# Sisters Unbound Is Required for Meiotic Centromeric Cohesion in *Drosophila melanogaster*

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**ABSTRACT** Regular meiotic chromosome segregation requires sister centromeres to mono-orient (orient to the same pole) during the first meiotic division (meiosis I) when homologous chromosomes segregate, and to bi-orient (orient to opposite poles) during the second meiotic division (meiosis II) when sister chromatids segregate. Both orientation patterns require cohesion between sister centromeres, which is established during meiotic DNA replication and persists until anaphase of meiosis II. Meiotic cohesion is mediated by a conserved four-protein complex called cohesin that includes two structural maintenance of chromosomes (SMC) subunits (SMC1 and SMC3) and two non-SMC subunits. In *Drosophila melanogaster*, however, the meiotic cohesion apparatus has not been fully characterized and the non-SMC subunits have not been identified. We have identified a novel *Drosophila* gene called *sisters unbound* (*sunn*), which is required for stable sister chromatid cohesion throughout meiosis. *sunn* mutations disrupt centromere cohesion during prophase I and cause high frequencies of non-disjunction (NDJ) at both meiotic divisions in both sexes. SUNN co-localizes at centromeres with the cohesion proteins SMC1 and SOLO in both sexes and is necessary for the recruitment of both proteins to centromeres. Although SUNN lacks sequence homology to cohesins, bioinformatic analysis indicates that SUNN may be a structural homolog of the non-SMC cohesin subunit stromalin (SA), suggesting that SUNN may serve as a meiosis-specific cohesin subunit. In conclusion, our data show that SUNN is an essential meiosis-specific *Drosophila* cohesion protein.

**M**EIOSIS is a specialized cell division that generates haploid gametes from diploid precursor cells and is essential for sexual reproduction. Segregation of chromosomes during meiosis occurs in two stages called meiosis I and meiosis II that follow a single round of DNA replication. During meiosis I, homologs pair and orient toward opposite poles of the spindle (bi-orient) with sister centromeres oriented toward the same pole (mono-oriented). As a result, homologous chromosomes segregate to opposite poles at the onset of anaphase I in a reductional segregation pattern. In meiosis II, as in mitosis, the sister centromeres are bi-oriented and sister chromatids segregate to opposite poles at the onset of anaphase II, a pattern referred to as equational segregation (Page and Hawley 2003; Petronczki *et al.* 2003).

In most eukaryotes, pairing of homologs during meiosis I is facilitated and reinforced by synapsis and recombination. Synapsis involves formation of elaborate zipper-like structures, called synaptonemal complexes (SCs), which hold homologs tightly together during prophase I. SCs are composed of the tightly paired sister chromatid axes of the two homologs, known as axial elements (AEs) before synapsis or as lateral elements (LEs) after synapsis, cross-linked by multiple transverse filament (TF) proteins. Synapsis initiates at a limited number of discrete sites of homolog alignment during zygotene and subsequently spreads until SCs form continuous chromosome-length structures during pachytene (Page and Hawley 2004). Most synapsis initiation sites appear to be in the euchromatin but in some eukaryotes, including yeast, several plant species, and female *Drosophila*, synapsis also initiates at centromeres and is preceded by homologous and/or non-homologous pairing of centromeres (Khetani and Bickel 2007; Stewart and Dawson 2008; Takeo *et al.* 2011; Tanneti *et al.* 2011).

Recombination overlaps temporally with synapsis and involves programmed formation and repair of double-strand breaks, resulting in high levels of exchange (crossing over) between homologous chromatids. After SC disassembly at the end of pachytene, homolog crossovers, stabilized by cohesion between sister chromatid arms, serve as stable interhomolog linkers known as chiasmata (Page and Hawley 2003; Kleckner 2006). Some eukaryotes achieve stable homolog pairing and regular homolog segregation during meiosis I without SCs or recombination. In *Drosophila* males, which lack meiotic recombination, stable homolog connections are provided by a male-specific "homolog conjunction complex" that serves as a functional substitute for chiasmata and is removed at anaphase I (Thomas *et al.* 2005).

Proper chromosome segregation at both meiotic divisions, as well as in mitosis, requires cohesion between sister chromatids provided by conserved four-protein complexes called cohesins. The mitotic cohesin complex is composed of SMC1, SMC3, SCC1/MCD1/RAD21 (henceforth called RAD21), and SCC3/stromalin/SA (henceforth called SA). SMC1, SMC3, and RAD21 form a tripartite ring structure that is thought to embrace the newly formed sister chromatids during S phase. Cleavage of the RAD21 subunit by the conserved protease Separase at anaphase releases cohesion and allows sister chromatids to segregate to the poles. SA is an all α-helical protein that binds to RAD21. It is essential for cohesion but its precise role in cohesion remains unclear. Meiotic cohesins are similar in composition to mitotic cohesin but frequently contain one or more paralogous meiosis-specific subunits that replace their mitotic counterparts. Most such paralogs are restricted to fairly narrow taxonomic lineages and have specialized functions, but REC8 replaces RAD21 in most meiotic cohesins and is required for nearly all meiotic chromosome interactions in most eukaryotes (Lee and Orr-Weaver 2001; Nasmyth 2001; Nasmyth and Haering 2009).

In meiosis I, cohesin is abundant all along the chromosome axes but arm and centromere cohesion play distinct roles in meiosis. During meiosis I, arm cohesion stabilizes the chiasmata that provide resistance to poleward forces required for homologs to biorient on the meiosis I spindle. Release of arm cohesion at the onset of anaphase I, by separase-mediated cleavage of REC8, destabilizes chiasmata and serves as the triggering event for homolog segregation (Petronczki *et al.* 2003; Nasmyth and Haering 2009). Arm cohesins also play important roles in synapsis and recombination during meiosis I although it remains unclear to what extent those roles are related to arm cohesion (Nasmyth 2001; Brar *et al.* 2009; Nasmyth and Haering 2009). Cohesion between sister centromeres enables sister chromatids to biorient on the meiosis II spindle and is preserved until a second round of separase activation at anaphase II cleaves centromeric cohesins and triggers sister chromatid separation. Preservation of centromeric cohesins during anaphase I is mediated by shugoshins, which are centromere proteins that inhibit separase cleavage of cohesin (Watanabe 2005; Clift and Marston 2011).

Centromere cohesion is also required during meiosis I, to enable sister centromeres to mono-orient. Mono-orientation is thought to require a "side-by-side" alignment of sister centromeres (rather than the "back-to-back" alignment characteristic of mitosis or meiosis II) enabling them to form a functionally single kinetochore that binds microtubules from only one pole (Hauf and Watanabe 2004). In *Schizosaccharomyces pombe*, this specialized centromere orientation entails establishing cohesion within the kinetochore-forming centromere core domain and requires both REC8 cohesin and a specialized meiosis-specific centromere protein called Moa1 (Watanabe and Nurse 1999; Yokobayashi and Watanabe 2005). In *S. cerevisiae*, both cohesin and a meiosis I-specific centromere complex called monopolin are required for regular mono-orientation (Toth *et al.* 2000). In several higher eukaryotes, mutations in *rec8* or other cohesion genes have also been found to disrupt mono-orientation (Klein *et al.* 1999; Pasierbek *et al.* 2001; Cai *et al.* 2003; Wang *et al.* 2003; Chelysheva *et al.* 2005; Golubovskaya *et al.* 2006; Severson *et al.* 2009). However, no specific mono-orientation factors have been identified in higher eukaryotes and the mechanism of mono-orientation remains unclear.

*Drosophila* has been a major model for meiotic studies for more than a century. However, insight into the mechanism and roles of cohesion in *Drosophila* meiosis has been hampered by limited data on the composition of the meiotic cohesion apparatus. Recent findings have pointed to meiotic roles for the cohesin SMC proteins. SMC1 localizes to centromeres during meiosis in both sexes and persists on centromeres until anaphase II in male meiosis (Khetani and Bickel 2007; Yan *et al.* 2010). Both SMC1 and SMC3 localize to LEs in female prophase I and loss of either protein completely ablates formation of LEs and SCs (Khetani and Bickel 2007; Tanneti *et al.* 2011; Yan and McKee 2013). However, as yet there is only indirect evidence for roles of SMC1 and SMC3 in arm or centromere cohesion. Moreover, the non-SMC subunits of meiotic cohesins in *Drosophila* remain poorly characterized. There are no published reports relevant to a meiotic role of SA. Although RAD21 was recently shown to localize to lateral elements in female meiosis and to be required for SC stability, neither premature cleavage or degradation of RAD21 during prophase I nor mutation of its Separase cleavage sites had any effect on sister chromatid cohesion or on chromosome segregation at either meiotic division (Urban *et al.* 2014). In addition, unlike all other characterized eukaryotes, *Drosophila* appears to lack a true REC8 homolog. The *Drosophila* genome does encode a meiosis-specific RAD21 paralog, C(2)M, that localizes to LEs and is required for synapsis, SC formation, and normal levels of recombination (Manheim and McKim 2003). However, C(2)M does not form centromeric foci and is not required for either centromere or arm cohesion during the meiotic division stages in female meiosis or for any aspect of male meiosis (Manheim and McKim 2003; Heidmann *et al.* 2004) .

Curiously, the best-characterized meiotic cohesion genes in *Drosophila* are two genes with no apparent homology to

any of the cohesins: *orientation disruptor* (*ord*) and *sisters on the loose* (*solo*) (Bickel *et al.* 1996; Yan *et al.* 2010). Mutations in both genes cause premature loss of sister centromere cohesion, accompanied by absence of centromeric SMC1 foci, leading to very high frequencies of both homolog and sister chromatid non-disjunction (NDJ) (Miyazaki and Orr-Weaver 1992; Bickel *et al.* 1997; Balicky *et al.* 2002; Yan *et al.* 2010). Both ORD and SOLO co-localize with SMC1 on centromeres in both sexes and persist there until anaphase II in male meiosis, disappearing simultaneously with SMC1 (Balicky *et al.* 2002; Khetani and Bickel 2007; Yan *et al.* 2010). Consistent with a cohesin-related role, SOLO was recently shown to reciprocally coimmunoprecipitate with SMC1 from ovary extracts (Yan and McKee 2013). These findings have led to suggestions that, despite their lack of sequence homology to cohesins, ORD and SOLO might be functional homologs of REC8 (Khetani and Bickel 2007; Yan *et al.* 2010; Yan and McKee 2013).

Here we describe a third *Drosophila*-specific, meiosis-specific cohesion gene, *sunn* (*sisters unbound*), with properties remarkably similar to those of *ord* and *solo*. *sunn* mutations cause high levels of both meiosis I and meiosis II NDJ in both sexes. In male meiosis, SUNN localizes primarily to centromeres until anaphase II and is required for centromeric cohesion, for mono-orientation of sister centromeres during meiosis I and for stable centromere recruitment of SMC1 and SOLO. In female meiosis, SUNN also localizes to centromeres during prophase I and is required for centromere pairing and cohesion during pachytene. These data identify SUNN as a major component of the meiotic cohesion apparatus in *Drosophila*. Although no sequence homologs of SUNN were identified in genome searches, structure-based bioinformatic analysis revealed similarity between SUNN and the *Drosophila* cohesin subunit stromalin (SA), suggesting SUNN's possible role as a meiosis-specific cohesin subunit.

## Materials and Methods

### Fly stocks and Drosophila culturing

*sunn* mutations were obtained from the Zuker-3 (Z3) collection of EMS mutagenized third chromosomes (Koundakjian *et al.* 2004). The Z3- lines used in this study were identified in a screen for loss of paternal 4th chromosomes (Wakimoto *et al.* 2004). *Dp (1:1) sc^{v1}* was obtained from Kim McKim (Rutgers University). All of the chromosome 3 deficiency stocks and compound chromosome stocks used in the crosses were obtained from the Bloomington Stock Center (Indiana University). Details about markers and special chromosomes can be found in FlyBase and the Bloomington Stock Center webpage (http://flystocks.bio.indiana.edu/).

Flies were cultured at 22° on a food mix containing cornmeal, malt, corn syrup, yeast, and propionic acid (antifungal agent) and crosses were set and maintained at 22°. Progeny from the cross were scored between 14 and 21 days after the cross was set.

### Measuring NDJ

The methods for measuring male NDJ are explained in the *Results* section and in the legends to Table 1 and Table 2. To measure female NDJ, *Dp(1;1)sc^{v1}, y .y^+/+; sunn/Df* females were crossed singly to two males of the genotype *YSX.YL, In (1)EN, y B/Y*. Regular segregation yields B females and B^+ males. Progeny from diplo-X and nullo-X- non-disjunctional eggs are B^+ females and yellow body y B males, respectively. The B^+ daughters carry two maternal X chromatids and were classified as resulting from sister chromatid NDJ if they were y. *Dp(1;1) sc^{v1}* has a duplication of the tip of the X chromosome on the right arm that carries the dominant $y^+$ marker. Both X chromosomes have recessive y alleles at the native locus near the tip of XL. There is no recombination between the duplicated $y^+$ allele and the X centromere so a NDJ female lacking both copies of the $y^+$ allele is expected to carry two sister centromeres.

### Mapping and identification of sunn mutations

Mapping of *sunn* alleles was performed by deficiency complementation against the 3rd chromosome deficiency kit (Bloomington Stock Center) using the X-Y NDJ phenotype. *sunn* location was narrowed down to a critical region, 68C8–68D6, on chromosome arm 3L using the following chromosome deficiencies (deleted region in parenthesis)—*Df(3L)vin6* (68C8–69A5), *Df(3L)vin2* (67F2–68D6), *Df(3L)vin3* (68C5–68E4), *Df(3L)vin5* (68A2–69A1), *Df(3L)vin4* (68B1–68F6), *Df(3L)vin7* (68C8–69B5), *Df(3L)ED4470* (68A6–68E1), and *Df(3L)BK9* (68E2–69A1). Exons of candidate genes from the critical region were amplified by PCR using the genomic DNA of *sunn* mutants and sequenced (Cycle Sequencing Kit, Life Technologies) to identify single nucleotide polymorphisms (SNPs).

### Generation of sunn cDNA clone and UAS–SUNN:: Venus transgene

*sunn* cDNA was amplified from total ovary RNA of *y w* (*yellow white*) females using Superscript^R III Reverse Transcriptase (Invitrogen) and Pfx polymerase (Invitrogen). Total RNA for reverse transcription was extracted using TRI-reagent (Sigma-Aldrich) and treated with DNase I (Invitrogen) before reverse transcription. *sunn* cDNA was amplified in two overlapping fragments, the first fragment stretching from the 1st exon to the 6th exon and the second fragment extending from the 6th exon to the 10th exon using Pfx polymerase (Invitrogen) and the following primers: First fragment forward, ATGGAATTTGTAAGCGCCATTTCGA and reverse, CATCACACTCTGCTACTGAGTCAA; second fragment forward, GAATTGAGCCTTATTGCTGCGCAA and reverse, ATCAGTTAGATCTGTTGTATTATGAATAGTTTTAATCT. The two fragments were cloned separately into pJet 1.2/Blunt vector (Fermentas) using CloneJET PCR cloning kit (Fermentas) then ligated together into a pJet 1.2/Blunt vector using restriction sites common to the overlapping fragments. The cDNA was then transferred to pENTR4 (Invitrogen) and recombined into the Gateway *P*-element vector pPWV 1094 (*Drosophila* Genomics Resource Center), which contains a C-terminal Venus tag and UAS

**Table 1 Sex chromosome NDJ in *sunn* mutant males**

| | Sperm genotype | | | | | |
|---|---|---|---|---|---|---|
| Paternal genotype | X | Y | XY | O | n[a] | %NDJ[b] |
| *sunn*[Z3-1956]/Df[c] | 337 | 353 | 177 | 337 | 1204 | 42.7 |
| *sunn*[Z3-5839]/Df[c] | 388 | 416 | 181 | 419 | 1404 | 42.7 |
| *sunn*[Z3-4085]/Df[c] | 344 | 353 | 158 | 400 | 1255 | 44.5 |
| *sunn*[Z3-5839]/*sunn*[Z3-5839] | 286 | 266 | 92 | 311 | 955 | 42.2 |
| Total *sunn* | 1355 | 1388 | 608 | 1467 | 4818 | 43.1 |
| Gamete frequency (%) | 28.1 | 28.8 | 12.6 | 30.4 | — | — |
| Df[c]/+ (WT) | 803 | 735 | 0 | 2 | 1540 | 0.1 |

*w*/B[S]Yy[+] males with the indicated third chromosome genotype for *sunn* were each crossed to two *y w* females. The dominant B[S] marker causes Bar eyes and was used to determine whether progeny inherited the Y chromosome.
[a] Total number of progeny scored.
[b] %NDJ = 100 × (XY+ O)/n.
[c] Df (3L) ED4470.

sequences, using Gateway[R] LR Clonase II Enzyme Mix (Invitrogen). The resulting construct was transformed into *w*[1118] flies by BestGene.

### Determining the 5′ and 3′ ends of Sunn mRNA using RACE

Total RNA was extracted from ovaries of *y w* (yellow white) females using TRI-reagent (Sigma Aldrich) and treated with DNase I (Invitrogen). RACE was performed using First-Choice RLM-RACE kit (Ambion Inc). The length of the 5′ UTR determined by 5′ RLM-RACE was 69 bp. The 5′ UTR of CG32088 shown in FlyBase is 72 bp, longer by 3 bp at the 5′ end when compared to the sequence we determined. The 3′ UTR determined by 3′ RACE was found to be 75 bp long and expected to have the features that should be present in a 3′ UTR of the mRNA and the surrounding DNA sequence: a consensus polyadenylation sequence 10–25 bp upstream of the mRNA cleavage site and a conserved element located within 30 bp, downstream of the cleavage site (Retelska *et al.* 2006). The putative polyadenylation sites, AGUAAA and UAUAAA, are located 23 bp and 32 bp upstream of the cleavage site, respectively, and a U-rich downstream element is positioned 21 bp downstream of the cleavage site. The 3′ UTR for CG32088 shown in FlyBase is 105 bp long, but it lacks essential features of a 3′ UTR. Primer sequences used for 5′ RLM RACE and 3′ RACE are available upon request.

### Generation of Venus::SMC1 transgene

*smc1* was PCR amplified from a *smc1* cDNA clone using the following primers: forward, CACCATGACCGAAGAGGACGA CG and reverse, TTACGTGTCCTCGAACGTTGTC. The product was cloned into pENTR/D-TOPO vector (Invitrogen) and the entry clone was recombined with Gateway *P*-element vector pPVW (1093) (*Drosophila* Genomics Research Center) using Gateway[R] LR Clonase II Enzyme Mix (Invitrogen). This vector contains an N-terminal Venus tag and UAS. The construct was transformed into *w*[1118] flies (BestGene).

