* is also important for chromosome resolution. We also propose that female germ line stem cells have differential requirements for sister chromatid cohesion, since we could not detect mitotic defects when they were mutant for *san*.

san is required *in vivo* for mitosis of different types of somatic cells

Since the mitotic function of *san* had only been studied *in vivo* in *Drosophila* neuroblasts (Williams et al., 2003), we decided to characterize its function during different stages of *Drosophila* development. Our analysis shows that *san* is important for chromosome segregation during syncytial blastoderm nuclei division (maternal phenotype) and, as previously shown, in larvae neuroblast mitosis (zygotic phenotype). We also observe that imaginal discs from larvae mutant for *san* are extremely small. Clonal analysis confirms *san* function is required within larvae imaginal discs and adult ovary follicle cells.

san is required for chromosome resolution

Defects in mitotic sister chromatid cohesion in yeast and higher eukaryotes have similar defects in chromosome alignment during metaphase and chromosome lagging during anaphase (Dorsett et al., 2005; Sonoda et al., 2001; Toyoda and Yanagida, 2006; Vass et al., 2003). *Drosophila* embryos depleted for Scc1 have dramatic defects in chromosome congression and alignment during metaphase (Pauli et al., 2008). In contrast, *san* mutant embryos have clear defects in chromosome segregation, but only show remarkably mild abnormalities during metaphase. These phenotypic discrepancies suggest that loss of *san* function causes other problems beyond sister chromatid cohesion defects.

Embryos mutant for *san* show a high frequency of chromosome bridges during anaphase. Sister chromatid cohesion defects can cause a high frequency of anaphase bridging (Pauli et al., 2008; Vass et al., 2003). Yet a large proportion of chromosome bridges in *san* embryos are negative for Cid staining. Bridges involving distal regions of the chromosomes were previously described in mutants with chromosome condensation and/or resolution defects (Bhat et al., 1996; Cobbe et al., 2006; Dej et al., 2004; Steffensen et al., 2001).

Chromosome condensation during mitosis relies on the condensin multisubunit protein complex (Hirano, 2005). Together with topoisomerase II, this complex has an important role in organizing the individual axes of sister chromatids prior to their segregation during anaphase (Hirano, 2006). Mutants for the condensin complex *SMC4* or *gluon* and *Barren* show chromosome segregation defects with the formation of chromosome bridges during anaphase. After depletion of SMC4, TopoII fails to localize to a clearly defined chromatid axial structure and there is a significant decrease in TopoII DNA decatenation activity (Coelho et al., 2003). TopoII is important for chromosome resolution, and inactivation of *topoII* in *Drosophila* embryos and S2 cells results in chromosome bridges (Buchenau et al., 1993; Chang et al., 2003).

Given the phenotypic similarities between *san*, *topoII*, and *barren* mutant embryos we investigated if Barren and TopoII expression was abnormal in *san* mutant embryos. In *san* mutant embryos a subset of mitotic nuclei show a dramatic decrease in Barren levels, suggesting defects in chromosome condensation. Interestingly, we also observe a subset of the chromosome bridges connecting the interphase nuclei showing reduced levels of TopoII. We hypothesize that *san* is important not only for sister chromatid cohesion but also for chromosome resolution, which implies a more general function of this acetyltransferase.

Several lines of evidence argue against a cross talk between the cohesin and condensin complexes: 1) cohesins and condensins were isolated as separate complexes in solution (Losada et al., 1998). 2) Condensin DmSMC4 depletion did not alter the localization or removal of cohesins from mitotic chromatin in *Drosophila* S2 cells (Coelho et al., 2003). 3) In yeast has been shown that although sister chromatid separation did not occur normally in condensin *Ycs4* mutants (Cap-D2 in *Drosophila*), cohesin MCD1/SCC1 was released normally from chromosomes at the metaphase-anaphase transition (Bhalla et al., 2002). 4) In higher eukaryotes cohesion depletion did not appear to affect chromosome condensation (Losada et al., 1998; Sonoda et al., 2001; Vass et al., 2003).

Nevertheless, previous reports in budding yeast have suggested mechanistic interactions between cohesins and condensins (Castano et al., 1996; Lavoie et al., 2002). We show that *san* is genetically upstream of both sister chromatid cohesion and chromosome resolution/condensation. Our current interpretation is that San acetyltransferase activity is necessary for sister chromatid cohesion and chromosome resolution/condensation without being a core component of these processes. To what extent we are uncovering a cross talk is still unclear, but this is a plausible possibility.

During oogenesis *san* is not required for germ line mitosis

The female ovary is composed of 16–20 ovarioles (Gilboa and Lehmann, 2004; Spradling, 1993). The germarium is at the anterior tip of each ovariole and is responsible for egg chamber formation. Two to three germ line stem cells are positioned at the anterior tip of each germarium. These cells divide asymmetrically: the most anterior daughter cell keeps a germ line stem cell fate whereas the most posterior daughter cell (the cystoblast) starts a differentiation program that ultimately produces an egg. The cystoblast divides mitotically four times in order to form a cyst of 16 cells connected by ring-canals and a highly branched cytoskeletal structure called fusome. One of these cells becomes the oocyte, the other 15 nurse cells.

In accordance with previously published work (Williams et al., 2003), we clearly demonstrate that *san* is necessary *in vivo* for normal mitosis in *Drosophila melanogaster*. It was therefore surprising that analysis of germ line clones mutant for *san* did not detect any obvious mitotic defects during oogenesis. 1) The mitotic divisions of the germ line stem cells and cysts mutant for *san* are normal based on morphological analysis of the DNA, number of nurse cells, and

fusome organization. 2) Egg-laying from females whose germ line is mutant for *san* is equivalent to the control females, even two weeks after pupa eclosion (hatching). This suggests that loss of *san* function does not reduce the mitotic rate or impair the viability of the germ line stem cells. 3) Egg chambers mutant for *san* show a normal determination and positioning of the oocyte, and a normal condensed karyosome. 4) *Drosophila* eggs mutant for *san* have a normal size and a normal dorsal ventral (DV) patterning (absence of *spindle* phenotype). If *san* activity were necessary within the germ line for chromosome segregation we would expect defects in the subsequent stages of oogenesis. Consistent with this expectation, defects in the repair of DNA double strand breaks (DSB) during meiosis activates the DNA damage checkpoint (Ghabrial and Schupbach, 1999), delaying meiosis, inhibiting karyosome condensation, and mutant females lay eggs with DV patterning defects.

A possible explanation for the lack of mitotic abnormalities in females whose germ line is mutant for *san* is that San protein is extremely stable. We do not favor this hypothesis for four separate reasons: 1) we induce germ line clones for *san* during larvae development (2^{nd} and 3^{rd} instar larvae) and analyze their effect at least 12 days later in adult females that were 7/8 and 15 days old (after pupa eclosion). 2) We consistently detect mitotic defects in embryos laid by females whose germ line is mutant for *san*. 3) *san* zygotic mutants reaching larvae stage is a typical phenotype associated with several previously characterized cell cycle mutants (Gatti and Baker, 1989; Krause et al., 2001). 4) *san* mutant clones in larvae imaginal discs show defects only three days after clone induction.

