Studies on SOLO Working Mechanism in the Meiosis of *Drosophila melanogaster*

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ABSTRACT

In eukaryotes, sister chromatids are closely aligned due to cohesion, a process essential for chromosome pairing and segregation during both mitosis and meiosis. A conserved cohesin complex in a ring structure is composed of four subunits, including each of these four members or their homologs, SMC1, SMC3, SCC1/RAD21/REC8, and SCC3/SA. Up to now, no REC8 homolog has been identified in the meiosis of *Drosophila*. SOLO is a meiotic protein required for accurate chromosome segregation, centromere cohesion, and cohesin complex localization in *Drosophila* meiosis. In addition, SOLO is required for synapsis and recombination in *Drosophila* female meiosis.

In this study, we further analyzed the working mechanism of SOLO and the SOLO-containing cohesion complex in *Drosophila* meiosis. SOLO C-terminal residues conserved with SCC1/ RAD21/ REC8 are essential for its chromosome localization, accurate chromosome segregation and centromere cohesion. Furthermore, yeast two-hybrid revealed that SOLO interacts with SA; in addition, it interacts with SMC1 and SMC3 with its C and N terminus, respectively. Similar to RAD21, SOLO interacts with *Drosophila* Separase homolog SSE based on the results from yeast two-hybrid. These results taken together support that SOLO might work as the SCC1/REC8 homolog in *Drosophila* meiosis.

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Chapter 1 - INTRODUCTION AND GENERAL INFORMATION

Overview of meiosis

Meiosis, in all eukaryotes, is a specialized cell division in which one diploid cell divides into four haploid cells called gametes. Meiosis I and Meiosis II are similar to their analogous subphases in the mitotic cell cycle, both divide into prophase, prometaphase, metaphase, anaphase, and telophase. Meiosis I is a reductional division in which homologous chromosomes pair, undergo recombination, and segregate to opposite spindle poles (Roeder, 1997; Page and Hawley, 2003; McKee, 2004). In Meiosis I, proper homolog segregation requires recombination to generate chiasmata, which derives from crossovers between homologous chromatids and are stabilized by sister chromatid arm cohesion (Carpenter, 1994; Page and Hawley, 2003; McKee, 2004). Meiotic recombination is initiated by the endonuclease Spo11 programmed double-strand break (DSBs) (Keeney et al., 1997). A meiosis specific ubiquitous homologous recombination pathway ensures adequate numbers of homolog crossovers in the DSB repair (Ehmsen and Heyer, 2008; Szekvolgyi and Nicolas, 2010). However, in meiosis I of some eukaryotes like *Drosophila melanogaster* males, homologues do not go through recombination or chiasmata but they pair and are stabilized via a “conjunction complex”. The components are unclear except that two male meiosis specific proteins Stromalin in Meiosis (SNM) and Mod (mdg4) in Meiosis (MNM) have been identified (Thomas et al., 2005; McKee et al., 2012). In meiosis I, homologs segregate but sister centromeres adopt a side by side orientation and form a single functional kinetochore to ensure that the sister chromatids coorient in meiosis I (Hauf and Watanabe, 2004). In contrast, meiosis II is a mitosis-like equational division in which sister chromatids segregate to opposite spindle poles. Sister chromatids are connected mainly by cohesion between

sister centromeres. After metaphase II, sister centromeric cohesion get dissolved by the cleavage of cohesin by Separase, thus sister chromatids orient back to back and establish separate kinetochores that attach to spindles respectively (**Figure 1-1**).