### Testis immunostaining

Testes were dissected and fixed according to Cenci *et al.* (1994). Immunostaining was performed using the protocol described

in Bonaccorsi (2000) with modifications. Testes were dissected in 1× phosphate buffered saline (PBS) (137 mM Nacl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.7 H_2O$, 1.4 mM $KH_2PO_4$) and covered with Sigmacote (Sigma Aldrich) treated cover slips and frozen in liquid nitrogen. Cover slips were removed and slides were immersed in −20° ethanol for 20 min, followed by 10 min in PBS solution containing 4% formaldehyde. Slides were washed twice with PBT (PBS with 0.2% Triton X-100) and blocked with 1% BSA–PBT solution. Primary antibodies were diluted in 1% BSA–PBT solution and secondary antibodies were diluted in PBT solution. Primary antibody incubations were done for 12–16 hr at 4° and secondary antibody incubations were done for 1 hr at room temperature. Antibody incubations were followed by PBT washes and finally DAPI stain was incubated for 20 min followed by PBS washes and slide mounting using Vectashield (Vector Laboratories). For identifying centromere cohesion phenotypes, a rabbit anti-centromere identifier (CID) primary antibody (Active Motif) and Alexa Fluor 555, a donkey anti-rabbit IgG secondary antibody (H+L, Invitrogen) were used at 1:1000 dilutions. For the Venus::SMC1 and GFP–LacI localization experiments, native fluorescence was used to detect the tagged proteins. Slides were prepared according to the above protocol without the antibody staining steps. For the anti-tubulin/DAPI experiment, immunostaining was performed according to Thomas *et al.* (2005) using FITC-conjugated monoclonal anti-tubulin antibody (Sigma) at a 1:150 dilution. Meiosis I and meiosis II cells were discriminated on the basis of number of cells per cyst (16 or 32, respectively) and size of DAPI-stained masses. The criteria for meiosis I and II substages are described in Cenci *et al.* (1994).

### Fluorescent in situ hybridization

Fluorescent *in situ* hybridization (FISH) experiments were performed according to Balicky *et al.* (2002) with modification (Thomas *et al.* 2005). The 359 bp satellite-repeat probe was amplified by PCR according to Thomas *et al.* (2005) and labeled using the Fluorescein-High Prime kit (Roche). The AATAC repeat probe was synthesized as a single-stranded oligonucleotide (IDT Biophysics) and labeled with Alexa Fluor 546 (Invitrogen) using terminal deoxynucleotidyl transferase (Promega).

### Ovary immunostaining

Virgin females were placed in a food vial with yeast paste and males. After 2 days, their ovaries were dissected, fixed, and stained using the protocol described in Page and Hawley (2001). Slides were mounted using Prolong Gold Antifade reagent (Invitrogen). To determine centromeric clustering and cohesion phenotypes, rabbit anti-CID (Active Motif) and mouse anti-C(3)G (Scott Hawley, Stowers Institute for Medical Research) primary antibodies were used at 1:1000 dilutions. Alexa Fluor 488 donkey anti-rabbit IgG (H+L, Invitrogen) and Alexa Fluor 555 donkey anti-mouse IgG (H+L, Invitrogen) secondary antibodies were used at 1:1000 dilutions. For the CID spot counts, C(3)G positive cells in germaria and stage 2 were identified as oocytes/pro-oocytes.

**Table 2 Sister chromatid *vs.* homolog NDJ in *sunn* mutant males**

| Paternal genotype | Sperm genotype | | | | | $n^a$ | %NDJ[b] | %sis[c] |
| | X | Y | XY | XX | O | | | |
| | Progeny phenotype | | | | | | | |
| | w B$^+$ ♂ | su-w$^a$ B$^S$ ♀ | w B$^S$ ♂ | w B$^+$ ♀ | su-w$^a$ B$^+$ ♀ | | | |
|---|---|---|---|---|---|---|---|---|
| *sunn$^{Z3-5839}$/Df* | 388 | 234 | 270 | 64 | 265 | 1221 | 54.3 | 32.2 |
| *sunn$^{Z3-1956}$/Df* | 366 | 265 | 223 | 56 | 279 | 1189 | 51.6 | 33.4 |
| *sunn$^{Z3-4085}$/Df* | 256 | 188 | 138 | 50 | 264 | 896 | 56.0 | 42.0 |
| Total *sunn* | 1010 | 687 | 631 | 170 | 808 | 3306 | 53.8 | 35.0 |
| Gamete frequency (%) | 30.6 | 20.8 | 19.1 | 5.1 | 24.4 | — | — | — |
| *Df*/+ (WT) | 730 | 489 | 0 | 1 | 1 | 1221 | 0.3 | 1 |

*w/B$^S$Yy$^+$* males with the indicated third chromosome genotype for *sunn* were each crossed to two *C(1)RM, y$^2$ su (w$^a$) w$^a$/O* females. These females produce only diplo-X and nullo-X eggs and permit recovery of viable progeny derived exclusively from sister chromatid NDJ sperm (XX), homolog NDJ sperm (XY), and nullo-XY (O) sperm (which result from both types of NDJ). The diplo-X eggs yield viable progeny when fertilized by Y or nullo-XY (O) sperm. These progeny exhibit a suppressed white-apricot (light brown) eye color (su-w$^a$) caused by the *su (w$^a$)* and *w$^a$* alleles on *C(1)RM*. The nullo-X eggs yield viable progeny when fertilized by X, XY, XX, or XXY sperm. These progeny all have white eyes because of the null *w* allele carried on the paternal X chromosome. Progeny were classified by sperm genotype as described above in column labels.

[a] Total number of progeny scored.

[b] % NDJ = 100 × ((2 × XX) + XY + O)/ n.

[c] % sister chromatid NDJ = 100 × (2 × XX)/ ((2 × XX) + XY).

Notes: (1) Progeny with one or two copies of *B$^S$Yy$^+$* cannot be discriminated, so some progeny scored as derived from Y or XY sperm could have been YY or XYY. (2) In the % NDJ and %sis formulae, the XX sperm-derived progeny are doubled to account for the YY sperm-derived progeny, which cannot be discriminated from regular Y sperm-derived progeny and are poorly viable. (3) Two, seven, and two progeny derived from XXY non-disjunctional sperm were recovered from *sunn$^{Z3-5839}$/Df*, *sunn$^{Z3-1956}$/Df*, and *sunn$^{Z3-4085}$/Df* hemizygotes, respectively (not shown in table).

CID foci were counted to be part of an oocyte if they were within the C(3)G-stained and DAPI-stained boundary of the cell. [Note: C(3)G is a transverse filament protein that provides a useful marker of the SC (Page and Hawley 2004)]. For quantification, non-overlapping CID foci were counted as separate spots. To determine SMC1 localization to centromeres, guinea pig anti-SMC1 (Sharon Bickel, Dartmouth University) and rabbit anti-CID (Active Motif) primary antibodies were used at 1:2000 and 1:1000 dilutions, respectively. Alexa Fluor 488 goat anti-guinea pig IgG (H+L, Invitrogen) and Alexa Fluor 647 donkey anti-rabbit IgG (H+L, Invitrogen) were used as secondary antibodies at 1:1000 dilutions. Classification of oocyte stages was done according to Matthies (2000).

### Microscopy

All micrographs were obtained using an Axioplan microscope (Zeiss), which is equipped with a HBO 100 W mercury lamp. This microscope is fitted with a high-resolution charge-coupled device camera (Roper Industries). Metamorph software (Universal Imaging) was used to acquire pictures, pseudocolor them, and merge them together. For all immunostaining and FISH images, Z-series pictures were taken, deconvolved, and merged/stacked using sum algorithm. Images and figures were prepared using Adobe Photoshop (CS2), Adobe Illustrator, and Microsoft PowerPoint.

## Results

### Mutation of sunn causes homologous and sister chromatid NDJ in both male and female meiosis

Three alleles of *sunn* were identified in a screen of the Zuker-3 collection of EMS-treated third chromosomes for mutants that showed increased rates of fourth chromosome loss in male meiosis (Koundakjian *et al.* 2004; Wakimoto *et al.* 2004). Males hemizygous for each *sunn* allele and that carried a genetically marked Y chromosome (*B$^s$Yy$^+$*) were tested for X and Y chromosome NDJ in crosses to chromosomally normal females. Progeny from XY and nullo-XY (O) sperm were recovered at frequencies of 42–45% in all three *sunn* mutants compared to <0.2% in wild-type (WT) controls (Table 1). A similar NDJ frequency was obtained in homozygous *sunn$^{Z3-5839}$* males (Table 1). Taken together, these data suggest, but do not prove, that all three *sunn* alleles are genetic null alleles.

The results in Table 1 show that *sunn* mutations cause high frequencies of homolog NDJ but do not address whether *sunn* mutations also cause sister chromatid NDJ. The diagnostic sperm class for NDJ of X sister chromatids is XX sperm, which yield inviable XXX progeny in crosses to chromosomally normal females. To detect XX sperm and compare the frequencies of homolog and sister chromatid NDJ, *sunn* males were crossed to females carrying an attached X chromosome [*C(1)RM/O*], which produce only diplo-X and nullo-X eggs in roughly equal proportions. In such crosses, all major sperm classes, including the XX, XY, and nullo-XY (O) NDJ classes, yield viable progeny in combination with one of the egg classes (see Table 2 legend for detailed explanation). Males hemizygous for the three *sunn* alleles produced XX, XY, and O non-disjunctional sperm at average frequencies of 5.1, 19.1, and 24.4%, respectively (Table 2), indicating that *sunn* mutations cause NDJ of both homologous and sister chromatids. The average NDJ frequency was 53.8% and the average relative frequency of sister chromatid NDJ out of total NDJ was 35%. In this assay, as in the previous one, differences among the three alleles were minor and insignificant. These results are consistent with random sex chromatid assortment through both meiotic

**Table 3 X chromosome NDJ in *sunn* mutant females**

| Maternal genotype | Progeny types | | | | | $n^d$ | %NDJ[e] | %sis[f] | P/F[g] |
|---|---|---|---|---|---|---|---|---|---|
| | DJ[a] B♀ | DJ[a] B[+]♂ | NDJ[b] y[+] B[+]♀ | NDJ(Sis)[b,c] y B[+]♀ | NDJ[b] y B♂ | | | | |
| *sunn*[Z3-5839]/Df | 219 | 198 | 146 | 19 | 124 | 706 | 58.1 | 23.0 | 11.6 |
| *sunn*[Z3-4085]/Df | 264 | 174 | 136 | 19 | 123 | 716 | 55.9 | 24.5 | 10.9 |
| *sunn*[Z3-1956]/Df | 268 | 149 | 123 | 21 | 128 | 689 | 56.6 | 29.2 | 11.9 |
| Total *sunn* | 751 | 521 | 405 | 59 | 375 | 2111 | 56.9 | 25.4 | — |
| Gamete freq. (%) | 35.6 | 24.7 | 19.2 | 2.8 | 17.8 | — | — | — | — |
| Df/+ | 1033 | 879 | 0 | 0 | 1 | 1913 | 0.1 | NA[h] | 79.7 |

Dp(1;1)sc[v1], y .y[+]/y females with the above third chromosome genotypes were crossed with two YSX.YL, In(1)EN, y B/Y males.
[a] DJ: progeny from normal (disjunctional) eggs.
[b] NDJ: progeny from NDJ eggs. The B[+] daughters result from diplo-X eggs and the y B sons from nullo-X eggs.
[c] NDJ(Sis): The y B[+] daughters derive from diplo-X eggs carrying two sister chromatids lacking the y[+] centromere marker, so represent sister chromatid NDJ only. The other two NDJ categories reflect a mix of sister chromatid and homolog NDJ.
[d] n: total number of progeny counted.
[e] %NDJ = 100 × 2 (NDJ)/ (n + NDJ).
[f] %sis = % sister chromatid NDJ = 2 × (y B[+]♀)/(y B[+]♀ + y[+] B[+]♀).
[g] P/F (progeny/female) = average number of progeny a single female produces when crossed to two males. [h]Not applicable.

divisions, as might result from loss of sister chromatid cohesion prior to meiosis I. Similar NDJ frequencies and patterns have been reported for null alleles of *ord* and *solo* (Miyazaki and Orr-Weaver 1992; Bickel *et al.* 1997; Yan *et al.* 2010). Mutation of *sunn* also causes high frequencies of both sister chromatid and homologous chromosome NDJ of the autosomal second (Supporting Information, Table S1) and fourth chromosomes (data not shown).

To determine whether *sunn* mutations also cause sex chromosome NDJ in female meiosis, *sunn* hemizygous females were crossed with males carrying a dominant *Bar* (*B*) mutation on their X chromosomes. The regular progeny from this cross are B females and B[+] males; the NDJ progeny are B[+] females and B males. The results showed that 56.9% of progeny from *sunn* females resulted from X-X NDJ compared to 0.1% in sibling WT control females (Table 3). Analysis of centromere-linked markers revealed that 25.4% of the B[+] females carried two maternal sister chromatids and the remainder carried two maternal homologous chromatids, indicating that both homologous and sister chromatids nondisjoin in *sunn* females (Table 3). Thus, like *ord* and *solo*, *sunn* is required for proper chromosome segregation in both meiotic divisions in both sexes (Miyazaki and Orr-Weaver 1992; Bickel *et al.* 1997; Yan *et al.* 2010; Yan and McKee 2013).

### sunn mutations disrupt sister chromatid cohesion during male meiosis

For an in-depth study of the NDJ mechanism in *sunn* mutants, we surveyed chromosome and nuclear morphology throughout male meiosis by staining spermatocytes with DAPI to label chromosomes and with a α-tubulin antibody to label spindles. WT male meiosis I occurs synchronously in interconnected cysts of 16 primary spermatocytes derived from a single germline stem cell. Although axial elements and synaptonemal complexes are absent and the chromosomes are decondensed, *Drosophila* spermatocytes traverse a series of prophase I substages, labeled S1–S6, during which the chromosomes undergo distinctive changes, the most prominent of which are the separation of the four bivalents into distinct nuclear territories near the end of stage S2 and their condensation during stage S6 to form four compact and roughly spherical bivalents. The bivalents then congress during prometaphase I to form a tight metaphase I bundle and segregate reductionally at anaphase I to form daughter nuclei with equal staining intensity. After a brief prophase II, meiosis II univalents recondense, congress, and segregate equationally at anaphase II, yielding cysts of 64 spermatids with round nuclei of uniform size (Cenci *et al.* 1994).

Despite the high rates of meiosis I NDJ in genetic crosses, DAPI-stained *sunn* spermatocytes appeared remarkably normal during meiosis I (Figure S1A). As in WT, three large DAPI-stained territories, corresponding to the X-Y, 2nd and 3rd chromosome bivalents, were present throughout mid- and late-prophase I, indicating that homolog pairing and territory formation are intact in *sunn* mutants. Sometimes, a small fourth territory is observed in WT and *sunn* mutants, which corresponds to 4th chromosome bivalent. The territories condensed into compact "blobs" by prometaphase I, congressed normally and segregated at anaphase I to form daughter nuclei that in most cases appeared to contain roughly equal amounts of chromatin. However, prematurely separated sister chromatids were common during and after anaphase I, and meiosis II was chaotic in *sunn* mutants. We frequently observed single chromatids during prometaphase II and metaphase II, defective metaphase II congression, and unequal segregation at anaphase II (Figure S1B).

In light of the genetic evidence for high rates of both meiosis I and meiosis II NDJ, the absence of obvious abnormalities in homolog pairing and segregation during meiosis I in the DAPI analysis would have been surprising had the same behavior not been previously observed in *ord* and *solo* mutants (Miyazaki and Orr-Weaver 1992; Bickel *et al.* 1997; Yan *et al.* 2010). As in those cases, abnormalities in prometaphase I and metaphase I bivalent morphology
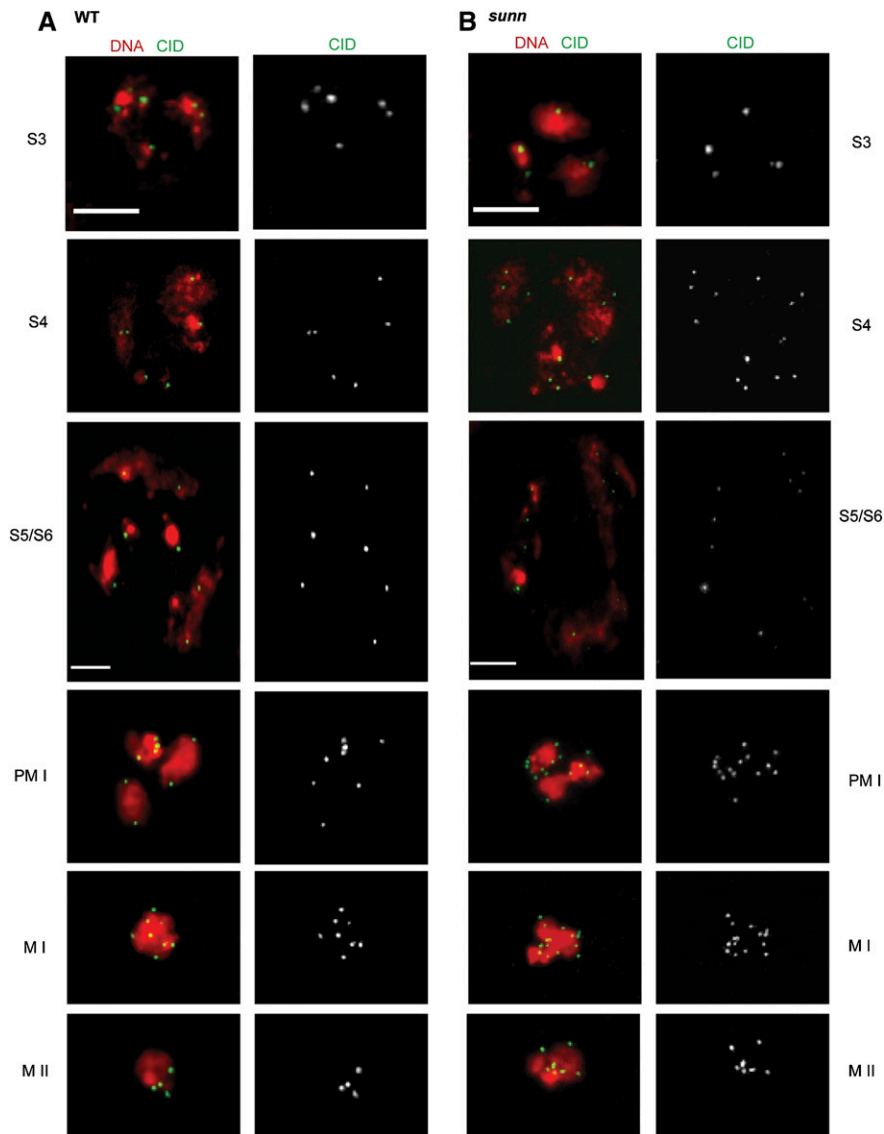
**Figure 1** Sister centromere cohesion is lost during prophase I in *sunn* spermatocytes. Immunostaining was performed using anti-CID antibody, which marks centromeres (green). DNA was stained with DAPI (red). PM I, prometaphase I; M I, metaphase I; M II, metaphase II. (A) In WT (*Df*/+) spermatocytes, spot numbers never exceeded eight during meiosis I or four during meiosis II. Representative images of S3, S4, S5/S6, PM I, M I, and M II show 6, 6, 7, 8, 8, and 4 CID spots, respectively. (B) In *sunn* (*sunn*[Z3-5839]/*Df*) spermatocytes, CID spot numbers exceeded 8 in most meiosis I spermatocytes from stage S4 onwards and exceeded 4 in most meiosis II spermatocytes. Representative images of S4, S5/S6, PM I, M I, and M II stages show 14, 12, 15, 14, and 7 spots, respectively. S3 bars apply to S4, PM I, M I, and M II. Bars, 5 μM. See Table 4 for quantification. *Df* = *Df(3L)ED4470*.

consistent with premature loss of sister chromatid cohesion—loose chromatid packing, extruded single kinetochore regions and, occasionally, fully separated chromatids—were seen in acetic–orcein preparations of mutant chromosomes, presumably because of harsher fixation procedures than are normally used in DAPI staining (Figure S1C). These observations suggested that although homologs remain paired throughout meiosis I in *sunn* mutants, defects in sister chromatid cohesion might underlie the abnormal segregation patterns.