The isolated alleles of *san* contain nonsense mutations within the open reading frame (ORF) and since they are protein-null alleles, the lack of oogenesis defects is not the result of a putative hypomorphic nature of the isolated alleles. Similar results were also obtained with san^2, a previously isolated loss-of-function allele of *san* (Williams et al., 2003). We conclude that during oogenesis *san* is not required for germ line mitosis.

san is possibly not required during oogenesis due to a functional redundancy with *deco* acetyltransferase. This is improbable since these two proteins are thought to have different substrates: San was predicted to be an N-acetyltransferase (Williams et al., 2003), and Deco was predicted to acetylate internal lysines (Ivanov et al., 2002). Additionally, San was shown to localize to the cytoplasm during the interphase of *Drosophila* Kc cells and human HeLa cells (Hou et al., 2007; Williams et al., 2003), whereas members Eco1/Ctf7 family (that includes *Drosophila* Deco) localize to the nucleus (Hou and Zou, 2005; Skibbens et al., 1999; Toth et al., 1999). Nevertheless, even if the observed results are a consequence of a redundancy between *san* and *deco* specifically within the germ line, this is consistent with the hypothesis that during oogenesis female germ line cells have differential requirements for mitotic sister chromatid cohesion (see bellow).

Female germ line cells have differential requirements for mitotic sister chromatid cohesion

Here we have shown that during mitosis different types of cells can have differential requirements for chromosome segregation. We cannot discard the possibility that some somatic cells might not require the mitotic function of *san*, but our data suggest that female germ line cells have differential requirements for mitotic sister chromatid cohesion. Consistent with this hypothesis a previous analysis of Smc1 germ line clones (focused primarily on prophase I cysts) has not reported mitotic defects during oogenesis (Khetani and Bickel, 2007). Identification of the *in vivo* substrates of *san* acetyltransferase should help a better understanding of the molecular nature of these differences.

Materials and Methods

Fly Work and Genetics

atado/*san* alleles were isolated from a maternal screen previously done in the laboratory of Ruth Lehmann (Barbosa et al., 2007). From this screen we identified 9 complementation groups on the right arm of the second chromosome. These mutants fail to form embryonic cuticle or have scraps of cuticle, however the primordial germ cells are formed properly at the posterior pole of the embryo. Complementation Group 2 contained two alleles that initially were named $atado^{1}$ and $atado^{2}$. Later on these alleles were respectively renamed to san^{3} and san^{4}. All flies were raised at 25°C unless otherwise indicated, using standard techniques.

Germ line clones were induced using the FLP/FRT ovo^{D} system (Chou and Perrimon, 1992). Germ line clones of san^{3} and san^{4} were made by crossing FRT42B *san*/CyO virgins to *hs flp*; FRT42B ovo^{D}/CyO males and heat shocking the progeny once at 37°C for 1 hour during second and third larval instar.

Mosaic ovaries with the nuclear GFP clone marker were generated by FLP-FRT-mediated mitotic recombination as described (Caceres and Nilson, 2005; Chou and Perrimon, 1992). FRT42B san^{4}/CyO virgins were crossed with *yw* P[w^{+}, hsFLP]^{1}; P[mini- w^{+}, FRT42B] P [w^{+}; ubi-nls-GFP]/CyOhshid males. The P[w^{+}; ubi-nls-GFP] FRT chromosomes bear a polyubiquitin promoter that drives ubiquitous nuclear green fluorescent protein (GFP) expression. Recombination was induced by a 1 hour heat shock at 37°C during the second and third instar stage. Adult ovaries were harvested from females with 7–8 days and 15 days old, and were subsequently processed for immunofluorescence. Germ line and follicle cell clones were identified by the absence of nuclear endogenous GFP.

To generate clones marked by the absence of GFP in imaginal discs, FRT42B san^{4}/CyOhshid males were crossed with *yw* P[w^{+}, hsFLP]^{1}; P[mini- w^{+}, FRT42B] P[w^{+}; ubi-nls-GFP]/ CyOhshid virgins. The offspring were heat-shocked for 2 hours at 37°C at both 24 and 48 hours after a 24 hour egg collection, corresponding to the first and second larval instar.

To test the egg laying, 1 to 3 days old females from both *hs flp;* FRT 42B san^{4}/FRT42B ovo^{D} germ line clones and control *hs flp;* FRT42B/ovo^{D} FRT 42B, where germ line clones had been induced by heat-shock during the second and third larval instar, were mated in parallel to 30 wild-type (Oregon R) males. Egg laying was counted every 24 hours during 15 days.

Both san^{3} and san^{4} alleles were balanced over a CyO Actin-GFP to enable isolation of transheterozygous mutant larvae. Mutant larvae were harvested on the basis of lack of GFP and transferred to fresh tubes. Development was followed until pupae stage where *san* mutant larvae died.

In order to compare *san* mutant phenotypes with a known lethal P-element insertion on the *separation anxiety gene* (*san*) (Williams et al., 2003), we recombined san^{2} allele with a FRT42B chromosome. This allowed us to generate san^{2} germ line clones and confirm that they are phenotypically indistinguishable from san^{3} *and* san^{4} germ line clones.

Cloning *atado*

Both alleles of *atado*/*san* gene were mapped using the Bloomington 2R deficiency kit. Deficiencies were crossed with both *atado*/*san* alleles and F1 progeny scored for zygotic lethality. The following seven deficiencies failed to complement *atado* alleles: Df(2R)en-A, Df(2R)en-B, Df(2R)E3363, Df(2R)Exel6060, Df(2R)ix[87i3], Df(2R)ED2219 and Df(2R) ED2155. This allowed us to map *atado*/*san* mutation to the cytological interval 47E3-47F5, comprising 47 genes. By a candidate gene approach we concluded that both alleles of *atado*/

san failed to complement a known lethal P-element of the *san* gene, *san*^{2} (Williams et al., 2003).

To molecularly characterize the isolated *san* mutations, genomic PCR was carried out from heterozygous mutants of *san* gene (*san*/Cyo). As a control, and in order to detect DNA polymorphisms, we used a mutant from a different complementation group isolated in the same screen. Two independent genomic PCR fragments from each allele were sequenced and compared with each other, and with the control. Both *san* alleles have distinct nonsense mutations within *san* open reading frame that are predicted to cause a truncation of the San protein.

Immunohistochemistry

For phenotypic analysis of *san* embryos, at 2–3 hours of age, embryos were collected and fixed (after dechorionation in 50% bleach for 5 min) by gentle shaking for 1 hour in 4 mL heptane, 0.125 mL 37% formaldehyde and 0.875 mL PEMS. Fixation was followed of devitellinization by addition of 4 mL methanol and shaking vigorously during 1 minute. Following rehydration, embryos were blocked in phosphate buffered saline (PBS, pH 7.4) containing 0.1% Tween-20, 0.1% bovine serum albumin and 5% serum (BBS), at 4°C overnight. To analyze nuclei arrested in metaphase, *san* embryos were incubated 15 minutes with 250 μM colchicine, PBS and heptane, prior to fixation.