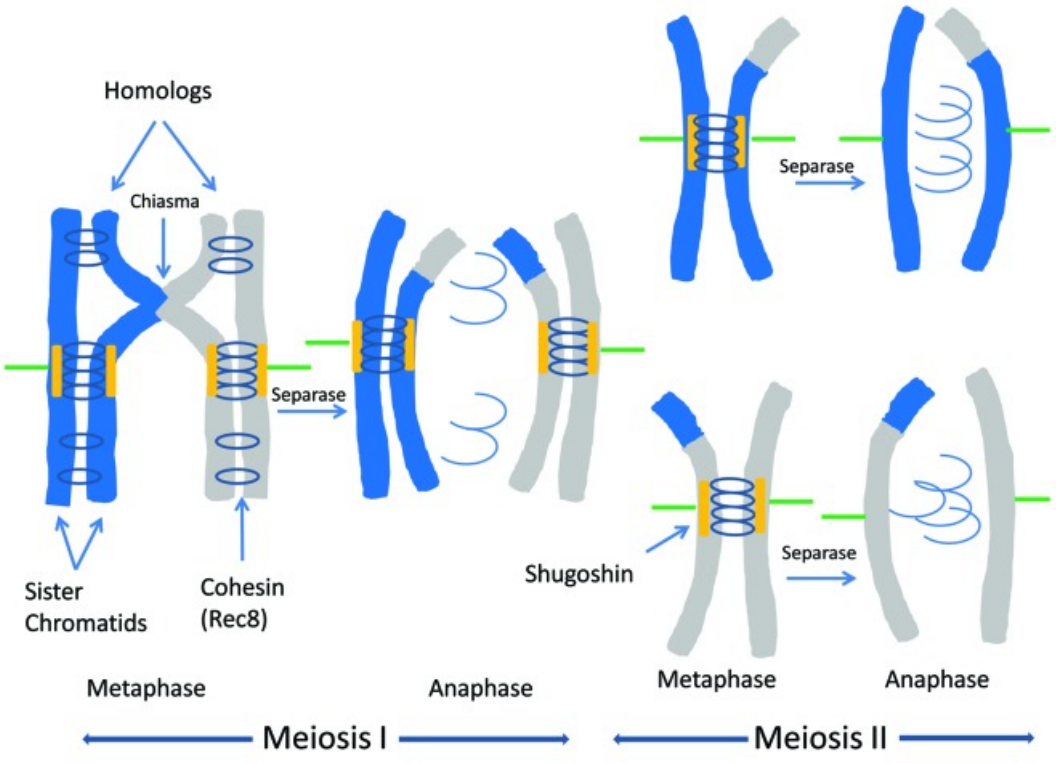


Figure 1-1 Meiotic chromosome segregation pattern in most eukaryotes

Homologous chromosomes segregate during meiosis I; while sister chromatids segregate during meiosis II. Sister chromatids are held in pairs by cohesin complexes containing the meiosis-specific REC8 subunit. In meiosis I, homologous chromosomes are linked by chiasmata initiated by homologous chromatids crossovers. At the same time, sister kinetochores attach to microtubules emanating from the same spindle pole. At anaphase I, Separase cleaves cohesin subunit REC8 only at chromosome arms, thus releasing chiasmata; cohesin at centromeres is protected by Shugoshin. In meiosis II, the residual centromeric cohesion facilitates sister kinetochores attachment to microtubules emanating from opposite spindle poles. Sister chromatid separation at anaphase II is triggered by Separase cleavage of the remaining REC8. This figure is adopted from (McKee et al., 2012).

Meiosis in *Drosophila*

In all eukaryotes, meiotic cell division entails sorting and segregation of both homologous and sister chromatids. In general, two mechanisms regulate the chromosome pairing and segregation during meiosis, a specialized chromatid cohesin complex to encircle sister chromatids and a homolog synapsis/recombination pathway to yield regular crossovers between homologous chromatids. Like most eukaryotes including baker's yeast, maize, mice, and human, *Drosophila* females utilize a "standard meiotic script" involving both sister chromatid cohesin and homologous synapsis as well as recombination. However, mutations of genes affecting female meiosis exchange segregation pathway, including spo11 homolog mei-W68, spnA, spnB, spnD or okr, do not affect male meiosis. In addition, mutations in genes required for the non-exchange distributive pathway including nod (no distributive disjunction) and ncd (nonclaret disjunctional) do not affect male meiosis either (Knowles and Hawley, 1991; Orr-Weaver, 1995). Thus, neither genes affecting recombination nor genes affecting distributive segregation in female meiosis exhibit any effect in male meiosis. Furthermore, mutations in the synaptonemal complex (SC) protein C(3)G (Crossover suppressor on 3 of Gowen) alters female but not male homologs segregation pattern (Walker and Hawley, 2000; McKim et al., 2002; McKee, 2004). Taken together, *Drosophila* male utilizes a unique meiotic system lacking recombination, synapsis and the associated structures. Nevertheless, in *Drosophila* male meiosis, non-exchange homologs pair and segregate faithfully during meiosis I. A unique protein complex including SNM and MNM provides stable connections between homologs in meiosis I (Thomas et al., 2005). In addition, the sex chromosomes pair at 240 bp rDNA repeats

with “intergenic spacer” (IGS) (Gershenson, 1933; Sandler and Braver, 1954; Peacock, 1965; McKee and Lindsley, 1987; McKee and Karpen, 1990; McKee et al., 1992; McKee, 1996; Ren et al., 1997; McKee et al., 2012). No autosomal site-specific pairing sites have been identified despite various approaches utilized, but it has been proposed that major autosomes adopt genome-wide homolog pairing instead of site-specific pairing to establish homologs (Yamamoto, 1979; McKee et al., 1993; Tsai et al., 2011; McKee et al., 2012).