To examine sister chromatid cohesion directly, we immunostained *sunn* and WT spermatocytes with an antibody against the centromere marker CID (Blower and Karpen 2001) (Figure 1). The cohesion status of sister centromeres was determined by counting the number of discrete CID spots per nucleus (Table 4). When homologous centromeres are unpaired but sister centromere cohesion is intact, as is generally the case after stage S3, spermatocytes are expected to show maxima of 8 CID spots during meiosis I

and 4 CID spots during meiosis II (Vazquez *et al.* 2002; Yan *et al.* 2010). As expected WT spermatocytes rarely exhibited >8 CID spots per nucleus during meiosis I (mean CID spot numbers of 6.1–7.2 from stage S4 through metaphase I) or >4 CID spots during meiosis II. *sunn* mutants did not differ from WT in early prophase I but began to diverge from WT by stage S4 when 34% of spermatocytes showed >8 CID spots. By late prophase I (stages S5 and S6) and throughout the division stages, >90% of *sunn* spermatocytes showed >8 spots, with a mean of ~11–12 spots per spermatocyte (Table 4). A total of 14–16 CID spots were seen in a substantial fraction of *sunn* spermatocytes at prometaphase I and metaphase I, indicating that *sunn* function is required for cohesion of all eight *Drosophila* chromosomes. In meiosis II, 82% of *sunn* spermatocytes showed >4 CID spots. Thus the data show that *sunn* mutants begin losing centromere cohesion by stage S4 and exhibit extensive cohesion loss by stage S5, long before chromosomes begin orienting on the meiosis I spindle.

**Table 4 Quantification of CID spots in *sunn* mutant spermatocytes**

| | *sunn*[a] | | | WT[b] | | |
|---|---|---|---|---|---|---|
| | ≤8 spots | >8 spots | Mean spot no. | ≤8 spots | >8 spots | Mean spot no. |
| Meiosis I stages | | | | | | |
| S1 | 75 (100) | 0 | 3.96 | 71 (100) | 0 | 3.31 |
| S2 | 55 (100) | 0 | 3.82 | 60 (100) | 0 | 3.14 |
| S3 | 96 (98.97) | 1 (1.03) | 4.12 | 53 (100) | 0 | 3.96 |
| S4 | 60 (65.9) | 31 (34.1) | 7.62 | 58 (98.25) | 1 (1.75) | 6.08 |
| S5, S6 | 9 (6.2) | 138 (93.8) | 10.41 | 134 (99.3) | 1 (0.7) | 6.92 |
| PM I | 5 (3.9) | 122 (96.1) | 10.75 | 68 (100) | 0 | 6.89 |
| Metaphase I | 1 (9) | 10 (91) | 12.20 | 11 (100) | 0 | 7.23 |

| | *sunn*[a] | | | WT[b] | | |
|---|---|---|---|---|---|---|
| | ≤4 spots | >4 spots | Mean spot no. | ≤4 spots | >4 spots | Mean spot no. |
| Meiosis II stage | | | | | | |
| Metaphase II | 8 (17.4) | 38 (82.6) | 6.22 | 79 (93) | 6 (7) | 3.80 |

Number in parentheses indicates percentage values calculated from the total number of nuclei scored at each spermatocyte stage. PM I, prometaphase I.
[a] *sunn*[Z3-5839]/Df.
[b] Df/+.

### *sunn* mutants disrupt sister centromere mono-orientation

The absence of cohesion between most sister centromere pairs during prometaphase I might impair sister centromere mono-orientation and thereby disrupt reductional segregation. To track the segregation of the X and Y chromatids at anaphase I, we performed FISH using probes that bind to the 359 bp satellite repeats in the pericentromeric region of the X chromosome and to a block of AATAC satellite repeats in the long arm of the Y chromosome. Signals were scored both during anaphase I and prometaphase II/metaphase II. In WT, as expected, only reductional segregations (XX-YY) were observed, as shown by a complete absence of anaphase I poles or prometaphase II/metaphase II nuclei with both X and Y signals or with no signals (Figure 2A and Table 5). The segregation pattern in *sunn* mutants was completely different. Only 31% of the 553 *sunn* poles/nuclei scored exhibited the reductional segregation pattern. Most (60%) of the *sunn* poles/nuclei exhibited one X signal and one Y signal, reflecting an XY-XY equational segregation pattern (Figure 2B; Table 5). The remaining 9% of *sunn* nuclei exhibited either three signals (2 X and 1 Y or vice versa) or one signal (either X or Y, reflecting unbalanced XXY-Y or XYY-X segregations (Table 5). No completely unbalanced (XXYY-O) segregations were observed.

Absence of sister centromere cohesion in *sunn* mutants was also evident in the FISH data. Unlike in WT in which the two X signals were usually fused or overlapping (due to cohesion of pericentric regions), sister X signals in *sunn* nuclei were usually separate even when they co-segregated (Figure 2B). The presence of two separate AATAC (Y chromosome arm) spots in most WT spermatocytes reflects the fact that arm cohesion is lost early in male meiosis—by stage S3 (Vazquez *et al.* 2002; Yan *et al.* 2010). The complete absence of sister chromatid cohesion in *sunn* mutants was also apparent in many prometaphase II and metaphase II

nuclei in which sister 359 bp or AATAC signals were present in separate DAPI-stained masses (Figure 3B). In conclusion, *sunn* mutations perturb the segregation pattern of the X and Y chromosomes at anaphase I by prematurely eliminating sister centromere cohesion, thereby disrupting sister centromere mono-orientation.

### *sunn* is not required for mitotic segregation or for arm cohesion

In WT male meiosis, arm cohesion is established in meiotic S phase but, unlike centromere cohesion, it is lost during midprophase I (late S2/S3). Consequently, when a single chromosome arm site is labeled by FISH or the GFP–LacI/*lacO* assay, only one spot is observed during early prophase I but two separate (sister) spots are generally observed at later stages of meiosis I (Vazquez *et al.* 2002; Yan *et al.* 2010). Thus the effect of a mutation on arm cohesion can be assayed by counting spots (one *vs.* two) during early prophase I. We examined arm cohesion in *sunn* spermatocytes by labeling a heterozygous *lacO* array inserted in the euchromatin of chromosome 2 with GFP–LacI expressed under control of the *hsp83* promoter. In both WT and *sunn* mutants, a single spot was observed in the great majority of stage S1 and S2 spermatocytes, indicating that *sunn* is not required for arm cohesion in early prophase I (Figure S2). Similar results were reported for *solo* mutants (Yan *et al.* 2010).

In later stages of meiosis I (after arm cohesion is lost), the number of spots in single-locus arm labeling experiments provides a reliable measure of chromatid copy number, useful to diagnose aneuploidy due to mitotic NDJ. The Y chromatids are particularly useful for such studies because they are normally present in two copies but can be absent altogether (XO) or present in four copies (XYY) without blocking spermatocyte development. Thus, in the FISH analysis reported above (Figure 2 and Table 5), mitotic NDJ of the Y chromosome in *sunn* spermatogonia would
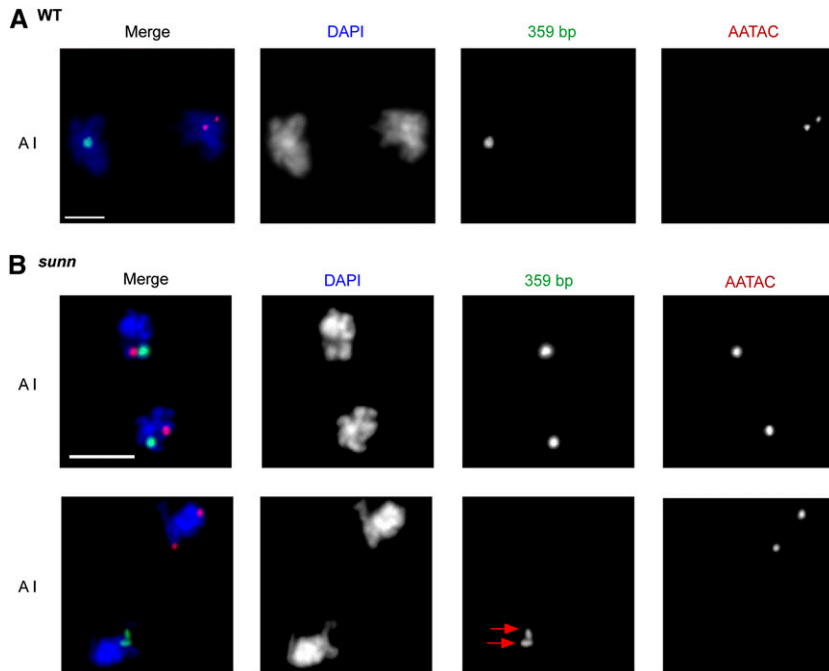
**Figure 2** X and Y chromatids segregate both equationally and reductionally at meiosis I in *sunn* spermatocytes. FISH was performed using probes for the 359-bp repeats on the X chromosome (green) and a block of AATAC repeats on the Y chromosome (red). DNA was stained with DAPI (blue). A I, anaphase I. (A) Meiosis I segregation is exclusively reductional in WT (*Df*/+) spermatocytes. Representative image of a reductional anaphase I segregation. The single 359 bp spot reflects maintenance of cohesion at and near the X centromere. Bar, 3 μM. (B) Both reductional and equational segregation occur at meiosis I in *sunn* (*sunn^{Z3-5839}*/*Df*) spermatocytes. Representative images at anaphase I showing normal reductional segregation (bottom) and abnormal equational segregation (top). The two pericentromerically located 359 bp signal spots are separated in *sunn* mutants (red arrows), reflecting premature loss of X centromere cohesion. Bar, 5 μM. See Table 5 for quantification.

be expected to generate XYY or XO spermatocytes exhibiting four or no AATAC signals, respectively, by late prophase I. However, 100% of the anaphase I *sunn* spermatocytes (*N* = 156) reported in Table 5 showed two AATAC spots (sometimes at opposite poles, sometimes at the same pole) as did 100% of prometaphase I and metaphase I *sunn* spermatocytes (*N* = 68, data not shown). These results strongly suggest that there is no significant mitotic NDJ in *sunn* mutants.

### sunn mutations disrupt centromere clustering, pairing, and cohesion in female meiosis

To determine if centromere cohesion is also lost prematurely in female meiosis, CID spot numbers were scored in pro-oocytes and oocytes from *sunn^{Z3-5839}*/*Df* females and WT sibling controls. Analysis of CID spot numbers in *sunn* oocytes was also of interest because of recent evidence for clustering of centromeres throughout prophase I in WT female meiosis and for the dependence of that clustering on *ord* and *solo* as well as on the genes encoding SC components, *c(3)G* and *cona* and kinetochore components, *cenp-c* and *cal-1* (Khetani and Bickel 2007; Takeo *et al.* 2011; Tanneti *et al.* 2011; Unhavaithaya and Orr-Weaver 2013; Yan and McKee 2013).

In female *Drosophila*, meiosis occurs in ovaries, which contain 10–30 ovarioles, each consisting of linear arrays of oocytes of increasing developmental age from stem cells to metaphase I arrested oocytes. Meiosis initiates in the germarium, the anterior-most compartment of each ovariole. Region 1, at the anterior end of the germarium, contains stem cells and pre-meiotic cysts undergoing mitotic amplification. Regions 2A, 2B, and 3 of the germarium contain 16-cell cysts in the zygotene (region 2A) or pachytene (regions 2A, 2B, and 3) stages of meiosis. Meiosis initiates and SCs begin

forming in up to four pro-oocytes in each cyst in region 2A but by region 3, only a single oocyte retains SC. The other 15 germ cells in each cyst develop as polyploid nurse cells that support the oocyte during its development. The maturing cysts leave the germarium and continue developing in the vitellarium. SC is disassembled in vitellarial stages 5–7, marking the end of pachytene (McKim *et al.* 2002; Lake and Hawley 2012). The oocyte subsequently enters an arrested late prophase I state termed the karyosome, in which the chromosomes are highly compact. Nuclear envelope breakdown in stage 12 is followed by prometaphase I and metaphase I in stages 13 and 14.

In agreement with the reports cited above, we found 1–3 CID foci in nearly all (~90–97%) nuclei in WT pro-oocytes/oocytes in germarial regions 2A, 2B, and 3 nuclei (average of 2.1–2.6 CID foci/nucleus), indicative of pairing and clustering of centromeres. Clustering was also present in stage 2 of WT vitellaria (Figure 4A, Table 6). However, in *sunn* mutants, 1–3 CID foci were observed in only 11.5, 0, 5, and 0% of nuclei in regions 2A, 2B, 3, and stage 2, respectively, indicating an absence of centromere clustering during prophase I. Most *sunn* pro-oocytes in regions 2A (89%) and 2B (85%) exhibited 4–8 CID spots, with means of 5.1 and 7 spots per nucleus, respectively, indicating that pairing of homologous centromeres was also compromised, somewhat more completely in region 2B than in region 2A.

Cohesion was intact in region 2A since no pro-oocytes with >8 CID spots were observed. However, more than 8 CID spots were observed in 16% of region 2B pro-oocytes, 50% of region 3 oocytes, and 56% of stage 2 oocytes in *sunn* mutants (Figure 4B, Table 6). Thus, cohesion begins deteriorating by region 2B and is extensively compromised by region 3 in *sunn* mutants. As in male meiosis, this cohesion loss

**Table 5 Quantification of X-Y chromatid segregation patterns in *sunn* mutant spermatocytes**

| Chromatid pattern | *sunn*[a] | WT[b] |
|---|---|---|
| Anaphase I | | |
| XX/YY | 47 (30%) | 62 (100%) |
| XY/XY | 95 (61%) | 0 |
| XXY/Y | 9 (6%) | 0 |
| XYY/X | 5 (3%) | 0 |
| **Total** | **156 (100%)** | **62 (100%)** |
| Prometaphase II or metaphase II | | |
| XX or YY | 78 (32%) | 121 (100%) |
| XY | 143 (59%) | 0 |
| XXY or Y | 11 (5%) | 0 |
| XYY or X | 9 (4%) | 0 |
| XXYY or O | 0 | 0 |
| **Total** | **241 (100%)** | **121 (100%)** |

X and Y chromatids were identified by FISH using probes for the 359 bp and AATAC loci as described in legend of Fig. 2 and *Materials and Methods*.
[a] *sunn*[Z3-5839]/*Df*.
[b] *Df/+*.

occurs long before centromeres must orient on the meiosis I spindle and thus provides a likely explanation for the high levels of meiosis I NDJ.

### SUNN is a novel protein produced from the CG32088 locus

Using deficiency complementation and candidate gene sequence analysis, *sunn* was mapped to the CG32088 locus in region 68D3 of chromosome arm 3L. We note that a gene named *mei(3)M20* that exhibited mutant phenotypes similar to those of *sunn* was previously reported and mapped to the 68C8–11;69A4–5 interval (Hirai *et al.* 2004). Complementation analysis will be required to determine whether *sunn* and *mei(3)M20* are allelic. Genomic DNA sequencing of CG32088 exons from the three alleles of *sunn* revealed single mutations in each line: a nonsense mutation predicted to truncate the protein 132 amino acids from the C terminus (Z3-1956); a missense mutation predicted to substitute arginine for a conserved glycine (G170) (Z3-4085); and a 8-bp deletion (Z3-5839) which creates a frameshift that leads to a predicted in-frame stop codon near the middle of the coding sequence (Figure 5). As no full-length cDNAs for *sunn* were available, a cDNA was obtained by reverse transcription and PCR from ovary RNA using primers designed on the basis of genomic sequence. The resulting *sunn* cDNA consists of 10 exons and contains a predicted coding sequence of 2856 bp corresponding to a protein 952 amino acids in length (Figure 5). Rapid amplification of cDNA ends (RACE) revealed short 5′ and 3′ UTRs, 69 bp and 75 bp in length, respectively, that contained no potential alternative start or stop codons.

To verify that CG32088 corresponds to *sunn*, we cloned the full-length cDNA-derived coding sequence of *sunn* (without its 5′ and 3′ UTRs) into an upstream activator sequence (UAS) vector in frame with C-terminal Venus, (enhanced yellow fluorescent protein, eYFP). Transgenic insertions of UAS–SUNN::Venus were generated and found to complement the

meiotic NDJ phenotypes of *sunn* mutants in both sexes when expressed under control of the germline specific driver *nos-GAL4::VP16* (Table S2 and Table S3). In addition to verifying the identity of *sunn* and CG32088, these results show that SUNN::Venus functions similarly to wild-type SUNN protein and that the *sunn* cDNA sequence used for the construct likely represents the true *sunn* coding region.