Primary antibody incubations were carried in BBS at 4°C overnight. Antibodies used were anti-Cid at 1:500 kindly provided by David Glover's laboratory, anti-Neurotactin at 1:133 (BP106 Hybridoma Bank), anti-pSer10-Histone H3 at 1:1000 (Upstate Biotechnology), anti-Topoisomerase II at 1:400 (Buchenau et al., 1993), anti-Barren at (1:2000) (Bhat et al., 1996), anti-Cnn at 1:1000 kindly provided by Jordan Raff, Anti-α Tubulin clone YL1/2 at 1:50 (Serotec UK). The embryos were washed extensively in PBS containing 0.1% Tween-20 (PBT), reblocked in BBS and incubated with the appropriate secondary antibody for two hours at room temperature (RT). Secondary antibodies were Cy3- and Cy5-conjugated at 1:1000 (Jackson ImmunoResearch Laboratories, West Grove, PA). After extensively washed in PBT, DNA was stained with OliGreen (Molecular Probes, Eugene, OR) at 1:5000 with the addition of 5 μg/mL RNAse A. For scoring the number of cortical nuclei with reduced levels of Barren we focused in embryos where the remaining nuclei (positive for Barren) were at metaphase or early anaphase stages. These are the stages when Barren localization in the chromosomes is the highest.

Ovaries were processed for immunofluorescence as described (Navarro et al., 2004), with exception of DNA staining. Primary antibody was rabbit polyclonal anti-San (Williams et al., 2003) at 1:1000 and secondary antibody was Cy5-conjugated at 1:1000. For DNA staining, ovaries were incubated with 100μg of RNAse/mL for 30 min following incubation with 0.17 μg/mL propidium iodide. Ovaries were 2 times 5-min washed in PBT and 2 rinses in PBS, following mounting. Endogenous GFP was used to distinguish mutant clone egg chambers from chambers that did not have clones.

For ovary staining with anti-Smc1 antibody, ovaries were processed as described (Song et al., 2002). Primary antibody was anti-Smc1 at 1:2000 (Khetani and Bickel, 2007) and anti-Hts at 1:50 (1B1 Hybridoma Bank). Secondary detection was done using rodamin red at 1:1000 (Jackson ImmunoResearch Laboratories) and Cy5-conjugated at 1:1000.

Imaginal discs were dissected in PBS from crawling late third instar larvae. Discs were fixed in 4% formaldehyde with PEMS during 30 minutes on ice, following a 15 min wash in PBS with 0,2% Triton X-100. DNA was stained with propidium iodide as previously described.

Whole brains from transheterozygous mutants of *san* (*san*^{3}/*san*^{4}) were dissected from third instar larvae in PBS and fixed for 20 minutes in 3.7% formaldehyde, 2 mM EGTA in PBS. Briefly, brains were three times 5-min washed with PBS, permeabilized for 10 min in PBS + 0,3% Triton X-100 and blocked in PBS containing 0.1% Triton X-100 and 1% BSA for 1 hour at RT. Anti-α Tubulin at 1:50 and anti-pSer10 Histone H3 at 1:300 were incubated with PBS + 0.1% Triton X-100 and 1% BSA overnight at 4°C. After three 5-min washes, Cy3- and Cy5-conjugated secondary antibodies were incubated for 4 hours at RT. Brains were rinsed with PBS and DNA was stained with propidium iodide as previously described.

Embryos and all tissues were mounted in Fluorescent Mounting Medium (DakoCytomation, Inc) and immunostainings were visualized using a Leica SP5 confocal microscope. All images are confocal sections, with the exception of Fig. 5J–O, Sup. Fig. 4, and Sup. Fig. 6, which are Z-stacks. The Z-stacks projections were obtained using Image J program (Grouped ZProjector, maximum pixel intensity).

Western Blotting

Embryos were collected on apple juice agar after 3 hour egg laying. Each protein sample was collected by lysing 10 embryos in SDS-PAGE sample buffer. Samples were boiled for 5 minutes and loaded on 5×8 cm 12.5% polyacrylamide gels.

Ovaries were dissected from 3- to 8-day-old Oregon females. Ovaries were homogenized in buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 0,1% NP-40, 2 mM DTT, 10 mM NaF and protease inhibitor (Roche). A Bio–Rad™ Bradford protein microassay ensured loading of 5, 10 and 15 μg of protein onto 12 % SDS polyacrylamide gels. As for embryo extracts, samples were boiled for 5 minutes in SDS-PAGE sample buffer before loading.

Proteins were then transferred onto Hybond-ECL membranes (Amersham) and western blotting was performed with standard procedure. Briefly, the membrane was blocked in 5% non-fat milk/PBT (0,1% Tween-20, 1xPBS) overnight at 4°C. Primary antibodies were incubated with the membrane overnight at 4°C. Following washes with PBT secondary antibodies were incubated for 4 hours at RT. After washes with PBT, the membranes were detected with an ECL Plus western blotting detection system (GE Healthcare). Primary antibodies used were anti-Cid (1:2000), rabbit polyclonal anti-San (1:1000), rabbit anti-Smc4 (1:1000) (Steffensen et al., 2001), rat anti-Tubulin (1:250). Secondary detection was performed with rabbit and rat HRP-conjugated antibodies used at a final concentration of 1:5000.

Recombinant San^{4} protein expression and detection

We expressed in bacteria a recombinant C-terminal polyhistidine (6xHis)-tagged fragment of San corresponding to residues 1–113 by cloning a cDNA EcoRI-XhoI fragment into the pET22b vector (Novagene) and expressing it in *E. coli* BL21. This is the truncated protein predicted to be encoded by *atado*^{2}/*san*^{4}. Since the San antibody was generated against a GST-San fusion protein, we cloned *san*^{4} cDNA in a His-tag expression vector to avoid cross-reactivity with GST. The San^{4}-6xHis protein was induced with 10mM IPTG in *E. coli* BL21. Bacterial samples were collected at 90, 120 and 180 minutes from both induced and non-induced cultures. The bacterial pellets from each time point were ressuspended in SDS-sample buffer, boiled for 5 minutes and loaded on two 15% polyacrylamide gels. One gel was stained with brilliant coomassie and the other gel was used for western blotting using the San antibody.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Arnesen T, Anderson D, Torsvik J, Halseth HB, Varhaug JE, Lillehaug JR. Cloning and characterization of hNAT5/hSAN: an evolutionarily conserved component of the NatA protein N-alpha-acetyltransferase complex. Gene 2006;371:291–5. [PubMed: 16507339]

Barbosa V, Kimm N, Lehmann R. A maternal screen for genes regulating Drosophila oocyte polarity uncovers new steps in meiotic progression. Genetics 2007;176:1967–77. [PubMed: 17507684]

Bhalla N, Biggins S, Murray AW. Mutation of YCS4, a budding yeast condensin subunit, affects mitotic and nonmitotic chromosome behavior. Mol Biol Cell 2002;13:632–45. [PubMed: 11854418]

Bhat MA, Philp AV, Glover DM, Bellen HJ. Chromatid segregation at anaphase requires the barren product, a novel chromosome-associated protein that interacts with Topoisomerase II. Cell 1996;87:1103–14. [PubMed: 8978614]

Bickel SE, Wyman DW, Miyazaki WY, Moore DP, Orr-Weaver TL. Identification of ORD, a Drosophila protein essential for sister chromatid cohesion. Embo J 1996;15:1451–9. [PubMed: 8635478]