### SUNN exhibits structural similarity to the cohesin protein SA

Predicted secondary structure for SUNN indicated a primarily α-helical protein (Figure S3). To identify homologs of SUNN, the complete amino acid sequence of SUNN was used to search the protein sequence database using the BLASTp tool offered by FlyBase (http://flybase.org/blast/). We found single SUNN homologs in all of the sequenced species of *Drosophila* (see File S1 for details). No homologs of SUNN were found in other eukaryotes and no conserved domains were identified by searching a conserved domain database at National Center for Biotechnology Information (NCBI). However, structure-based searches, described in detail in File S1, proved more informative. In particular, the fold-recognition/threading programs MUSTER and I-TASSER revealed statistically significant similarity of SUNN to multiple templates, all of which belong to the HEAT repeat protein family (Table 7 and Table S4) (Wu and Zhang 2008; Roy *et al.* 2010). HEAT repeats are conserved domains that form all-α superhelices and are involved in protein–protein interactions. They are particularly abundant in chromosomal proteins involved in cohesion and condensation, including the cohesin cofactors Nipped-B and Pds5 and the condensin subunits Cap-G and Cap-D2. The cohesin subunit SA also exhibits weak similarity to HEAT repeats (Neuwald and Hirano 2000; Andrade *et al.* 2001; Nasmyth and Haering 2009; Hirano 2012).

Detailed comparisons of the MUSTER and I-TASSER analyses revealed a stronger similarity of SUNN to SA than to the other *Drosophila* chromosomal HEAT repeat proteins. In the MUSTER analysis 5 of the top 6 matches for SA were also among the top 6 matches for SUNN (Table 7). Similarly, 8 of the top 10 matches for SUNN and SA in the I-TASSER analysis overlapped, and 6 of these common templates overlapped with the top common templates hit by SUNN and SA in MUSTER (Table S4). Although some of the top-matching templates for the other *Drosophila* chromosomal HEAT repeat proteins, Nipped-B, Pds5, Cap-G, and Cap-D2, overlapped with the templates matched by SUNN, the overlap was much less extensive than for SA. For example, in the MUSTER analysis, the highest-scoring and second-highest scoring templates for Cap-G, Cap-D2, Nipped-B, and Pds5 were not among the 10 best matches for SUNN (Table 7). Taken together, these results suggest that SUNN is a distant member of the HEAT-repeat family and exhibits stronger structural similarity to the cohesin subunit SA than to other *Drosophila* chromosomal
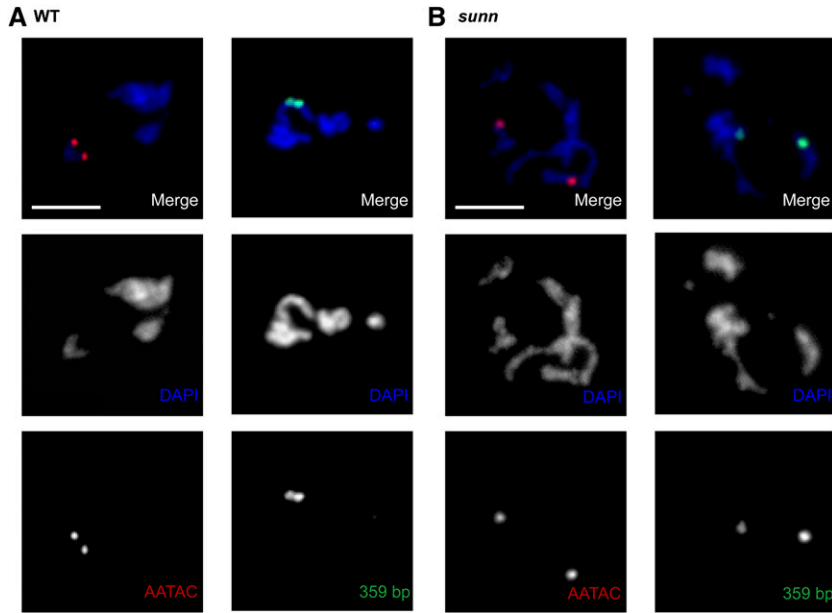
**Figure 3** Sister chromatid separation in prometaphase II spermatocytes of *sunn* mutants. X and Y chromatids were identified by the presence of 359 bp (green) and AATAC (red) probes, respectively. (A) Sister chromatid cohesion is maintained in WT (*Df/+*). In two prometaphase II spermatocytes bearing a Y chromosome (left) or an X chromosome (right), both AATAC signals and both 359 bp signals are situated in the same chromosome territory. (B) Premature sister chromatid separation in *sunn* (*sunn^{Z3-5839}/Df*) spermatocytes. In two prometaphase II spermatocytes bearing two separated Y chromatids (left) or two separated X chromatids (right), the two AATAC signals and the two 359 bp signals are located in completely separate chromosome territories. See Table 5 for quantification. Bars, 5 µM.

HEAT-repeat proteins. Structure-based pairwise alignments of SUNN and SA with the most similar template protein (1qgkA) from the MUSTER search are shown in Figure S4 and a graphical alignment of the α-helices is shown in Figure S5.

### SUNN co-localizes with CID during meiosis

Immunostaining of spermatocytes expressing SUNN::Venus with anti-CID antibody revealed bright Venus foci that co-localized with CID spots (Figure 6A and Figure S6B). These centromeric SUNN::Venus foci were present at all stages of meiosis through metaphase II but were absent at later stages. The SUNN::Venus foci sometimes showed small extensions, suggesting that SUNN localizes to heterochromatic domains that extend beyond the centromeres. However, no localization of SUNN::Venus to chromosome arms in male germ cells was detected at any stage. SUNN::Venus also co-localized with CID in premeiotic 8-cell cysts, but these centromeric signals were weaker than in spermatocytes. In addition, SUNN::Venus formed large foci and bright smears outside the DNA in premeiotic 8-cell cysts (Figure S6A). These non-chromosomal staining patterns were still present but greatly attenuated in the earliest 16-cell cysts, and were not seen in later stages (Figure 6A and Figure S6). The significance of this non-chromosomal staining is unknown; the possibility that it is an artifact of ectopic expression cannot be excluded. We conclude that SUNN localizes to centromeric regions prior to the beginning of male meiosis and persists at centromeres through metaphase II but is removed by anaphase II.

To evaluate the localization pattern of SUNN in female meiosis, we immunostained ovaries expressing SUNN::Venus with anti-CID. Discrete SUNN::Venus foci that co-localized with CID foci were observed in the germ cells, but not the somatic follicle cells, in the proximal half of region 1, where

four- and eight-cell premeiotic gonial cysts reside, throughout regions 2A, 2B, and 3 of the germarium (Figure 6B) and in the vitellarium at least up to stage 3 (not shown). In addition to the centromeric foci, SUNN::Venus also exhibited diffuse localization in female germ cells, particularly in regions 2A and 2B (Figure 6B). The nature of this non-centromeric localization could not be ascertained from the whole-mount squash preparations and remains under investigation. SUNN's localization to the centromeres in both sexes could explain the phenotypes of NDJ, centromeric cohesion, and centromeric clustering defects associated with *sunn* mutants

### Mutual co-dependence of SUNN, SOLO, and SMC1 centromere foci

The cohesion proteins SMC1, ORD, and SOLO also localize to centromeric regions in spermatocytes and SMC1 centromeric foci have been shown to depend on both *ord* and *solo* (Balicky *et al.* 2002; Thomas *et al.* 2005; Khetani and Bickel 2007; Yan *et al.* 2010). To test for dependence of SMC1 centromeric foci on *sunn*, we expressed Venus::SMC1 using the *nos-GAL4::VP16* driver in both WT and *sunn* mutant backgrounds. Although bright Venus::SMC1 foci were observed throughout WT meiosis, no Venus::SMC1 foci were seen at any stage of meiosis in *sunn* spermatocytes (Figure 7A). In WT females, SMC1 forms bright foci at the centromeres and it localizes to the chromosome arms in oocytes/pro-oocytes. However, in *sunn* mutants, SMC1 is absent from the centromeres but is still present (although weakly) on the chromosome arms (Figure 7B). Thus stable centromere localization of SMC1 requires wild-type function of *sunn*.

To investigate whether SUNN localization depends on *solo*, SUNN::Venus was expressed using the *nos-GAL4::VP16* driver in both WT and *solo* mutant backgrounds. Although SUNN::Venus foci that co-localized with CID were readily observed throughout meiosis in WT spermatocytes, no
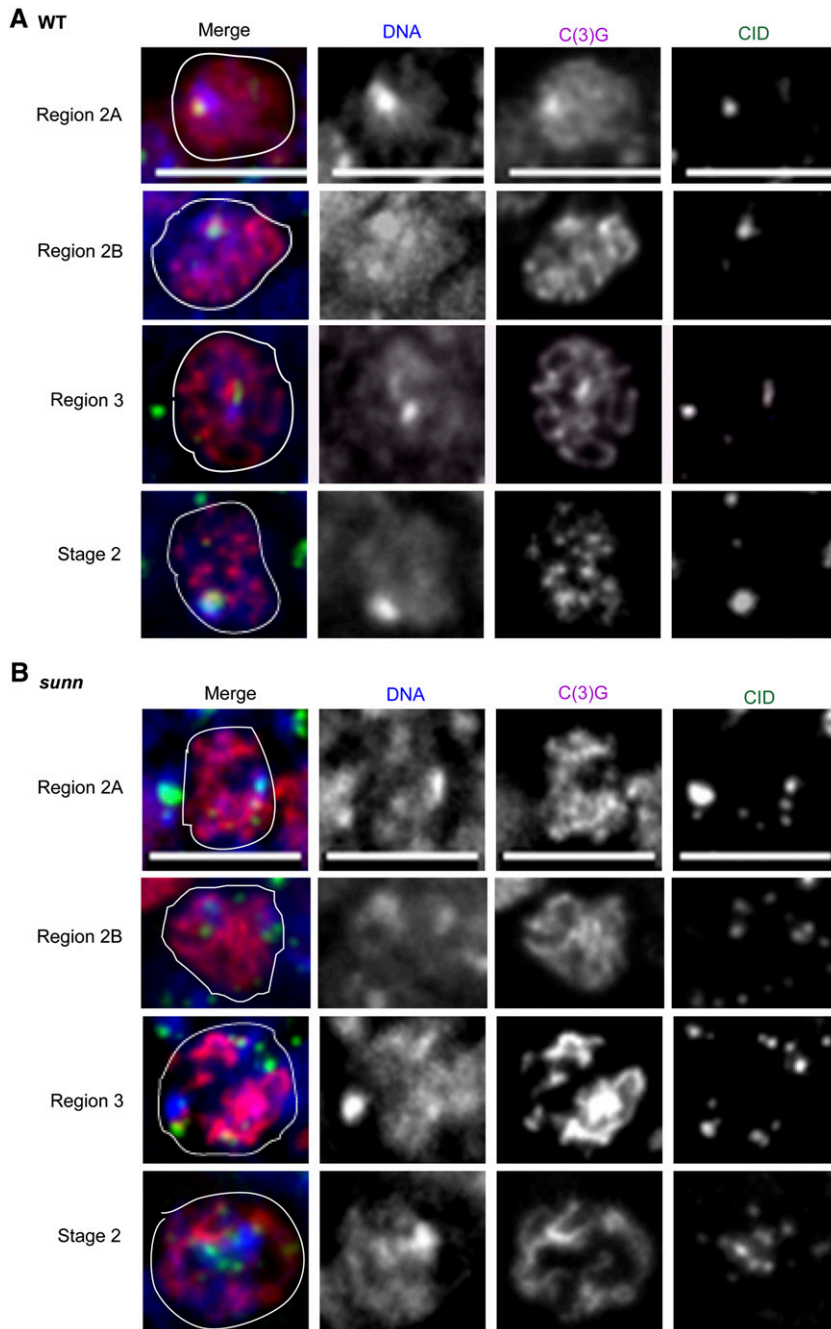
**Figure 4** Centromeric clustering is disrupted in *sunn* mutant females. Whole-mount ovaries were immunostained with anti-CID and anti-C(3)G, which serve as markers for centromeres and SCs, respectively (Blower and Karpen 2001; Page and Hawley 2001). (A) Centromeres are paired and clustered in WT (*Df /TM3*) oocytes. Pro-oocytes/oocytes showed one to three CID foci throughout the germarium in regions 2A, 2B, and 3 and in the vitellarium at stage 2. Representative oocyte/pro-oocyte images all show 2 large CID foci in region 2A, region 2B, region 3, and stage 2. (B) Loss of centromere clustering, pairing, and cohesion in *sunn* (*sunn^{Z3-5839}/Df*) oocytes. Pro-oocytes/oocytes averaged >4 CID spots throughout pachytene. Representative images show 5, 8, 10, and 13 CID foci in region 2A, region 2B, region 3, and stage 2, respectively. Bars, 5 μM. See Table 6 for quantification.

SUNN::Venus foci were observed at any stage of meiosis in *solo* spermatocytes. Premeiotic SUNN–Venus foci were also absent in *solo* spermatocytes (Figure 7C). We conclude that localization of SUNN to centromere regions requires wild-type *solo* function.

To determine whether SOLO localization requires *sunn* function, we expressed Venus::SOLO (Yan *et al.* 2010) in WT and *sunn* mutant backgrounds in male meiosis. Venus:: SOLO foci are visible on chromosomes in WT males throughout meiosis, but no Venus::SOLO foci were detected at any stage of meiosis in *sunn* mutants (Figure S7). Thus, SOLO and SUNN foci are reciprocally codependent. This pattern is consistent with SUNN and SOLO participating in the same cohesion complex.

## Discussion

### SUNN is a Drosophila-specific meiotic cohesion protein

Several components and/or regulators of the meiotic cohesion machinery in *Drosophila* have been identified, but critical questions about meiotic cohesion remain unanswered. Chief among these are the composition of meiotic cohesin(s) and the role(s) of cohesion factors in pairing,

**Table 6 Quantification of CID spots in WT and *sunn* mutant oocytes/pro-oocytes**

| Genotype | Oocyte stages | | | |
| --- | --- | --- | --- | --- |
| | Region 2A | Region 2B | Region 3 | Stage 2 |
| WT[a] | 2.14 ± 0.91 (N = 21) | 2.19 ± 0.79 (N = 36) | 2.6 ± 1.07 (N = 10) | 3 ± 1.12 (N = 12) |
| <4 CID (%) | 95.2 | 97.2 | 90 | 75 |
| ≥4, ≤ 8 CID (%) | 4.8 | 2.8 | 10 | 25 |
| >8 CID (%) | 0 | 0 | 0 | 0 |
| *sunn*[b] | 5.15 ± 1.43 (N = 26) | 6.96 ± 1.96 (N = 32) | 8.5 ± 2.8 (N = 20) | 8.8 ± 2.6 (N = 9) |
| <4 CID (%) | 11.5 | 0 | 5 | 0 |
| ≥4, ≤ 8 CID (%) | 88.5 | 84.4 | 45 | 44.4 |
| >8 CID (%) | 0 | 15.6 | 50 | 55.6 |

[a, b] Table entries in the "WT" and "*sunn*" lines are mean CID spots per oocyte with standard deviations. *N* indicates the total number of oocytes counted in the indicated region of the ovariole.
[a] *Df*/TM3.
[b] *sunn*[Z3-5839]/*Df*.

synapsis, and recombination. The core cohesin subunits SMC1 and SMC3 are required for SC formation and have been implicated in centromere cohesion (Khetani and Bickel 2007; Yan *et al.* 2010; Tanneti *et al.* 2011; Yan and McKee 2013). The non-SMC components of meiotic cohesin remain uncharacterized. No meiotic role of SA has been reported, and although RAD21 has recently been shown to localize to SCs and to play a role in SC stability, it appears to be dispensable for meiotic cohesion and for proper chromosome segregation at both meiotic divisions (Urban *et al.* 2014). The *Drosophila* genome encodes meiosis-specific paralogs of both SA and RAD21, SNM, and C(2)M, respectively, but neither protein is required for meiotic cohesion in either sex (Manheim and McKim 2003; Heidmann *et al.* 2004; Thomas *et al.* 2005).

Heretofore, the best-characterized meiotic cohesion factors are two meiosis-specific proteins, ORD and SOLO, not found outside of the genus *Drosophila* but required for all aspects of meiotic cohesion. This report adds a third protein, SUNN, to this group of *Drosophila*-specific meiotic cohesion factors. Like fluorescently tagged versions of ORD and SOLO, SUNN::Venus localizes to centromeres of pre-meiotic gonial chromosomes (most clearly in eight-cell cysts) and meiotic chromosomes in both sexes. The disappearance of SUNN from spermatocyte centromeres at anaphase II is similar to the timing of ORD and SOLO removal and coincident with the disappearance of SMC1. Like *solo* and *ord*, mutations in *sunn* abolish SMC1 centromere foci and disrupt centromere cohesion during prophase I, well in advance of prometaphase I when sister centromeres would normally mono-orient. The result is high frequencies of meiosis I and meiosis II NDJ in both sexes, as previously described for *ord* and *solo* mutants (Miyazaki and Orr-Weaver 1992; Bickel *et al.* 1997; Yan *et al.* 2010; Yan and McKee 2013). The similarities among the phenotypes and localization patterns are striking and suggest that ORD, SOLO, and SUNN play closely related roles in meiotic cohesion and cohesion-related processes.

### SUNN is required for centromere clustering and pairing

Both homologous and non-homologous centromere pairing have been described during meiosis in yeast, plants, and Dro-

*sophila* (Stewart and Dawson 2008). In *Drosophila* female meiosis, centromeres typically aggregate into one to three clusters throughout prophase I from zygotene until prometaphase I. This phenomenon is meiosis specific since it is not observed in pre-meiotic gonia or in nurse cells (Takeo *et al.* 2011). Centromere clustering requires the SC proteins C(3)G and CONA, the cohesion proteins ORD and SOLO (Khetani and Bickel 2007; Takeo *et al.* 2011; Tanneti *et al.* 2011; Yan and McKee 2013), and the centromere proteins CENP-C and CAL-1 (Unhavaithaya and Orr-Weaver 2013). Our data show that SUNN is also required for centromere clustering. The frequency of *sunn* oocytes with fewer than four CID spots (the hallmark of clustering) was <12% in region 2A and ≤5% in all later stages. Moreover, the great majority of oocytes in all stages exhibited more than four CID spots, indicating a substantial disruption of homologous centromere pairing as well (Table 6). It remains to be determined whether pairing and clustering of centromeres are somehow consequences of centromere cohesion or whether they reflect separate functions of SUNN.

### SUNN is required for centromere cohesion in both male and female meiosis

Sister centromeres normally remain tightly cohesive throughout meiosis until anaphase II, when the release of centromere cohesion triggers sister chromatid segregation. Sister centromere cohesion underlies not only the proper bipolar orientation of sister kinetochores during meiosis II but also their mono-orientation during meiosis I. Mutations in *ord* and *solo* were previously shown to disrupt centromere cohesion prior to prometaphase I in both sexes (Balicky *et al.* 2002; Bickel *et al.* 2002; Yan *et al.* 2010; Takeo *et al.* 2011; Tanneti *et al.* 2011; Yan and McKee 2013). In this article, we have shown that *sunn* mutations have similar effects. In spermatocytes, FISH analysis revealed substantial loss of cohesion at the pericentromeric X chromosome 359 bp repeat locus by prometaphase l, consistent with similar observations in *ord* and *solo* mutants (Balicky *et al.* 2002; Yan *et al.* 2010). CID spot counts showed that although centromere cohesion remains intact throughout stages S1–S3 of
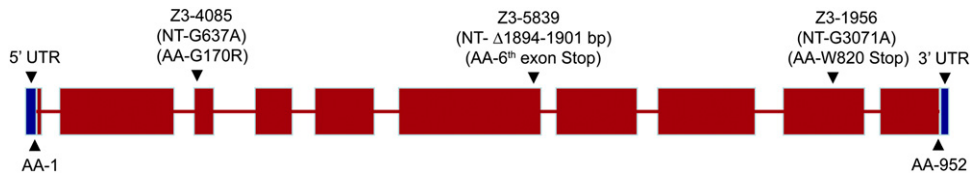
**Figure 5** Structure of *sunn* gene. Gene structure of *sunn* and location and identity of *sunn* mutations. Sequencing of ovary cDNAs showed that there are 10 exons in *sunn*. The red boxes show the predicted coding sequence of *sunn* and the blue boxes show the UTRs. The length of the predicted coding sequence of *sunn* is 2856 bp and the lengths of the 5′ and 3′ UTRs are 69 bp and 75 bp, respectively. The locations and identities of the sequenced *sunn* mutations discussed in the text are shown above. NT denotes genomic nucleotide position (introns included) from the start of the translation unit (position 1 is the first nucleotide of the predicted initiator AUG) and AA depicts the respective predicted amino acid numbers.

prophase I in *sunn* mutants, it begins deteriorating by stage S4 and is largely absent by stage S5–S6. Results of CID spot counts for *solo* mutants were similar except that no cohesion loss was detected until stage S5.

Results of CID spot counts in *sunn* oocytes were similar. No region 2A prooocytes with more than eight CID spots were seen, indicating that sister centromere cohesion remained intact in early pachytene. However, by region 2B, 16% of prooocytes exhibited more than eight CID spots and by region 3 and stage 2, at least half of oocytes did. Again, the progressive loss of cohesion during pachytene broadly parallels results of similar studies in *ord* and *solo* mutants. However, no pro-oocytes/oocytes with more than eight spots were observed in any region of the germarium in those mutants. Oocytes with more than eight spots were present in vitellarial stages 5–7 in *ord* and *solo* mutants (Takeo *et al.* 2011; Yan and McKee 2013). Thus cohesion is compromised at an earlier stage of pachytene in *sunn* oocytes than in *ord* or *solo* oocytes, paralleling the difference in timing of cohesion loss between *sunn* and *solo* spermatocytes.

Three conclusions seem warranted. First, SUNN, SOLO, and ORD work together during prophase I to maintain centromere cohesion in both sexes. In the absence of any of the three proteins, centromere cohesion is completely lost by the onset of prometaphase I. In light of the shared phenotype of loss of centromeric SMC1 foci in *ord*, *solo*, and *sunn* mutants, it seems likely that this cohesion pathway is mediated by a cohesin complex, although this inference remains to be verified. Second, since centromere cohesion is intact during early prophase in both spermatocytes and oocytes in all three groups of mutants, there must be at least one additional centromere cohesion mechanism that does not require ORD, SOLO, or SUNN. This mechanism may be independent of cohesin as well although the possibility that below-detection levels of SMC cohesins remain at centromeres in the absence of these proteins cannot be excluded. The nature of this alternative mechanism (or mechanisms) remains to be elucidated. Third, since there is an earlier onset of centromeric cohesion loss in *sunn* mutants than in *ord* or *solo* mutants in both male and female meiosis, SUNN may play a minor role in SOLO/ORD-independent early-prophase cohesion. Although we cannot completely exclude the possibility that the earlier onset of cohesion loss in *sunn* mutants resulted from a background genotype effect, we think this explanation is unlikely as the *sunn* and *solo* alleles used in these studies were derived on the same strain background.

### SUNN is required for sister centromere mono-orientation

Reductional segregation during meiosis I requires sister centromeres to mono-orient so that homologous centromeres can reliably biorient. Mono-orientation requires that sister centromeres form a functionally single kinetochore by adopting a side-by-side configuration instead of a back-to-back configuration, which is characteristic of meiosis II and mitosis (Hauf and Watanabe 2004; Yokobayashi and Watanabe 2005). How sister centromeres achieve this unique orientation is poorly understood but genetic studies in several model eukaryotes have pinpointed sister centromere cohesion as a necessary prerequisite for mono-orientation. Mutation of cohesin genes including *smc3*, *rec8*, and *scc3/sa* in budding yeast, fission yeast, *Arabidopsis*, and *Caenorhabditis elegans* have been found to disrupt mono-orientation and cause chaotic and/or equational meiosis I segregation (Klein *et al.* 1999; Watanabe and Nurse 1999; Pasierbek *et al.* 2001; Wang *et al.* 2003; Chelysheva *et al.* 2005; Golubovskaya *et al.* 2006; Severson *et al.* 2009).

In *Drosophila*, no cohesins have been shown directly to be required for mono-orientation, but the detailed FISH analyses of meiosis I segregation reported previously for *solo* mutants (Yan *et al.* 2010) and herein for *sunn* mutants show that the products of these essential centromere cohesion genes are also essential for mono-orientation, at least in male meiosis. The simplest interpretation is that the mono-orientation defect is a consequence of the cohesion defect, although the possibility that SUNN or SOLO has an independent role in mono-orientation cannot be excluded. The fact that SMC1 centromere foci are absent in *sunn* mutants, as in *ord* and *solo* mutants, is consistent with the idea that mono-orientation in *Drosophila* requires cohesin (again presumably derivative of its role in cohesion), but direct proof of this inference is lacking as yet. The data available so far also do not address the question of whether the known cohesion factors are sufficient for mono-orientation. It would not be surprising if additional factors were needed since the same proteins mediate cohesion during both meiosis I and II but mono-orientation is restricted to meiosis I. Specific mono-orientation factors have been identified in both *S. cerevisiae* and *S. pombe* but no such factors have been identified as yet in higher eukaryotes (Toth *et al.* 2000; Yokobayashi and Watanabe 2005).

**Table 7 Threading/fold recognition (Z scores) results of hits generated by SUNN, stromalin (SA), and some *Drosophila* chromosomal HEAT repeat proteins using MUSTER**

| PDB ID | Protein | SUNN | SA | Pds5 | Nipped-B | CapG | CapD2 |
|---|---|---|---|---|---|---|---|
| 1wa5C | Exportin CSE1P | **8.333** | **8.711** | 10.255 | — | **10.343** | 10.313 |
| 3m1iC | Exportin-1 | **8.116** | **8.839** | **11.174** | 7.733 | 9.980 | **11.181** |
| 1qgkA | Importin-β | **7.995** | **10.060** | **10.933** | 7.637 | **11.106** | **11.310** |
| 3ea5B | Importin-β1 | **7.969** | **9.981** | **11.422** | — | **11.083** | **11.386** |
| 4fgvA | Exportin 1 | **7.918** | — | 10.350 | 7.416 | 9.901 | 10.350 |
| 2x1gF | Importin-13 | **7.914** | **9.278** | 10.838 | — | 9.904 | 10.727 |
| 1u6gC | Cand1/TIP120 | — | **8.912** | **12.815** | **7.945** | **11.406** | **12.807** |
| 3icqT | Exportin-T | 7.480 | 8.494 | — | — | — | — |
| 2x19B | Importin-13 | 7.389 | 8.596 | 10.140 | — | — | 10.092 |
| 3a6pA | Exportin-5 | 7.386 | — | — | — | — | — |
| 3gjxA | Exportin-1 | 7.156 | — | — | — | 9.648 | — |
| 1b3uA | PP2A | — | 8.258 | — | — | — | — |
| 3nowA | UNC45 | — | 8.221 | — | — | — | — |
| 3w3tA | Importin-β3 | — | — | **12.645** | 8.222 | 12.213 | 12.645 |
| 1qbkB | Karyopherin-β2 | — | — | — | **7.759** | — | — |
| 4c0oA | Transportin 3 | — | — | 11.585 | **8.295** | 10.977 | 11.596 |
| 1vw1A | TcdA1 | — | — | — | **8.405** | — | — |
| 4acqA | α-2-Macroglobulin | — | — | — | **7.984** | — | — |
| 4jspB | mTOR | — | — | — | 7.580 | — | — |

Bold Z scores represent the top six PDB templates matched by the proteins tested. Above depicted proteins were derived from the following organisms: 1wa5C: Exportin CSE1P (*Saccharomyces cerevisiae*). 3m1iC: Exportin-1 (*S. cerevisiae*). 1qgkA: Importin-β (human). 3ea5B: Importin-β1 subunit (*S. cerevisiae*). 4fgvA: Exportin 1 (*Chaetomium thermophilum*). 2x1gF: Importin-13 (*Drosophila melanogaster*). 1u6gC: Cand1/TIP120 (human). 3icqT: Exportin-T (*S. pombe*). 2x19B: Importin-13 (human). 3a6pA: Exportin-5 (human). 3gjxA: exportin-1 (mouse). 1b3uA: PP2A (human). 3nowA: UNC45 (*D. melanogaster*). 3w3tA: importin subunit β3 (*S. cerevisiae*) (Kap121p). 1qbkB: karyopherin-β2 (human). 4c0oA: transportin 3 (human). 1vw1A: TcdA1 (*Photorhabdus luminescens*). 4acqA: α-2-macroglobulin (human). 4jspB: (human serine/threonine-protein kinase mTOR).

Is SUNN also required for mono-orientation during meiosis I in female *Drosophila*? Since we did not conduct cytological analysis of meiosis I segregation in females, our data do not provide direct evidence on this point. In principle, the observed combination of homolog and sister chromatid NDJ in the cross experiments could be explained without invoking any mono-orientation defects. The homolog NDJ products could result solely from dyad–dyad NDJ due to a failure of arm cohesion during meiosis I, and the sister chromatid NDJ products could result solely from meiosis II NDJ. However, we think this explanation is unlikely, mainly because of our data showing that centromere cohesion is extensively compromised during prophase I. It is difficult to see how prematurely separated sister centromeres could mono-orient on the meiosis I spindle. Although we cannot rule out the possibility that the dissociated sister centromeres somehow reassociate by prometaphase I, no such reassociation was seen in a FISH-based analysis of X chromatid segregation in *ord* females, which exhibit very similar centromere cohesion loss and chromatid missegregation phenotypes as *sunn* females. Instead, X chromatids often appeared fully separate after the onset of prometaphase I and segregated chaotically (Bickel *et al.* 2002). Thus, we suggest that meiosis I missegregation in both sexes in *sunn* mutants is likely due mainly to the premature loss of centromere cohesion and the resulting failure of sister centromeres to mono-orient.

### Balanced vs. unbalanced segregations in the absence of cohesion

Our FISH analyses showed that in >90% of sex chromosome bivalents in both *solo* (Yan *et al.* 2010) and *sunn* (this article, Figure 2, and Table 5) males, the chromatids segregate in numerical balance (two toward each pole) at anaphase I even though the cross data indicate very high rates of meiosis I NDJ. Our FISH data show that the explanation for the high meiosis I NDJ is random sister chromatid partner choice, which results in a 1:2 ratio of reductional to equational sister chromatid segregation. This "random $2 \leftarrow \rightarrow 2$" segregation pattern requires the homolog conjunction complex, since *snm* mutations in a *solo* mutant background result in completely random segregation (Yan *et al.* 2010).

Might the random $2 \leftarrow \rightarrow 2$ mechanism also apply to meiosis I segregation in *sunn* females? Unfortunately, cross data are not informative on this point because the predicted ratio of sister *vs.* homolog NDJ products among XX eggs is identical (1:2) whether segregation is completely random or random $2 \leftarrow \rightarrow 2$, and the unbalanced segregation products (XXX and XXXX) which are critical to distinguishing which mechanism is operative cannot be recovered. Nevertheless, we favor the fully random model in females for two reasons. First, the homolog conjunction complex that is essential for the random $2 \leftarrow \rightarrow 2$ mechanism in male meiosis is absent in female meiosis (Thomas *et al.* 2005). Although females also have a robust achiasmate segregation mechanism (Hawley *et al.* 1992), it bears little mechanistic resemblance to that in males. Second, the FISH analysis of meiotic segregation of X chromatids in *ord* females mentioned above found no indication of orderly segregation (Bickel *et al.* 2002). It seems likely, then, that the random $2 \leftarrow \rightarrow 2$ mechanism is male specific and that the uncohesive chromatids in *sunn* females segregate fully randomly during both meiotic divisions. It
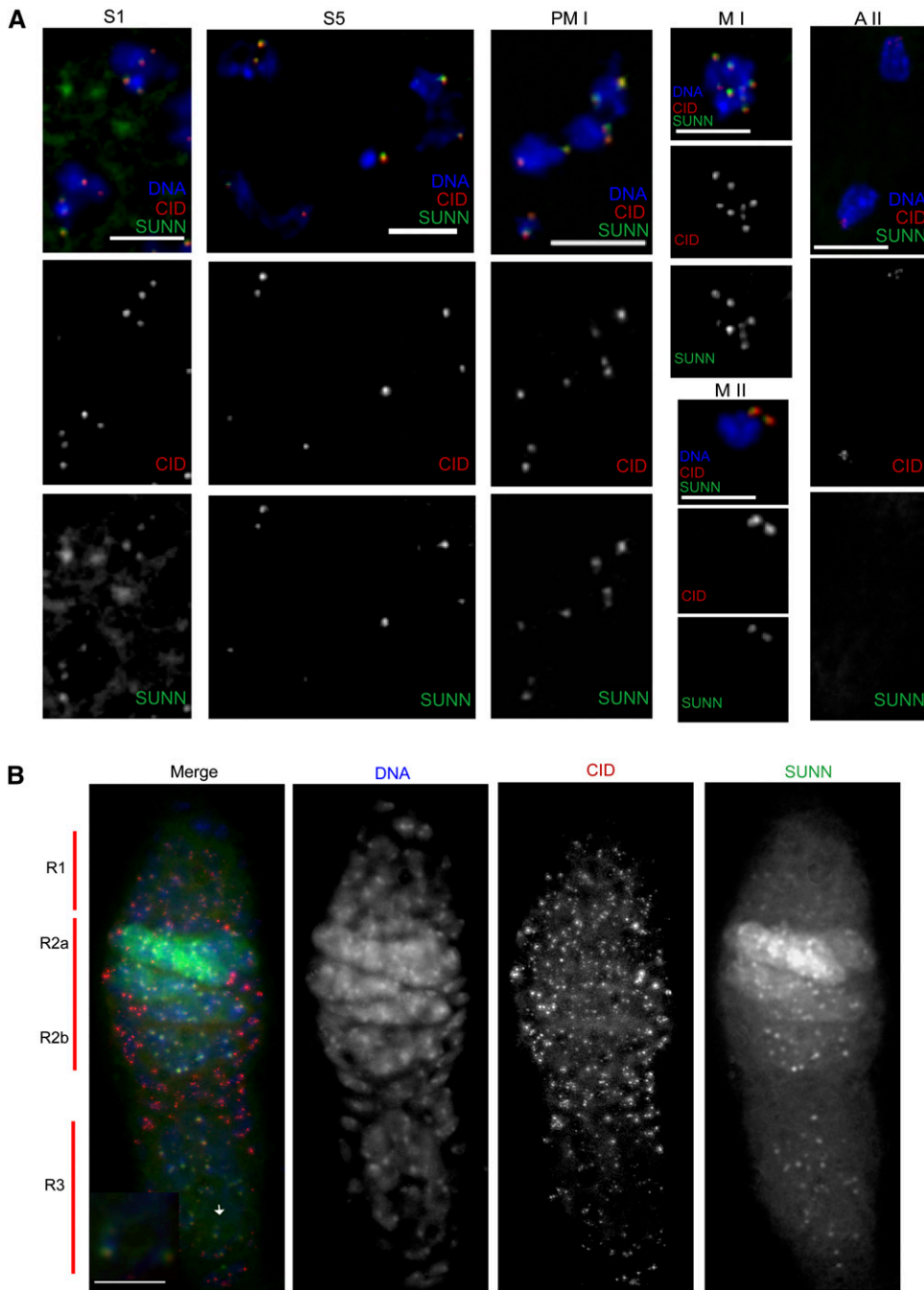
**Figure 6** Co-localization of SUNN:: Venus with CID. (A) SUNN and CID co-localize in male meiosis. Spermatocytes from *sunn[Z3-5839]/Df*, *nos-GAL4::VP16* males carrying *UASp-SUNN::Venus* were immunostained with anti-CID antibody. SUNN::Venus forms bright spots, which co-localize with CID spots. At S1 stage, SUNN::Venus also shows diffuse signals and large foci, which do not localize with CID and are present predominantly on the nuclear membrane and outside the nucleus in the cytoplasm. In representative images, SUNN::Venus and CID both form three to four CID spots, eight spots and four spots at S1, PM I, and M II, respectively. SUNN::Venus signals are absent at A II (anaphase II). (B) Co-localization of SUNN::Venus and CID in female germ cells. Immunostaining was performed using anti-CID antibody on whole-mount ovaries from *UASp-SUNN:: Venus*; *sunn[Z3-5839]/Df*, *nos-GAL4::VP16* females. SUNN::Venus foci were observed in germ cells in all regions of the germarium including region 1, and they co-localized with CID spots. SUNN::Venus expression was absent from the follicle cells due to germ cell-specific expression directed by the nos-GAL4 driver. Arrow shows enlarged germ cell used in the inset. Bars, 5 μM.

will be important to test this prediction experimentally. The mechanism underlying random $2 \leftarrow \rightarrow 2$ segregation in male meiosis also remains to be investigated.