Bickel SE, Wyman DW, Orr-Weaver TL. Mutational analysis of the Drosophila sister-chromatid cohesion protein ORD and its role in the maintenance of centromeric cohesion. Genetics 1997;146:1319–31. [PubMed: 9258677]

Buchenau P, Saumweber H, Arndt-Jovin DJ. Consequences of topoisomerase II inhibition in early embryogenesis of Drosophila revealed by in vivo confocal laser scanning microscopy. J Cell Sci 1993;104(Pt 4):1175–85. [PubMed: 8391015]

Caceres L, Nilson LA. Production of gurken in the nurse cells is sufficient for axis determination in the Drosophila oocyte. Development 2005;132:2345–53. [PubMed: 15829517]

Castano IB, Brzoska PM, Sadoff BU, Chen H, Christman MF. Mitotic chromosome condensation in the rDNA requires TRF4 and DNA topoisomerase I in Saccharomyces cerevisiae. Genes Dev 1996;10:2564–76. [PubMed: 8895658]

Chang CJ, Goulding S, Earnshaw WC, Carmena M. RNAi analysis reveals an unexpected role for topoisomerase II in chromosome arm congression to a metaphase plate. J Cell Sci 2003;116:4715–26. [PubMed: 14600258]

Chou TB, Perrimon N. Use of a yeast site-specific recombinase to produce female germline chimeras in Drosophila. Genetics 1992;131:643–53. [PubMed: 1628809]

Ciosk R, Shirayama M, Shevchenko A, Tanaka T, Toth A, Shevchenko A, Nasmyth K. Cohesin's binding to chromosomes depends on a separate complex consisting of Scc2 and Scc4 proteins. Mol Cell 2000;5:243–54. [PubMed: 10882066]

Cobbe N, Savvidou E, Heck MM. Diverse mitotic and interphase functions of condensins in Drosophila. Genetics 2006;172:991–1008. [PubMed: 16272408]

Coelho PA, Queiroz-Machado J, Sunkel CE. Condensin-dependent localisation of topoisomerase II to an axial chromosomal structure is required for sister chromatid resolution during mitosis. J Cell Sci 2003;116:4763–76. [PubMed: 14600262]

Dej KJ, Ahn C, Orr-Weaver TL. Mutations in the Drosophila condensin subunit dCAP-G: defining the role of condensin for chromosome condensation in mitosis and gene expression in interphase. Genetics 2004;168:895–906. [PubMed: 15514062]

Dorsett D, Eissenberg JC, Misulovin Z, Martens A, Redding B, McKim K. Effects of sister chromatid cohesion proteins on cut gene expression during wing development in Drosophila. Development 2005;132:4743–53. [PubMed: 16207752]

Foe, VE.; Odell, GM.; Edgar, BA. The development of Drosophila melanogaster. 1. C. s. h. l. press; 1993. Mitosis and Morphogenesis in the Drosophila embryo: point and counterpoint; p. 149

Gatti M, Baker BS. Genes controlling essential cell-cycle functions in Drosophila melanogaster. Genes Dev 1989;3:438–53. [PubMed: 2498166]

Ghabrial A, Schupbach T. Activation of a meiotic checkpoint regulates translation of Gurken during Drosophila oogenesis. Nat Cell Biol 1999;1:354–7. [PubMed: 10559962]

Gilboa L, Lehmann R. How different is Venus from Mars? The genetics of germ-line stem cells in Drosophila females and males. Development 2004;131:4895–905. [PubMed: 15459096]

Glynn EF, Megee PC, Yu HG, Mistrot C, Unal E, Koshland DE, DeRisi JL, Gerton JL. Genome-wide mapping of the cohesin complex in the yeast Saccharomyces cerevisiae. PLoS Biol 2004;2:E259. [PubMed: 15309048]

Hartman T, Stead K, Koshland D, Guacci V. Pds5p is an essential chromosomal protein required for both sister chromatid cohesion and condensation in Saccharomyces cerevisiae. J Cell Biol 2000;151:613–26. [PubMed: 11062262]

Hirano T. Condensins: organizing and segregating the genome. Curr Biol 2005;15:R265–75. [PubMed: 15823530]

Hirano T. At the heart of the chromosome: SMC proteins in action. Nat Rev Mol Cell Biol 2006;7:311–22. [PubMed: 16633335]

Holm C, Stearns T, Botstein D. DNA topoisomerase II must act at mitosis to prevent nondisjunction and chromosome breakage. Mol Cell Biol 1989;9:159–68. [PubMed: 2538717]

Hou F, Chu CW, Kong X, Yokomori K, Zou H. The acetyltransferase activity of San stabilizes the mitotic cohesin at the centromeres in a shugoshin-independent manner. J Cell Biol 2007;177:587–97. [PubMed: 17502424]

Hou F, Zou H. Two human orthologues of Eco1/Ctf7 acetyltransferases are both required for proper sister-chromatid cohesion. Mol Biol Cell 2005;16:3908–18. [PubMed: 15958495]

Ivanov D, Schleiffer A, Eisenhaber F, Mechtler K, Haering CH, Nasmyth K. Eco1 is a novel acetyltransferase that can acetylate proteins involved in cohesion. Curr Biol 2002;12:323–8. [PubMed: 11864574]

Khetani RS, Bickel SE. Regulation of meiotic cohesion and chromosome core morphogenesis during pachytene in Drosophila oocytes. J Cell Sci 2007;120:3123–37. [PubMed: 17698920]

Klein F, Mahr P, Galova M, Buonomo SB, Michaelis C, Nairz K, Nasmyth K. A central role for cohesins in sister chromatid cohesion, formation of axial elements, and recombination during yeast meiosis. Cell 1999;98:91–103. [PubMed: 10412984]

Krause SA, Loupart ML, Vass S, Schoenfelder S, Harrison S, Heck MM. Loss of cell cycle checkpoint control in Drosophila Rfc4 mutants. Mol Cell Biol 2001;21:5156–68. [PubMed: 11438670]

Lavoie BD, Hogan E, Koshland D. In vivo dissection of the chromosome condensation machinery: reversibility of condensation distinguishes contributions of condensin and cohesin. J Cell Biol 2002;156:805–15. [PubMed: 11864994]

Lengronne A, McIntyre J, Katou Y, Kanoh Y, Hopfner KP, Shirahige K, Uhlmann F. Establishment of sister chromatid cohesion at the S. cerevisiae replication fork. Mol Cell 2006;23:787–99. [PubMed: 16962805]

Losada A. Cohesin regulation: fashionable ways to wear a ring. Chromosoma 2007;116:321–9. [PubMed: 17333234]

Losada A, Hirano M, Hirano T. Identification of Xenopus SMC protein complexes required for sister chromatid cohesion. Genes Dev 1998;12:1986–97. [PubMed: 9649503]

Losada A, Yokochi T, Kobayashi R, Hirano T. Identification and characterization of SA/Scc3p subunits in the Xenopus and human cohesin complexes. J Cell Biol 2000;150:405–16. [PubMed: 10931856]