### What role does SUNN play in cohesion?

Several of the findings summarized above are consistent with SUNN functioning as a component of a cohesion-providing complex along with SOLO and ORD. One possibility is that all three proteins are subunits, along with SMC1 and SMC3, of a specialized meiotic cohesin complex, perhaps replacing either or both of the mitotic non-SMC subunits RAD21 and SA, neither of which has been shown as

yet to have a role in meiosis. This idea is supported by several lines of evidence. First, mutations in *ord*, *solo*, and *sunn* abolish detectable centromeric foci of SMC1 at all stages in both male and female meiosis (Khetani and Bickel 2007; Yan *et al.* 2010; Tanneti *et al.* 2011; Yan and McKee 2013). Second, centromeric foci of SOLO require both *ord* and *sunn* function and centromeric foci of SUNN require *solo* function, suggesting reciprocal co-dependence of the three proteins (Yan *et al.* 2010). Third, survival of centromeric foci of SMC1, ORD, and SOLO beyond metaphase I in male meiosis depends on *mei-S332* (Balicky 2005; Yan *et al.* 2010), the *Drosophila* Shugoshin homolog. We expect that
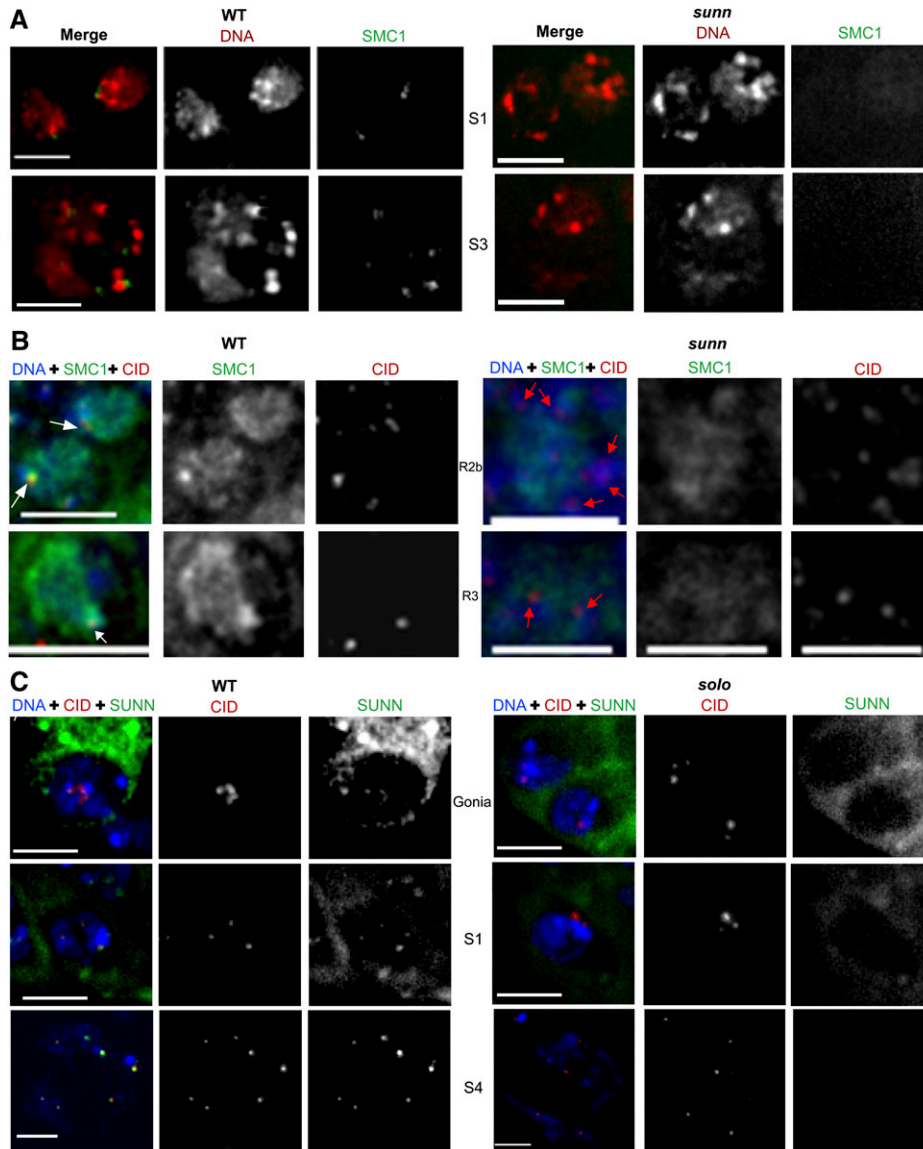
**Figure 7** Interactions among SUNN, SMC1, and SOLO. (A) SMC1 foci in spermatocyte nuclei require *sunn* function. Venus:: SMC1 expressed under the control of *nos-GAL4*::*VP16* formed DNA-associated foci in WT but not *sunn* (*sunn*[Z3-5839]/*Df*) spermatocytes. (B) SMC1 localization to centromeres in pro-oocytes/oocytes requires *sunn*. Whole-mount ovaries where immunostained with anti-SMC1 and anti-CID antibodies. In WT, SMC1 forms centromeric foci (white arrows) and localizes to chromosome arms, whereas in *sunn* (*sunn*[Z3-5839]/*Df*), SMC1 is lost from the centromeres (red arrows) observed as gaps in C (3)G staining but is weakly present on chromosome arms. R2b, region 2b; R3, region 3. (C) Centromeric SUNN::Venus foci in spermatocyte nuclei require *solo* function. Spermatocytes expressing SUNN::Venus under control of *nos-GAL4*::*VP16* were immunostained with anti-CID antibody. DNA-associated SUNN::Venus foci co-localized with CID in WT spermatogonia (eight-cell stage) and spermatocytes (S1 and S4 stages) but were absent at the same stages in *solo* (*Df(2L)A267*/*solo*[Z2-0198]) spermatogonia and spermatocytes. Bars, 5 μM.

SUNN centromere foci will prove to be similarly dependent on *mei-S332*, although this remains to be shown. Fourth, SOLO interacts physically with SMC1 in coimmunoprecipitation assays from ovarian extracts (Yan and McKee 2013) and with both SMC1 and SMC3 in yeast two-hybrid analysis (Q. Ma and B.D. McKee, unpublished data). At minimum, these data indicate very strong interactions of ORD, SOLO, and SUNN with each other and with the SMC cohesins and are consistent with roles as cohesin components. This idea does not exclude the possibility of other meiotic cohesin complexes, perhaps involving the mitotic non-SMC subunits and/or C(2)M. Multiple meiosis-specific cohesin complexes have been demonstrated in several higher eukaryotic systems (Nasmyth and Haering 2009; Severson *et al.* 2009; Llano *et al.* 2012).

The bioinformatic analysis of SUNN presented above is of interest in light of these considerations. Although no homologs of SUNN were identified in sequence- or profile-based searches, fold recognition and structural analysis indicated that SUNN may be a homolog of the cohesin protein SA. Although this line of reasoning is inconclusive, it suggests that the shared roles of SA and SUNN in sister chromatid cohesion may have a basis in a shared overall structure and raises the possibility that SUNN might serve as a meiosis-specific substitute for SA in some meiotic cohesin complexes. It will clearly be important to establish the role, if any, of SA in meiotic cohesion. More detailed biochemical and genetic studies of SUNN and its partners will be required to resolve the precise functions of these intriguing proteins in cohesion.

## Acknowledgments

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# GENETICS

# Sisters Unbound Is Required for Meiotic Centromeric Cohesion in *Drosophila melanogaster*

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and Bruce D. McKee**

**File S1**

**Bioinformatic analysis of SUNN**

A BLAST search (ALTSCHUL *et al.* 1997) of the non-redundant protein database at NCBI initiated with the SUNN protein sequence retrieved only 11 similar sequences, one each from the sequenced *Drosophila* species. PSI-BLAST (ALTSCHUL *et al.* 1997) searches with the full-length SUNN sequence or its parts retrieved no new sequences. Predicted secondary structure for SUNN indicated a primarily $\alpha$-helical protein (Fig. S3). No known domains were identified in the SUNN protein sequence (NCBI accession number NP_729739.3) by comparing it to profile models in Pfam (PUNTA *et al.* 2012) and CDD (MARCHLER-BAUER *et al.* 2013) databases. Profile-profile searches against CDD and PDB databases using HHpred (SODING *et al.* 2005) resulted in the identification of several sequences with potentially similar structure, albeit with low confidence. The highest scoring segment (probability of shared structure $P^{SS}$ = 58%) was located in the N-terminus (amino acid residues 21-64) showing similarity to a TPR (tetratricopeptide)-like repeat. Similarly, the highest scoring segment in fold recognition performed with Phyre2 (KELLEY and STERNBERG 2009) was in the N-terminus (amino acid residues 2-181), where structural similarity to the mouse plexin a3 intracellular domain (PDB code 3IG3) was detected (23% identity, 87.4% confidence). In addition to plexin domains that are all $\alpha$-helical, other low scoring Phyre2 matches included short segments from other $\alpha$-helical domains, such as ferredoxin, and repeats, such as ARM. The fold recognition program MUSTER (WU and ZHANG 2008) revealed structural similarity of SUNN to a family of exportins/importins – all $\alpha$-helical and HEAT repeat proteins – throughout the entire SUNN length (Table 7, Figs. S4 and S5).

The cohesin protein SCC3/SA is an all-$\alpha$-helical protein with HEAT-like repeats (NEUWALD and HIRANO 2000). Although BLAST analysis revealed no significant sequence similarity between SUNN and *Drosophila* SA (CG3423, NCBI accession number NP_477268.2), we performed bioinformatic analysis of SA in order to find its predicted structural features. Matching the SA sequence against Pfam and CDD yielded only one statistically significant hit to the cohesion-specific STAG domain (residues 187-207). HHpred searches revealed similarity to several ARM/HEAT-repeat containing proteins, such as cohesins and adaptins, as well as transportins. A substantial result regarding the possible relatedness of SUNN and SA was determined from fold recognition/threading analysis by MUSTER. Remarkably, among the six best (and statistically significant) template matches in the MUSTER analysis, five were the same for SUNN and SA (Table 7). Structural alignments of both the SUNN and SA sequences to the joint-top-matching 1qgkA template from the MUSTER analysis (human β-importin) are shown in Figs. S4 and S5. Using MUSTER, we also searched the structures of the following classes of proteins: 1) known HEAT repeat proteins such as the condensin subunits (CapG, CapD2), cohesin co-factors (Nipped-B, Pds5, Wapl) and PR65 (PP2A) (Table 7 and not shown); and 2) known ARM repeat proteins (Armadillo/$\beta$-Catenin 1, Importin-$\alpha$1, Importin-$\alpha$2 (data not shown) (NEUWALD and HIRANO

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2000; ANDRADE *et al.* 2001; NASMYTH and HAERING 2009; TEWARI *et al.* 2010; HIRANO 2012) (http://flybase.org). Wapl, PR65 and the ARM repeat proteins mostly matched to different templates from those matched by SUNN (data not shown). The templates matched by Nipped-B, Pds5, Cap-G and Cap-D2 overlapped with those matched by SUNN but none of them showed similarity comparable to that of SUNN and SA. The two highest-matching templates for Nipped-B, Pds5, Cap-G and Cap-D2 were not among the significant matches for SUNN (Table 7).

To be sure that the MUSTER results were meaningful, we employed another tool for fold recognition – I-TASSER (ROY *et al.* 2010). The results of the I-TASSER analysis confirmed the structural relatedness of SUNN and SA. Eight of the ten best-matching templates were the same for SUNN and SA and six of these shared templates were also common templates in the MUSTER analysis (Table S4). The structure of importin-$\beta$ (1qgkA) used for structural alignment of SUNN and SA (Figs. S4 and S5) is the joint-highest-scoring template found for SUNN and SA in the MUSTER analysis and is among the top-scoring templates for both proteins in the I-TASSER analysis (Tables 7 and S4).

We conclude that although SUNN and SA do not share any detectable primary sequence similarity, they may be structural homologs. Both proteins are predicted to be predominantly $\alpha$-helical, with structurally recognizable short $\alpha$-helical repeats that belong to the alpha-alpha superhelix fold (HEAT repeat, Sec7_domain, TPR-like). Both proteins can be confidently matched to multiple structural templates using MUSTER (1qgkA, 1wa5C, 3ea5B, 3m1iC, and 2x1gF) and I-TASSER (4c0oA, 2x19B, 1wa5C, 1qgkA, 3nbyA, 3icqT, 2x1gF and 2bptA (same as 3ea5B)), all of which are HEAT-repeat proteins belonging to the importin/exportin family. Although SUNN also exhibits structural similarity to other *Drosophila* HEAT-repeat proteins, the highest-matching templates for those proteins mostly differ from the highest-matching templates for SUNN and SA, suggesting that SUNN may be more closely related to SA than to other *Drosophila* members of the HEAT-repeat family. The complete bioinformatic analysis of SUNN is available on request.

**TABLE S1**  Second chromosome NDJ in *sunn* mutant males

| Sperm Genotype | NDJ type | Ova genotype | Progeny phenotype | No. of progeny Obtained |
|---|---|---|---|---|
| +/+ and bw/+ | Sister+Homologue | O | WT | 432 |
| O | Sister+Homologue | 2^2 b pr | b pr | 543 |
| bw/bw | Sister | O | bw | 72 |
| **Total** | **-** | **-** | **-** | **1047** |

+/Y; bw/+; *sunn*[Z3-5839/ Z3-1956] males were crossed with *C(2)EN, b pr* females in vials containing two males and four females each. In total, 76 males were tested, and they produced 1047 progeny.  The average number of progeny produced per male was 13.8. *C(2)EN b pr* females carry two copies of each arm of chromosome 2 attached to a single centromere and produce only diplo-2 (2^2 b pr) and nullo-2 (O) eggs. Fertilization of diplo-2 or nullo-2 eggs with sperm containing a single copy of chromosome 2 causes the production of inviable monosomic or trisomic embryos. However, paternal NDJ yields diplo-2 or nullo-2 sperm that can generate viable embryos. Thus, the level of second chromosome NDJ in males is proportional to the number of progeny produced per male. Parent males have bw/+ second chromosome genotype, so the presence of bw/bw progeny indicates the occurrence of sister chromatid NDJ. The following formula was used to calculate % sister chromatid NDJ – 100 x 2 (bw progeny) / (bw + WT progeny). % sister chromatid NDJ = 28.6. Note: None of the WT males tested produced any progeny.

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**TABLE S2**  UASp-SUNN::Venus rescues the *sunn* mutant NDJ phenotype in *Drosophila* males

| Male Genotype | X | Y | XY | O | n[a] | %NDJ[b] |
|---|---|---|---|---|---|---|
| *UASp-SUNN::Venus* ;  $\frac{sunn^{Z3-5839}}{Df^{\,c}, nos\text{-}GAL4::VP16}$ | 718 | 565 | 0 | 6 | **1289** | 0.46 |
| $\frac{sunn^{Z3-5839}}{Df, nos\text{-}GAL4::VP16}$ | 137 | 127 | 24 | 265 | **553** | 52.2 |
| $\frac{sunn^{Z3-5839}\;or\;Df, nos\text{-}GAL4::VP16}{+}$ | 586 | 447 | 1 | 4 | **1038** | 0.48 |

*w/B^S Yy^+* males with the indicated second and third chromosome genotypes were each crossed to 2 *y w* females. *nos-GAL4::VP16* drives expression of the UASp construct in germline cells. [a]total number of progeny scored. [b]%NDJ = 100 x (XY+O)/n. [c]*Df (3L) ED4470.*  Note: Although this cross does not specifically assay for sister chromatid NDJ, the O sperm class results from both homolog and sister chromatid NDJ.  Thus, the near absence of progeny from O sperm in line 1 indicates that the UASp-SUNN::Venus transgene suppresses sister chromatid as well as homolog NDJ.  In addition, a cross of the transgene males to *C(1)RM* females yielded no progeny from XX sperm (n=294), the class of sperm diagnostic of sister chromatid NDJ (data not shown).

**Table S3**  UASp-SUNN::Venus rescues *sunn* mutant NDJ phenotype in *Drosophila* females

| Female Genotype | X | XX | O | n[a] | %NDJ[b] |
|---|---|---|---|---|---|
| *UASp-SUNN::Venus* ;  <u>*sunn*[Z3-5839]</u> <br> *Df, nos-GAL4::VP16* | 593 | 10 | 5 | **608** | 4.9 |
| <u>*sunn*[Z3-5839]</u> <br> *Df, nos-GAL4::VP16* | 12 | 10 | 4 | **26** | 70 |
| <u>*sunn*[Z3-5839]</u> or <u>*Df, nos-GAL4::VP16*</u> <br> + | 269 | 0 | 0 | **269** | 0 |

*w/+* females with the indicated second and third chromosome genotype were each crossed to 2 *YSX.YL, In(1)EN, y B* males. [a]total number of progeny scored. [b]%NDJ = 100 x 2 (XX + O)/ (n + XX + O)).

**Table S4** Threading/fold recognition results (TM scores) for SA (NP_477268.2) and SUNN (CG32088, NP_729739.3) protein sequences by I-TASSER.

| PDB ID | Protein | I-TASSER (TM scores) | |
|---|---|---|---|
| | | SA | SUNN |
| 4c0oA | Transportin 3 | 0.538 | 0.875 |
| **2x19B** | **Importin-13** | **0.517** | **0.750** |
| **1wa5C** | **Exportin CSE1P** | **0.542** | **0.687** |
| **1qgkA** | **Importin-β** | **0.601** | **0.606** |
| 3nbyA | Exportin-1 | 0.494 | 0.710 |
| **3icqT** | **Exportin-T** | **0.519** | **0.663** |
| **2x1gF** | **Importin-13** | **0.479** | **0.701** |
| **2bptA*** | **Importin-β1** | **0.570** | **0.595** |
| 1w3tA | Importin-β3 | 0.816 | - |
| 4fgvA | Exportin-1 | - | 0.710 |
| 3m1iC | Exportin-1 | - | 0.697 |
| 4fddA | Transportin-1 | 0.541 | - |

The top 10 templates hit by SUNN and SA using I-TASSER (Roy *et al.* 2010) are shown ordered by total TM scores. Templates also hit by both SUNN and SA in the MUSTER analysis are highlighted in bold. PDB templates. 1 qgkA: Importin-β (Human). 2x1gF: Importin-13 (*Drosophila melanogaster*). 1wa5C: Exportin CSE1P (*Saccharomyces cerevisiae*). 2x19B: Importin-13 (*Saccharomyces cerevisiae*). 3icqT: Exportin-T (*Schizosaccharomyces pombe*). 4c0oA: Transportin 3 (Human). 2bptA: Importin-β1 subunit (*Saccharomyces cerevisiae*). 4fgvA: Exportin-1 *(Chaetomium thermophilum).* 3nbyA: Exportin-1 (Mouse). 3m1iC: Exportin-1 (*Saccharomyces cerevisiae*). 3w3tA: Importin subunit β3 (*Saccharomyces cerevisiae*) (Kap121p). 4fddA: Transportin-1 (Human). *2bptA represents the same protein chain as 3ea5B in Table 7.
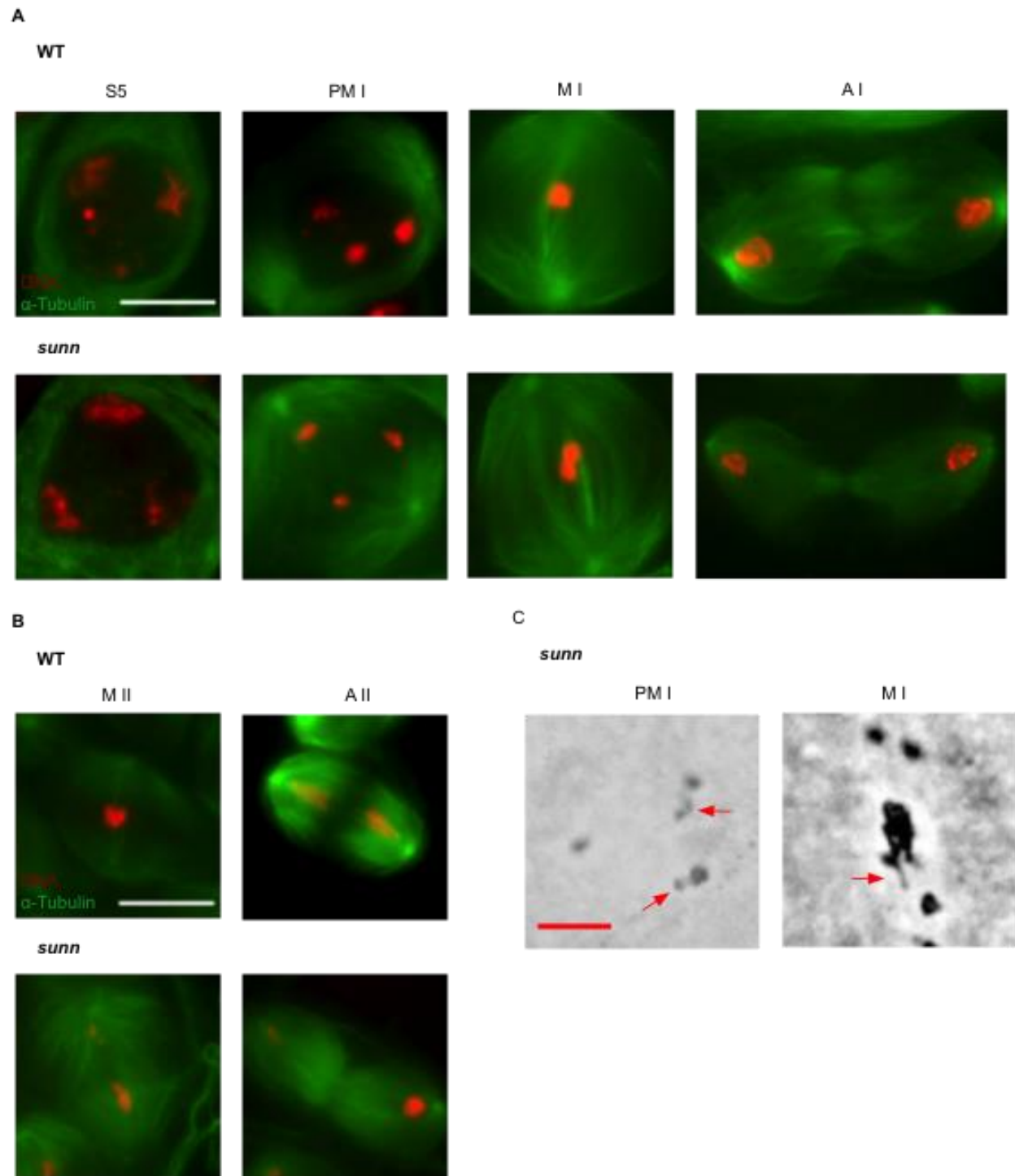
**Figure S1** Chromosome segregation during meiosis in *sunn* mutants. (A) and (B) Immunostaining was performed on WT and *sunn* (*sunn^{Z3-5839}/Df*) mutant spermatocytes using anti-α-Tubulin antibody conjugated with FITC (Fluorescein isothiocyanate) to visualize the spindle and DAPI to visualize DNA. PM I stands for prometaphase I, M I stands for metaphase I, A I stands for anaphase I, A II stands for anaphase II, M II stands for metaphase II. (A) Chromosome territory formation at S5 and PM I is normal in *sunn* mutants. At A I, roughly equal DAPI masses were observed at opposite poles in both *sunn* mutants and WT. (B) Metaphase II congression is defective and anaphase II segregation is unequal in *sunn* mutants. (C) Aceto-orcein staining of spermatocytes from *sunn* mutants revealed the presence of DNA territory extrusions (red arrows) in prometaphase I and metaphase I cells, which are diagnostic of loose packing of bivalent territories and loss of cohesion between sister chromatids. Aceto-orcein staining was performed according to (BONACCORSI 2000). Scale bars = 5 μM.

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A

| | Merge | DAPI | GFP-LacI |

B

| | Merge | DAPI | GFP-LacI |
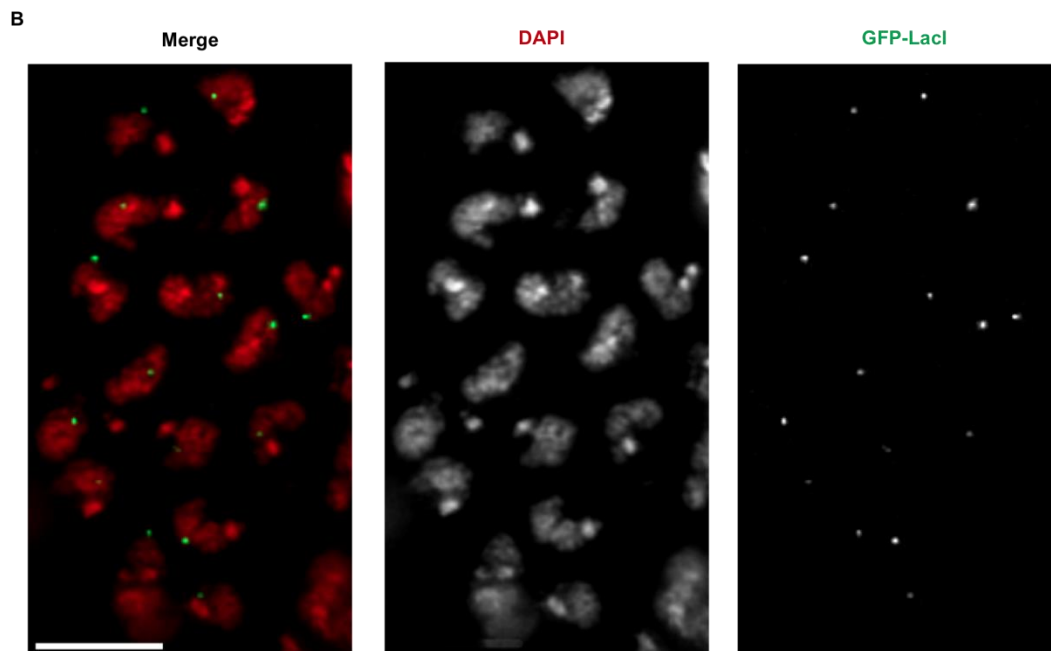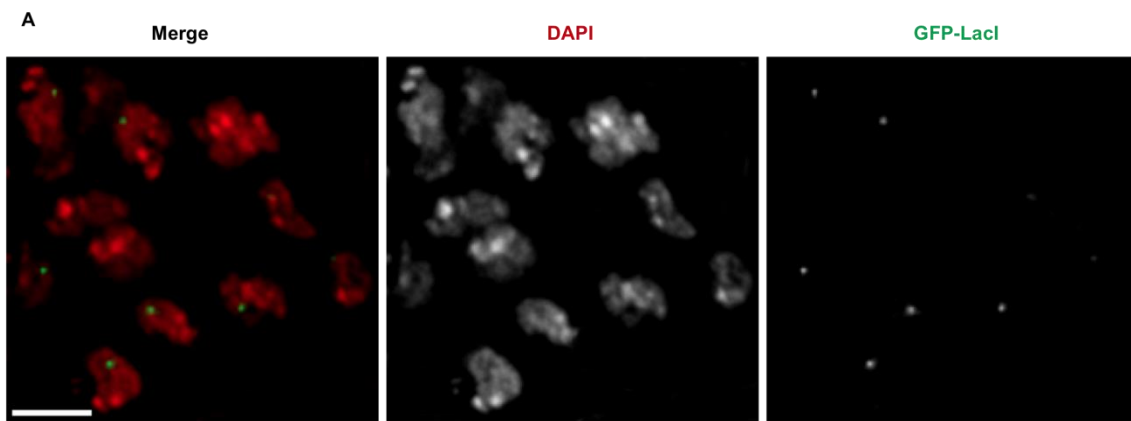
C

| Genotype | Stage | 1 spot | 2 spot | Total |
| --- | --- | --- | --- | --- |
| *sunn* | | | | |
| | Spermatogonia + S1 | 263 (92.6) | 19 (6.7) | 282 |
| | S2 | 38 (100) | 0 | 38 |
| WT | | | | |
| | Spermatogonia + S1 | 29 (87.9) | 4 (12.1) | 33 |
| | S2 | 15 (100) | 0 | 15 |

**Figure S2** Arm cohesion is not affected in *sunn* mutants. Single GFP foci were observed in nuclei from DAPI-stained spermatogonial 8-cell cysts (A) and stage S1 meiotic 16-cell cysts (B) from *sunn* [Z3-5839]/*Df* males heterozygous for a second chromosome insertion of a 256-mer lacO array and expressing GFP-Lac I under control of the *hsp83* promoter (VAZQUEZ *et al.* 2002). Nuclei exhibit one or two foci depending on whether sister chromatid arms are together or apart, respectively. Scale bars represent 5uM in (A), 10 uM in (B). In eight cell cysts (A) and S1 16 cell cyst (B), *sunn* mutants mostly display a single GFP-Lac I focus. (C) Quantification of GFP-Lac I foci in *sunn* and WT spermatogonia and S1 and S2 stage spermatocytes.

```
1:   MEFVSAISTVGNTETVAREFAIAFLQAVTSDPDNWIVKVSHQFVGQCFRVLGLNFNVSVQ
SS : CCHHHHHHCCCCHHHHHHHHHHHHHHHHHHCCCCCHHHHHHHHHHHHHHHHHHCCCCCEEHH

61:  AFSQTQDHPYRDANPNYIFDPVPLVMTELDKFLDRLPRLLAQVGELLAKDSQGTRFDELT
SS : HHHHHCCCCCCCCCCCCEEECCHHHHHHHHHHHHHHHHHHHHHHHHHHHCCCCCCCHHHHC

121: QPLPCYLNLILKWIKVVQAMCKNMQLSNSTSVDRYICEPLARMSFSILGGLAKLLPKYEN
SS : CCHHHHHHHHHHHHHHHHHHHHHHHCCCCCCCCCCHHHCCHHHHHHHHHHHHHHHHCCCCCC

181: FSGFKGICLALTVDVRYGIFYQETCDVVVPPMLDIFRKHYKTFCGPEKSALDFVYCILTV
SS : CCHHHHHHHHHHHHHHHHHHHHHHHCCCCCCHHHHHHHHHHHHCCCCCCCCCCEEEEEEEE

241: VMSEDDCYIKAYEFLESMIRQFAENSQHSHHLRCFTNELITVKYVVLQFETLKDSNSKPL
SS : EEECCCHHHHHHHHHHHHHHHHHHHCCCCCHHHHHHHHHHHHHHHHHHHHHHHHCCCCCHH

301: LLATLKYLMTLQRNVASAECFTKRFHEELLHLVLRMSVSSATVASMAAKVYITLSQRQHQ
SS : HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHCCCHHHHHHHHHHHHHHHHHHHHH

361: EQEIEQHILETYVKIPQNPRKNITYEQFRNELTRYLKTLIQYFPPLQEFDFYARVLTARN
SS : HHHHHHHHHHHHHHHCCCCCCCCCCHHHHHHHHHHHHHHHHHHCHHHHHHHHHHHHHHCCCH

421: VRIELSLIAAQCASIIFEMHMAEYTSLPVARDQVNELLRVWSRILKASSKQNGTRPLIYS
SS : HHHHHHHHHHHHHHHHHHHHHHHHHHHCHHHHHHHHHHHHHHHHHHHHHHHCCCCCCCEEEE

481: IYDLIDFDSVAECDADLLLNLESSCLENFLNDDTIIESEFQRLYVNISRSVGATGNIQIH
SS : HHHHHHHHHHHHHHHHHHHHHHHHHHHCCCCCCCCHHHHHHHHHHHHHEEECCCCCEEHH

541: TTTSRALRDEQAALQLRLNNTDPESPEMQQLLKEYAFSYMRIHAVLMVNKLHVRYVADIY
SS : HHHHHHHHHHHHHHHHHHHCCCCCCCCCHHHHHHHHHHHHHHHHHHHHHHCCCCHHHHHHHH

601: ETLAKFVLEMPTLNENITLYGSESLANMLVLLHGDLKDSDGEMIDKVSGLVKQLQDFCVS
SS : HHHHHHHHHCCCCCCHHHHHHHHHHHHHHHHHHHCCHHHCCHHHHHHHHHHHHHHHHHHHH

661: ELRKEKLNLKRAKFFVCSTLIMHINQLPYLSLDSAAYDKILEVLTSPPRESPPELPSNTY
SS : HHHHHHHHHHHHHHHHHHHHHHHHHHCCCCCCCCCHHHHHHHHHHCCCHHCCCCCCCCCCH

721: VADMHYMFRLLIKTEEFDLPTNRVWKLLMKYKMSSIKCLDTEIEDLINVFIKYRIESYIH
SS : HHHHHHHHHHHHHHCCEECCCCHHHHHHHHHHCCCCCCCCCHHHHHHHHHHHHHHHHHHHHH
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781: YMSVIQLHIYKDSNVNKKGSIALTAHLRLIDMNCSALDSWLLRYIIFKESLSLLIKNMRI
SS : HHHHHEEEECCCCCHHHCCHHHHHHHHHHHHHHHCCHHHHHHHHHHHHHHHHHHHHHHHHCEE


841: RRSEVTNSSQRNRLLPLKYILNMVVNLRLDEAQFLSIAKLLHVLKDETIGPQENSEIENF
SS : EECCCCCCCCCCCCCCHHHHHHHHHHHHCCCCHHHHHHHHHHHHHHHCCCCCCHHHHHHHHH


901: ITQISTYKYQAEDEHLEATKNEYYEGRLTPLPSGPLNLWQIKTIHNTTDLTD
SS : HHHHHHHHHCCCHHHHHHCCCCHHCCCCCCCCCCCCCHHHHHHHHHHHCCCCC
```

**Figure S3** SUNN (CG32088) secondary structure (SS) predicted by PSI-PRED (THORNE *et al.* 1996; JONES 1999). H, α-helices (highlighted in yellow), E, β-strands (highlighted in green), C, coils.