Miyazaki WY, Orr-Weaver TL. Sister-chromatid misbehavior in Drosophila ord mutants. Genetics 1992;132:1047–61. [PubMed: 1459426]

Moldovan GL, Pfander B, Jentsch S. PCNA controls establishment of sister chromatid cohesion during S phase. Mol Cell 2006;23:723–32. [PubMed: 16934511]

Molnar M, Bahler J, Sipiczki M, Kohli J. The rec8 gene of Schizosaccharomyces pombe is involved in linear element formation, chromosome pairing and sister-chromatid cohesion during meiosis. Genetics 1995;141:61–73. [PubMed: 8536990]

Nasmyth K, Haering CH. The structure and function of SMC and kleisin complexes. Annu Rev Biochem 2005;74:595–648. [PubMed: 15952899]

Navarro C, Puthalakath H, Adams JM, Strasser A, Lehmann R. Egalitarian binds dynein light chain to establish oocyte polarity and maintain oocyte fate. Nat Cell Biol 2004;6:427–35. [PubMed: 15077115]

Panizza S, Tanaka T, Hochwagen A, Eisenhaber F, Nasmyth K. Pds5 cooperates with cohesin in maintaining sister chromatid cohesion. Curr Biol 2000;10:1557–64. [PubMed: 11137006]

Pasierbek P, Jantsch M, Melcher M, Schleiffer A, Schweizer D, Loidl J. A Caenorhabditis elegans cohesion protein with functions in meiotic chromosome pairing and disjunction. Genes Dev 2001;15:1349–60. [PubMed: 11390355]

Pauli A, Althoff F, Oliveira RA, Heidmann S, Schuldiner O, Lehner CF, Dickson BJ, Nasmyth K. Cell-Type-Specific TEV Protease Cleavage Reveals Cohesin Functions in Drosophila Neurons. Dev Cell 2008;14:239–51. [PubMed: 18267092]

Skibbens RV, Corson LB, Koshland D, Hieter P. Ctf7p is essential for sister chromatid cohesion and links mitotic chromosome structure to the DNA replication machinery. Genes Dev 1999;13:307–19. [PubMed: 9990855]

Song X, Zhu CH, Doan C, Xie T. Germline stem cells anchored by adherens junctions in the Drosophila ovary niches. Science 2002;296:1855–7. [PubMed: 12052957]

Sonoda E, Matsusaka T, Morrison C, Vagnarelli P, Hoshi O, Ushiki T, Nojima K, Fukagawa T, Waizenegger IC, Peters JM, Earnshaw WC, Takeda S. Scc1/Rad21/Mcd1 is required for sister chromatid cohesion and kinetochore function in vertebrate cells. Dev Cell 2001;1:759–70. [PubMed: 11740938]

Spradling, AC. The development of Drosophila melanogaster. 1. C. S. H. L. Press; 1993. Development genetics of oogenesis; p. 1

Steffensen S, Coelho PA, Cobbe N, Vass S, Costa M, Hassan B, Prokopenko SN, Bellen H, Heck MM, Sunkel CE. A role for Drosophila SMC4 in the resolution of sister chromatids in mitosis. Curr Biol 2001;11:295–307. [PubMed: 11267866]

Sumara I, Vorlaufer E, Gieffers C, Peters BH, Peters JM. Characterization of vertebrate cohesin complexes and their regulation in prophase. J Cell Biol 2000;151:749–62. [PubMed: 11076961]

Tanaka K, Yonekawa T, Kawasaki Y, Kai M, Furuya K, Iwasaki M, Murakami H, Yanagida M, Okayama H. Fission yeast Eso1p is required for establishing sister chromatid cohesion during S phase. Mol Cell Biol 2000;20:3459–69. [PubMed: 10779336]

Tomonaga T, Nagao K, Kawasaki Y, Furuya K, Murakami A, Morishita J, Yuasa T, Sutani T, Kearsey SE, Uhlmann F, Nasmyth K, Yanagida M. Characterization of fission yeast cohesin: essential anaphase proteolysis of Rad21 phosphorylated in the S phase. Genes Dev 2000;14:2757–70. [PubMed: 11069892]

Toth A, Ciosk R, Uhlmann F, Galova M, Schleiffer A, Nasmyth K. Yeast cohesin complex requires a conserved protein, Eco1p(Ctf7), to establish cohesion between sister chromatids during DNA replication. Genes Dev 1999;13:320–33. [PubMed: 9990856]

Toyoda Y, Yanagida M. Coordinated requirements of human topo II and cohesin for metaphase centromere alignment under Mad2-dependent spindle checkpoint surveillance. Mol Biol Cell 2006;17:2287–302. [PubMed: 16510521]

Uemura T, Ohkura H, Adachi Y, Morino K, Shiozaki K, Yanagida M. DNA topoisomerase II is required for condensation and separation of mitotic chromosomes in S. pombe. Cell 1987;50:917–25. [PubMed: 3040264]

Uhlmann F, Lottspeich F, Nasmyth K. Sister-chromatid separation at anaphase onset is promoted by cleavage of the cohesin subunit Scc1. Nature 1999;400:37–42. [PubMed: 10403247]

Uhlmann F, Nasmyth K. Cohesion between sister chromatids must be established during DNA replication. Curr Biol 1998;8:1095–101. [PubMed: 9778527]

Vass S, Cotterill S, Valdeolmillos AM, Barbero JL, Lin E, Warren WD, Heck MM. Depletion of Drad21/Scc1 in Drosophila cells leads to instability of the cohesin complex and disruption of mitotic progression. Curr Biol 2003;13:208–18. [PubMed: 12573216]

Webber HA, Howard L, Bickel SE. The cohesion protein ORD is required for homologue bias during meiotic recombination. J Cell Biol 2004;164:819–29. [PubMed: 15007062]

Williams BC, Garrett-Engele CM, Li Z, Williams EV, Rosenman ED, Goldberg ML. Two putative acetyltransferases, san and deco, are required for establishing sister chromatid cohesion in Drosophila. Curr Biol 2003;13:2025–36. [PubMed: 14653991]