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(A)

```
SUNN    MEFVSAISTVGNTETVAREFAIAFLQAVTSDPDNWIVKVSHQFVGQCFRVLGL--NFNVS
1qgkA   MELITITILEKTVSPDRLELEAAQKFLERAAV--EN-----LPTFLVELSRVLA-NPGNSQV
        **:::: :.... ..: :  * *  **: .:   :*       *: :  ***.   . .

SUNN    VQAFSQTQDHPYRDANP--NYIFDPVPLVMTELDKFLDRL-PRLLAQVGELLAK--DSQG
1qgkA   ARVAAGLQ---------IKNSLTSKDPDIKAQYQQRWLAIDANARREVKNYVLHTLGTET
        .:. :  *         IKN  *  . * :: ::    : ..  :* : .: .::

SUNN    TRFDELTQPLPCYLNLIL---KWIKVVQAMCKNMQLSNSTSVDRYICEPLARMSFSILGG
1qgkA   YRPSSASQCVAGIACAEIPVNQWPELIPQLVANVTNPNST-------EHMKESTLEAIGY
         *  .. :* :.     :   :* ::: :  *:  .***        * : . ::. :*

SUNN    LAKLLPKYENFSGFKGICLALTVDVRY-----GIFYQETCDVVVPPMLDIFRKHYKTFCG
1qgkA   ICQDIDPEQLQDKSNEILTAIIQGMRKEEPSNNVKLAATN--ALLNSLEFTKANFDKE--
        :.:: .   :  : * *:  .:*    .:*     .:     *   .:   *:: .::.

SUNN    PEKSALDFV--YCILTVVMSEDDCYIKAYEFLESMIRQFAENSQHSHHLRCFTNELITVK
1qgkA   ---SERHFIMQVVCEATQCPDTRVRVAALQNLVKIMSLYYQ------YMETYMGPALFAI
           *  .*:      :.  .:     : * : * .::  : :       ::. : . : .

SUNN    YVVLQFETLKDSNSKPLLLATLKYLMTLQRNVASAECFTKRFHEELLHL-VLRMSVSSAT
1qgkA   TIEAMKS-----DIDEVALQGIEFWSNVCDEEMDLAIEASEAAEQ----GRPPEHTSKF-
         :    .       : . .: *  ::: .:  . . . :..  *:        .*.

SUNN    VASMAAKVYITLSQRQHQEQEIEQHILETYVKIPQNPRKNITYEQFRNELTRYLKTLIQY
1qgkA   -------------YAKGALQYLVPILTQTLTKQDENDDDDW--NPCKAAGVCLMLLATC
                     : *:  : :* .* :* .:   :  : :    *  *

SUNN    FP---PLQEFDFYARVLTARNVRIELSLIAAQCASIIFEMHMAEYTSLPVARDQVNELLR
1qgkA   CEDDIVPHVLPFIKEHIKNPDWRYRDAAVMAFGCIL-------EGPEPSQLKPLVIQAMP
         : : *   . :.   : * . ::: *  .:      *  ... :  * :

SUNN    VWSRILKASSKQNGTRPLIYSIYDLIDFDSVAECDADLLLNLESSCLENFLNDDTIIESE
1qgkA   TLIELMKDPS-----------------VVVRDTAAWTVGRICELLPEAAINDV-----Y
        .  ..::* .*                  * :  *   :.:.    *   :**

SUNN    FQRLYVNISRSVGATGNIQIHTTTSRALRDEQAALQLRLNNTDPESPEMQQLLKEYAFSY
1qgkA   LAPLLQCLIEGLSAEPRVASNVCWAFSSLAEAAYEAADVADDQEEPAT------------
        :  *   :  : ..:.*  .:  :.   :: * *    : : : *..

SUNN    MRIHAVLMVNKLHVRYVADIYETLAKFVLEMPTLNENITLYGSESLANMLVLLHGDLKDS
1qgkA   ---------YCLS-SSFELIVQKLLETTDRPDGHQNNLRSSAYESLMEIVKNSAKDCYPA
                 *    .  *  :.* : . .   ::*:  . *** :::    *    :

SUNN    DGEMIDKVSGLVKQLQDFCV--SELRKEKLNLKRAKFFVCSTLIMHINQLPYLSLDSAAY
1qgkA   VQKTTLVIMERLQQVLQME-SHIQSTSDRIQFNDLQSLLCATLQNVLRKVQH-QDALQIS
         :     :   ::*: ::       :  .:::::   : ::*:**   :.:: :  .

SUNN    DKILEVLTSPPRESPPELPSNTYVADMHYMFRLLIKTEEFDLPTNRVWKLLMKYKMSSIK
1qgkA   DVVMASLLRMFQSTAGSG---GVQEDALMAVSTLVEV----LGGE------------FLK
        * ::  *    :.:.  .       *     . *::.    *  :            :*

SUNN    CLDTEIEDLINVFIKY-RIESYIHYMSVIQ-LHIYKDSNVNKKGSIALTAHLRLID----
1qgkA   YMEAFKPFLGIGLKNYAEYQVCLAAVGLVGDLCRALQSNIIPFCDEVMQLLLENLGNENV
         :::    *   : :*  . : : :.: :   :.:: *   :**:    . .:    *. ..

SUNN    -MNCSALDSWLLRYIIFKE------SLSLLIKNMR-IRRSEVTNSSQRNRLLPLKYILNM
1qgkA   HRSVKPQILSVFGDIALAIGGEFKKYLEVVLNTLQQASQAQVDKSD--------YDMVDY
          . ..   :: : * :       *.:::::.:   :::* :*.             :::

SUNN    VVNLRLDEAQFLSIAKLLHVLKD--ETIGP------QENSEIENFITQISTYKYQAEDEH
1qgkA   LNELR--ESCLEAYTGIVQGLKGDQENVHPDVMLVQPRVEFILSFIDHIAGDEDHTDGVV
        : :** *: .: : : :::  **.  *.:       . . * .** :*: . : :::.

SUNN    LEATKNEYYEGRL-TPLPSGPL-----NLWQIKTIHNTTDLTD-----------------
1qgkA   ACAAG---LIGDLCTAFGKDVLKLVEARPMIHELLTEGRRSKTNKAKTLARWATKELRKL
            *:        * * *.:. .. *       .     : : :       .
```

```
SUNN          ----
1qgkA         KNQA
```

**(B)**

```
1qgkA         ------------------------------------------------------------
SA            MMARRGGKRIRMDDPPPDYEELHSDALNESTSDADSPTKRMTRLRARGGVRDKPPIIDDD


1qgkA         -------------------------------------------------MELITILEK
SA            EDDFFAPIARKRKTPATRKGPTERKERVERPRKEPVDKGHHERIDSEREITTDENSLYYI
                                                                  :   ::

1qgkA         TVSPDRLELEAAQKFLERAAVENLPTFLVELSRVLAN-----PGNSQVAR--VAAGLQIK
SA            VRHSKNPIASIVDQWIEQYKA-NRETALVALMQFFINASGCKGKISEDIQYPVDHTSIIR
              .  ...   . .::::*:  . *  * ** * .:. *      *:  :  *    *:

1qgkA         NSLT--------------SKDPDIKAQYQQRWLAI----------DANARREVKNYVLH
SA            RMTEEFDEESGEYPLIMSGTQWRKFKNNFCDFVQTLVKQCQYSIIYDQFLMDNVISLLTG
              .                 ::  .:* ::  :    ::         *      :* . :

1qgkA         TLGTETYRPSSASQCVAGIACAEIPVNQWPELIPQLVANVTN-----PNSTEHMKESTLE
SA            -LSDSQVRAFRHTATLAAMKLMTALVDVALLVSNNFDNAAKQFEAERVKSRDRRASDRLD
               *.. . *.  :  :*.:     *:   :  ::    ..:      :* ::   .. *:

1qgkA         AIGYICQDIDPEQLQDKSNEILTAIIQGM---RKEEPSNNVKLAATNALLNSLEFTKANF
SA            SLMTKRSEL--EENMDEIKSMLTYMFKSVFVHRYRDSLPDIRAICMAEIGIWMENYPQNF
              ::    .::  *:  *: :.:** :::.:   *  .:  :::   .   :*     **

1qgkA         DKESERHFIMQVVCEATQCPDTRVRVAALQNLVKIMSL--YYQYMETYMGPALFAITIEA
SA            LD----DSYLKYIGWTLHDKIGEVRLRCLQSLLPLYEKDELKGKLELFTS-KFKDRIVAM
                .    . :: : ::     .**: .**.*: .        :* : .  :    :

1qgkA         MKSDIDEVALQGIEFWSNVCDEEMDLAIEASEAAEQGRPPEHTSKFYAKGALQYLVPILT
SA            TLDKEFEVSVHAVKLVISILKIHPEI-LADKDCEIVYELVYSSHRGVAQAAAEFLNVRLF
               ..  **:::.:::  .: . .  :: .  .:.      : :  *:.* ::*    *

1qgkA         QTLTKQDENDDDDDWNPCKAAGVCLMLLATCCEDDIVPHVLPFIKEHIKNP----DWRYR
SA            HLTADMEETKTKRGKVRMPNTPLVRDLVQFFIESELHEHGAYLVDSFIDSNDMVRDWECM
              : :. :*... .       :: :    *:    *.:: *   :::..*..      **.

1qgkA         DAAVM---AFGCILEGPEPSQLKPLVIQAMPTLIELMKDPSVVVRDT--AAWTVGRICEL
SA            TDLLLEEP--GPNEEVLDNKQESTLIEIMVSSVKQSATGEVPVGRASNRKCTLSAKELKA
                ::      *   *   : .* ..*:   :.:: *   . .. * * :     .: :

1qgkA         LPEAAIN--DVYLAPLLQCLIEGLSAEPRVASNVCWAFSSLAEAAYEAADVADDQEEPAT
SA            IQDEKAKLT-EHFIVTLPSLLEKYQADSEKLANLLAVPQYFDLNLYTTNR----------
              : :   :      :: . * .*:* .*:..  :*: . . :      * :

1qgkA         YCLSSSFELIVQKLLETTDRPDGHQNNLRSSAYESLMEIVKNSAK-------DCYPAVQK
SA            --QEGNLQALLDRINQVMSMHTGRE--VLETCAKTLECLCAEGSATYTRCNIARSNIIES
                ...:: :::::: :. .    *:: : .:. ::* : :.:           ::.

1qgkA         TTLVIMERLQQVLQMESHIQSTSDRIQFNDLQSLLCATLQNVLRKVQHQDALQISDVVMA
SA            AVNKYKDAIEEWRNLIQGEETPNEDDIYNITITLKVLSILYSSHNLN---PWELFKSLFQ
              :.     : ::: ::  . ::..:    :*   :*     ::   ::::    : :

1qgkA         SLLRMFQSTAGSGGVQEDALMAVSTLVEVLGGEFLKYMEAFKPFLGIGLKNYAEYQVCLA
SA            DVEEAQSKENIDRCLPNEALVYCIEACYFS------ISWGLQYVENEC----ESVNV---
              .: .  ..    . : ::**:       .           .:: . .       . :*

1qgkA         AVGLVGDLCRALQSNIIPFCDEVMQLLLENLGNENVHRSVKPQILSVFGDIALAIGGEFK
SA            -----TEVVAEL----RNNLDTFMGACFELTRD-GPTVQIQEAAYQSICDLLIIFSDKLA
                   ::    *      * .* :* :    :  .   ::   : *  : :...::

1qgkA         KYLEVVLNTLQQASQAQVDKSDYDMVDYLNELRESCLEAYTGIVQGLKGDQENVHPDVML
SA            RSEIEHIRGLE----YKSR----------MDEHLILDNFVQHYVFSLKQDVAQDETRIEE
              :     :. *:     :            :: :    *  .** * :  .. :
```
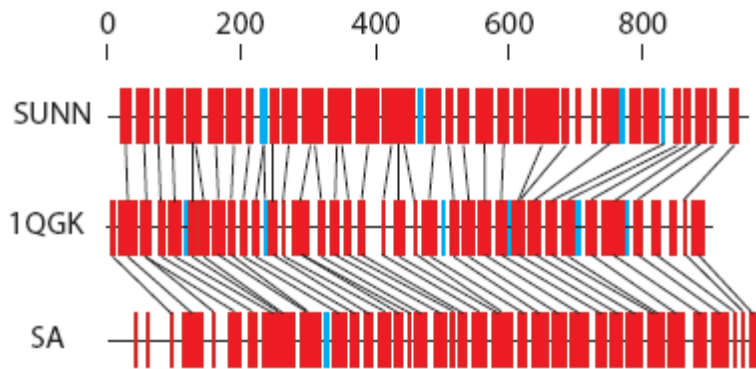
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```
1qgkA    VQPRVEFILSFIDHIAGDEDHTDGVVACAAGLIGDLCTAFGK-DVLKLVEARPMIHELLT
SA       LHKKRNFLACYCKLVVYNIIP----TMRAASIFKYYVKCYNDY--------GDIIKATLG
         :: : :*: .: . :. :       .  **.::    ..:..         :*:  *

1qgkA    EGRR-SKTNKAKTLARWATKELRKLKNQA----------
SA       KAREINKVNFAMTLLLSLITVFKSLQEQSEDGIVSKSSQ
         :.*. .*.* * **      . ::.*::*:
```

**Figure S4** Alignments of amino acid sequences of SUNN (A) and SA (B) with human β-importin (1qgkA) on the basis of structural similarities as determined by MUSTER. Dashes indicate gaps introduced by the alignment procedure. "*" indicates amino acid identities. ":" and "." indicate strong and weak amino acid similarity, respectively (Wᴜ and Zʜᴀɴɢ 2008).

**Figure S5** Structural similarity between SUNN and SA by MUSTER analysis. Predicted secondary structure organization is similar between SUNN and SA. Numbers on top indicate amino acid positions. 1QGK (human importin-β) was the most significant template hit in MUSTER analysis for both SUNN and SA. Red and blue boxes indicate predicted α-helices and β-sheets, respectively. Lines between α-helical segments of SUNN and 1QGK or between SA and 1QGK are based on structural alignments in Fig. S4.
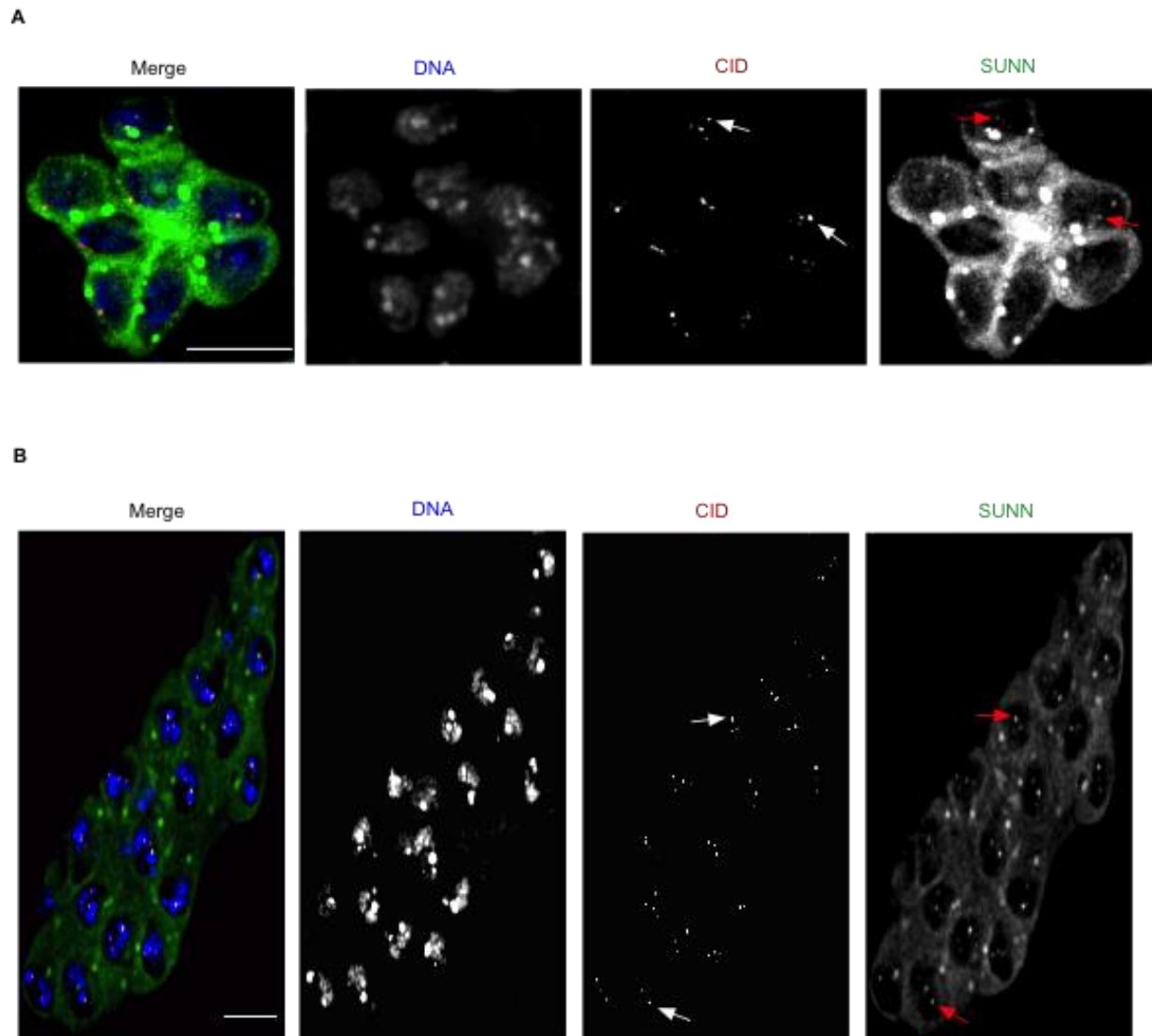
**Figure S6** SUNN co-localizes with CID in male germ cells. 8 cell spermatogonial (mitotic) cysts (A) and 16-cell stage S1 (meiotic) spermatocyte cysts (B) from w/$B^sYy^+$; *UASp-SUNN::Venus*; *sunn$^{Z3-5839}$ /Df, nos-GAL4::VP16* males were stained with anti-CID antibody and DAPI. Co-localizing SUNN::Venus and CID spots are indicated with red and white arrows respectively. The level of SUNN::Venus co-localizing with CID is higher at the sixteen-cell stage when compared to eight-cell stage. SUNN::Venus also localizes to the cytoplasm and between cells in the cyst during two-cell cyst, four-cell cyst stage, (not shown) and eight-cell cyst stages. This localization decreases in 16 cell cysts and is inconspicuous by S2. In pre-16 cell cysts, very large SUNN foci are also observed which do not co-localize with CID and are present on DNA periphery. The functional relevance of this localization is not clear. Scale bars = 10 μM.
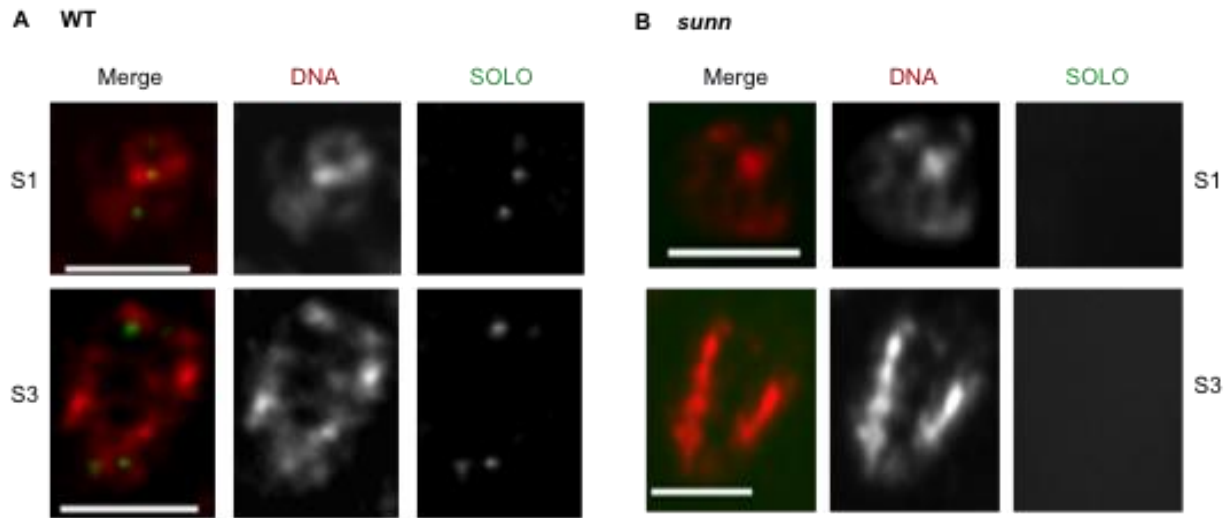
**Figure S7** SOLO fails to form foci in spermatocyte nuclei in *sunn* mutants. Venus::SOLO expressed under the control of nos-GAL4::VP16 formed DNA-associated foci in WT in S1 and S3 stages (A) but not in *sunn* (*sunn* [Z3-5839] */Df)* (B) spermatocytes.

**File S2**

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