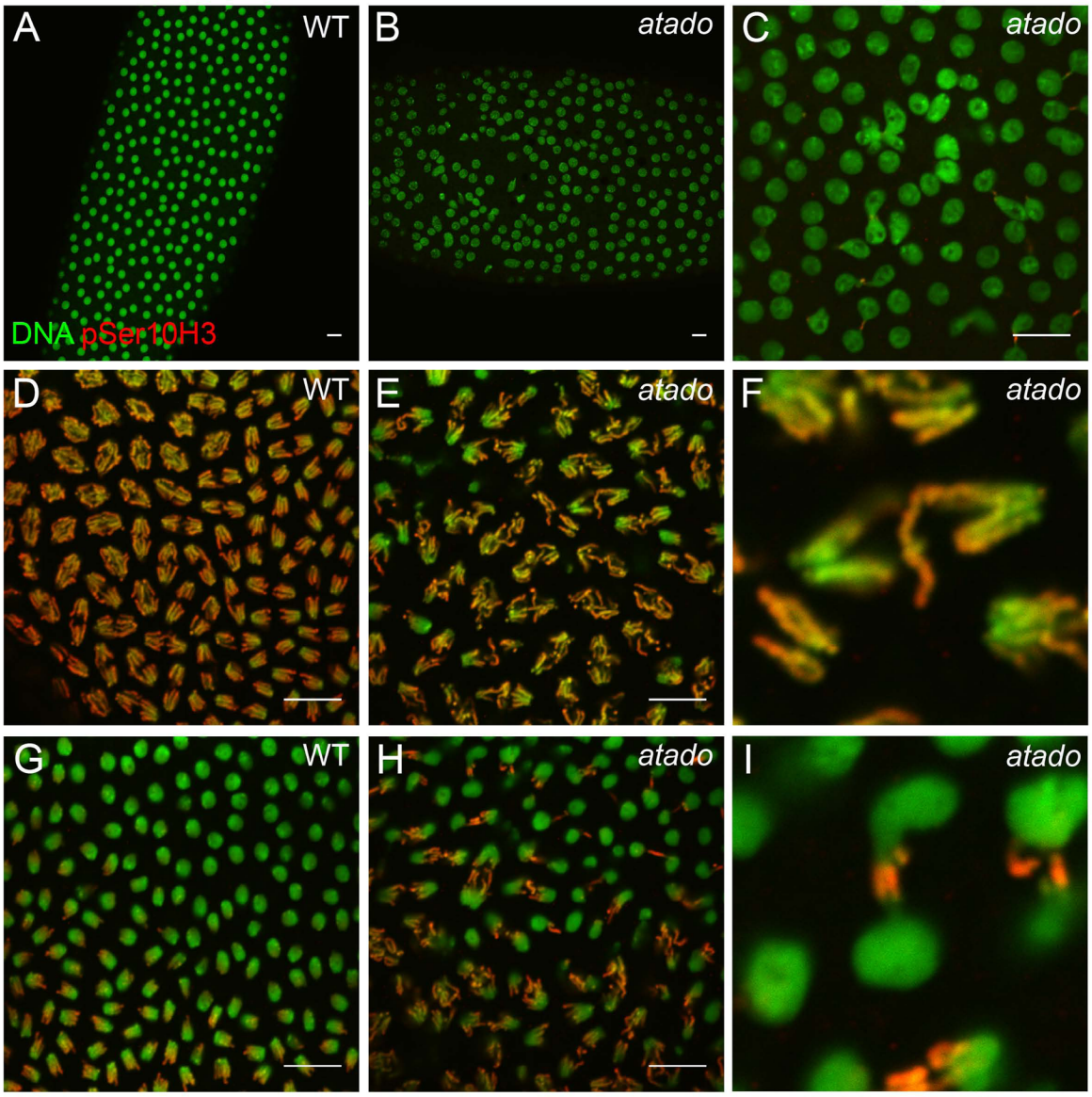


Figure 1. *atado* is required maternally for chromosome segregation during syncytial blastoderm

Embryos mutant for *atado*^{2} show abnormal anaphases (D, E), with a high frequency of chromosome bridges (A–C, G–I) and chromosome lagging (F). All panels show syncytial blastoderm embryos. *atado*^{2} mutant embryos were obtained after the induction of germ line clones (maternal mutants). All embryos were stained for DNA (green) and pSer10 Histone H3 (red). Low magnification of a wild type (A) and *atado*^{2} (B) embryo during interphase. (C) Shows a detail of the *atado*^{2} embryo shown in B. High magnification of a wild type (D) and *atado*^{2} (E) embryo during anaphase. (F) Shows a detail of the *atado*^{2} embryo shown in E. High magnification of a wild type (G) and *atado*^{2} (H) embryo during late anaphase/telophase. (I) Shows a detail of the *atado*^{2} embryo shown in H. pSer10 histone H3 staining marks condensed chromosomes. For quantitative data on the observed chromosome bridges, refer to Figure S1. Scale bars equals 10μm.

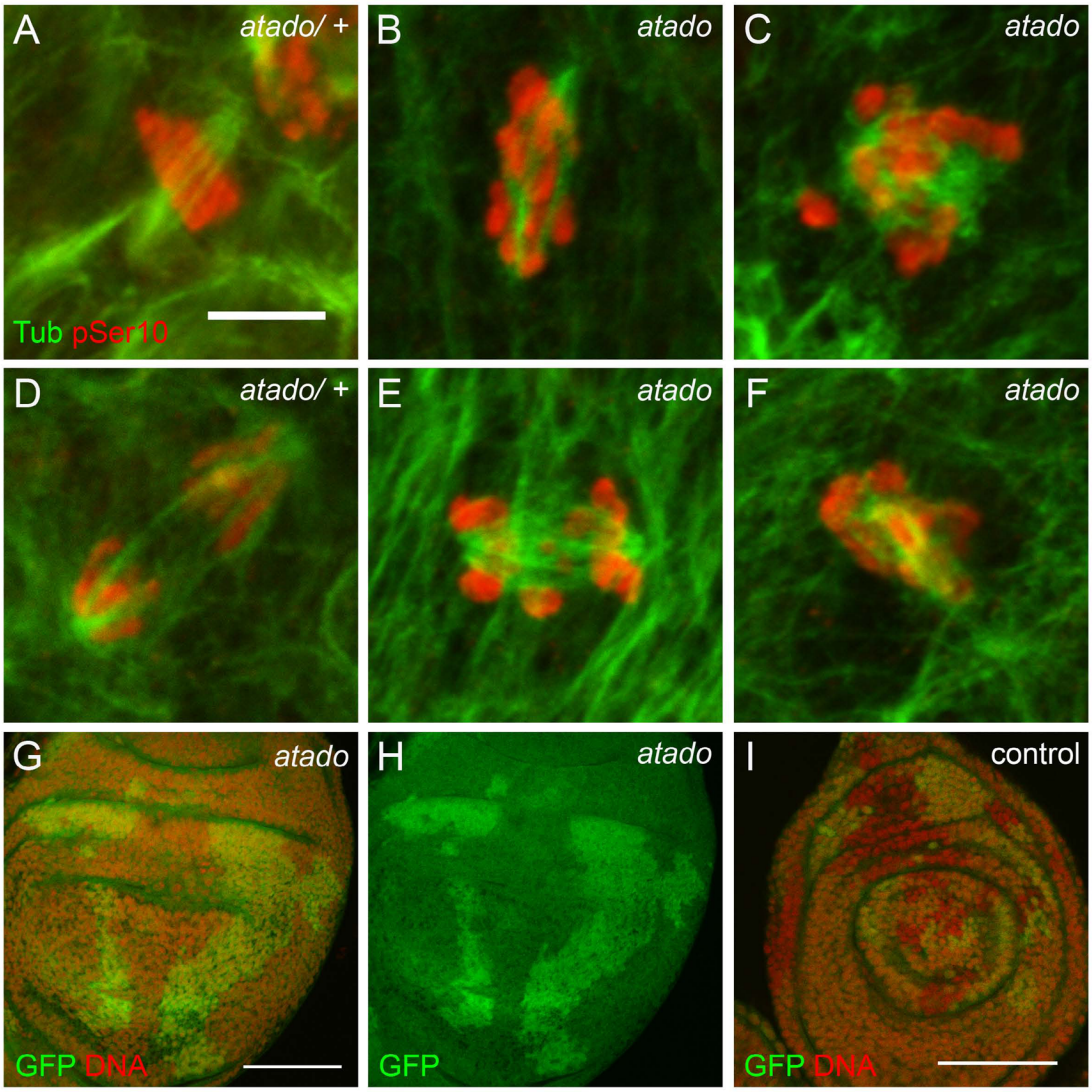


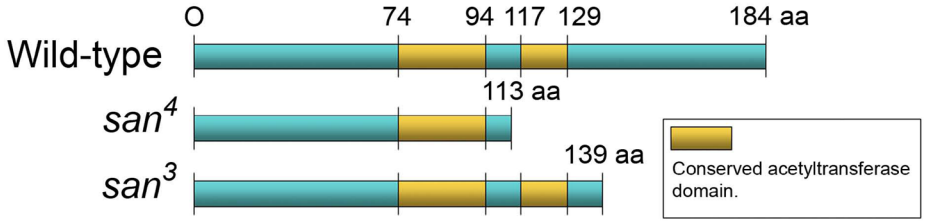
Figure 2. *atado* is required zygotically for normal mitosis of larvae neuroblasts and imaginal discs development

Larvae zygotically mutant for *atado*^{2} contain smaller brains (refer to Figure S3) and the neuroblasts show mitotic defects (A–F). Neuroblasts mutant for *atado*^{2} show chromosome congression defects during metaphase (A, B) and chromosome lagging during anaphase (D, E). In larvae imaginal discs, clones with two copies of GFP (control clones) are significantly bigger than the twin-clones without GFP (clones mutant for *atado*^{2}) (G–H). In control imaginal discs both types of clones have equivalent sizes (I). (A–F) Neuroblasts were stained for α-Tubulin (green) and pSer10 Histone H3 (red). (G–I) Imaginal discs were stained for DNA (red). Scale bars equals (A–F) 5μm, (G–H) 50μm, and (I) 30μm.

A

	san^3/ Cyo		san^4/ Cyo	
	Cy	Cy^+	Cy	Cy^+
san^2/Cyo	148	0	259	0

B



C

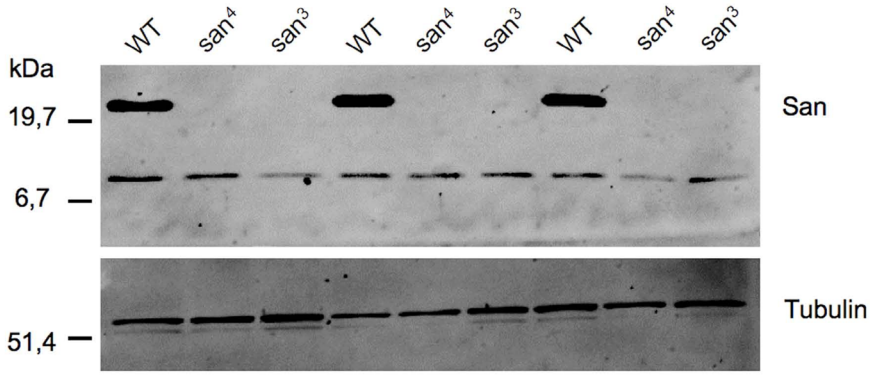


Figure 3. *atado* is allelic to *separation anxiety* (*san*)

Both alleles of *atado* contain nonsense mutations within *san* open-reading frame and are protein null for San. The mutations of both alleles of *atado* were mapped to a small cytological interval using the 2R deficiency kit. Using a candidate gene approach it was observed that both alleles of *atado* failed to complement a loss-of-function allele of the gene *separation anxiety* (san^2) (A). The *atado* alleles ($atado^1$ and $atado^2$) were therefore respectively renamed as san^3 and san^4. Both isolated alleles of *san* contain nonsense mutations within *san* open-reading frame, which will putatively lead to the truncation of San protein (B). Total protein extracts from embryos mutant for both alleles of *san* show absence or undetectable levels of San protein (C).

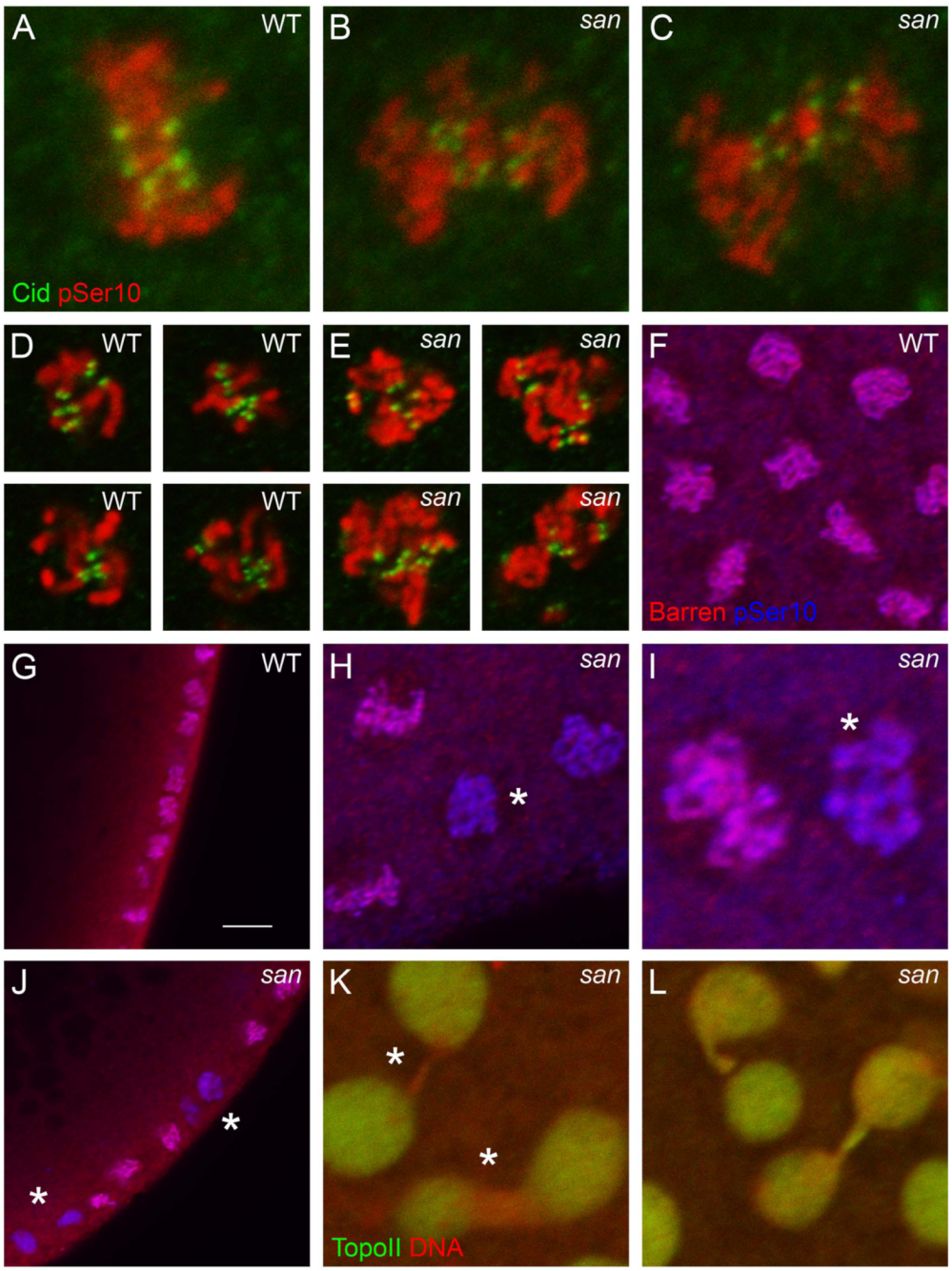


Figure 4. A subset of nuclei from syncytial blastoderm embryos mutant for *san* show reduced levels of Barren and Topoisomerase II

Embryos mutant for *san*^{4} show mild defects in chromosome congression and alignment during metaphase (A–C). *san*^{4} embryos incubated with colchicine arrest the cell cycle with condensed chromosomes and show a minor separation of the sister chromatid kinetochores (D, E). Subsets of mitotic nuclei in *san*^{4} embryos show a dramatic reduction in Barren localization (F–J; asterisks indicates reduction in Barren localization; see text for quantification). Subsets of interphasic nuclei in *san*^{4} embryos show reduced levels of TopoII localization specifically in the chromosome bridges (K, L; asterisk indicates reduction of TopoII). All panels show syncytial blastoderm embryos. (A–E) Embryos were stained for Cid (green) and pSer10

Histone H3 (red). (F–J) Embryos were stained for Barren (red) and pSer10 Histone H3 (blue). (K, L) Embryos were stained for Topoisomerase II (green) and DNA (red). (D, E) Embryos were incubated with colchicine for 15 min at room temperature. Scale bar equals 10μm.

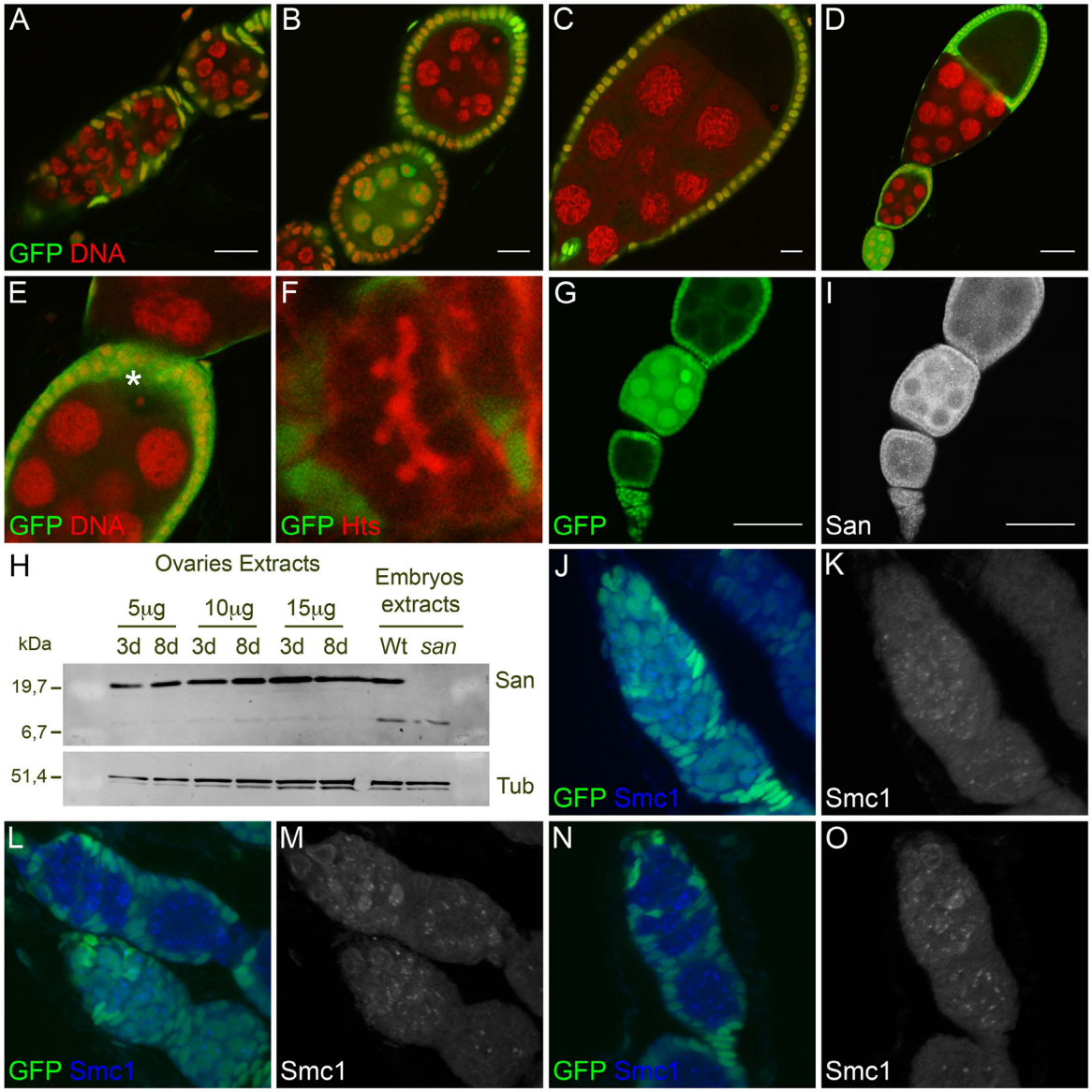


Figure 5. During oogenesis *san* is not required for germ line mitosis

Absence of endogenous GFP (green) indicates that the cells are homozygous for *san* mutations. Germ line mutant clones of *san*^{4} were induced at larvae stages using heat-shock-controlled flipase. Ovaries were dissected from adult females that were 7/8 (A–C, F, G, I–O) or 15 days (D, E) old after hatching (pupal eclosion). *Drosophila* germarium whose germ line is mutant for *san*^{4} develops normally (A). Germ line stem cells mutant for *san*^{4} divide normally since egg-laying is identical to the one observed in control females (refer to Figure S7) and egg-chambers mutant for *san*^{4} develop normally (A–D), with a normal condensed karyosome (E; asterisk indicates karyosome) and a normal fusome (F). San is expressed in the ovaries (H) and no obvious difference in the expression levels of San could be detected between young (3 days) and older females (8 days). San is expressed within the ovaries germ line (G, I). Germarium whose germ line is mutant for *san*^{4} (L–O) show Smc1 expression identical to the one observed in wild-type germarium (J, K). Absence of GFP indicates that the germ-line is mutant for *san*^{4}. (A–E) Ovaries were stained for DNA (red). (F) Ovary was stained for fusome marker Hts (red). (G, I) Ovary was stained for San (gray). (J–O) Ovaries were stained for Smc1 (blue

or gray). (H) Total ovaries and embryonic protein extracts were analyzed by SDS-PAGE and western blot using an anti-San polyclonal antibody. An anti-α-tubulin antibody was used as a loading control. Scale bars equals (A–C) 10μm, (D, G, I) 50μm. (E) Is a detailed view of the image shown in (D).