A number of proteins have been reported to mediate homolog segregation and cause meiosis I nondisjunction in *Drosophila* males including male specific protein TEFLON, MEI-S8 (Meiotic from via Salaria 8), MEI-O81 (Meiotic from Ostiense 81), SNM, and MNM. TEFLON is required for accurate autosomes segregation but is not necessary for sex chromosomes (Tomkiel et al., 2001). MEI-S8 cause nondisjunction of chromosome 4 while it has no effect on sex chromosomes and major autosomes. MEI-O81 works in the segregation of all four chromosomes (Sandler et al., 1968). SNM and MNM are also essential for segregation of all homolog pairs in male *Drosophila* meiosis I, but not for females or sister chromatids segregation (Thomas et al., 2005; Tsai et al., 2011).

In addition, three unique *Drosophila* meiotic proteins, Sisters On the LOose (SOLO), ORientation Disruptor (ORD) and Sisters UNbound (SUN), have been identified to work in both male and female meiosis in similar ways. SOLO, ORD, and SUN are required for cohesion and accurate chromosome segregation in both sexes and for recombination and SC stability in females. Although sequence analysis show no significant similarities to cohesin, the SOLO, ORD and SUN proteins colocalize with cohesin and are required for stable localization of cohesin to centromeres (Krishnan; Goldstein, 1980; Bickel et

al., 1996; Bickel et al., 1997; Balicky et al., 2002; Webber et al., 2004; Khetani and Bickel, 2007; Yan et al., 2010; Yan and McKee, 2013).

Cohesion and cohesin

In eukaryotes, sister chromatids are closely aligned from their establishment in prophase until anaphase (anaphase II in meiosis), due to cohesion, a process critical for chromosome pairing and segregation during both mitosis and meiosis. In addition to its function to associate sister chromatids, cohesion promotes homolog recombination and SC formation and stabilizes chiasmata in meiosis I (Petronczki et al., 2003). A conserved cohesin complex plays a pivotal role in this process by establishing and maintaining sister chromatid cohesion.

Cohesin complex is composed of four subunits including Structural Maintenance of Chromosomes 1 (SMC1), Structural Maintenance of Chromosomes 3 (SMC3), a kleisin subunit, which can be either the mitotic SCC1/RAD21 protein or its meiosis-specific paralog REC8, and a SCC3/SA-family subunit. SMC1 and SMC3 are long intramolecular coiled-coil proteins that form extended hairpin structures with the N- and C-terminal globular ATPase domains at one end and globular hinge domains at the other. SMC1 and SMC3 bind at their hinge domains and to opposite ends of the kleisin subunit SCC1/RAD21/REC8 at their ATPase domains, forming a tripartite ring that embraces pairs of sister chromatids. The SA subunit binds to the kleisin subunit and contributes to cohesin chromosome binding (Klein et al., 1999; Watanabe and Nurse, 1999; Haering et al., 2002; Gruber et al., 2003; Schleiffer et al., 2003; Nasmyth

and Haering, 2009) (**Figure 1-2**).

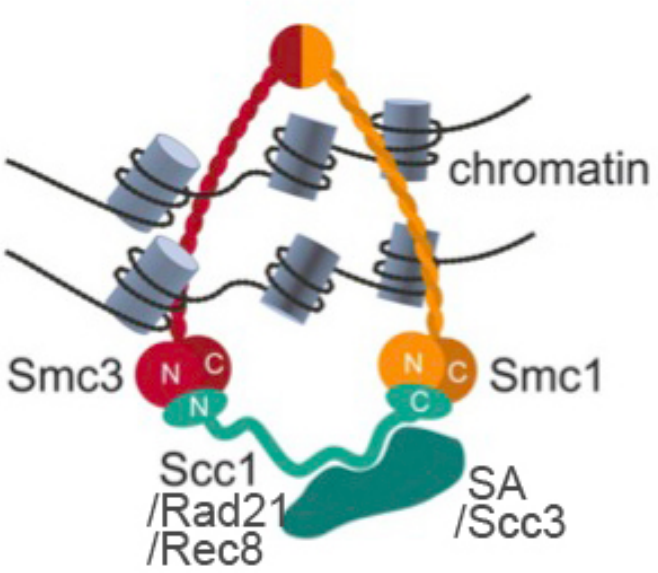


Figure 1-2 Model of the cohesin ring protein complex

Cohesin complex is composed of four subunits including SMC1, SMC3, a kleisin subunit SCC1/RAD21 or its meiosis-specific paralog REC8, and a SCC3/SA-family subunit. SMC1 and SMC3 are long intramolecular coiled-coil proteins that form extended hairpin structures with the N- and C-terminal globular ATPase domains at one end and globular hinge domains at the other. SMC1 and SMC3 bind at their hinge domains and to opposite ends of the kleisin subunit SCC1/RAD21/REC8 at their ATPase domains, forming a tripartite ring that embraces pairs of sister chromatids. The SA subunit binds to the kleisin subunit and contributes to cohesin chromosome binding.

REC8, a meiosis specific cohesin subunit

Many species utilize one or more meiosis-specific components to substitute the mitotic subunit of the cohesin ring (Petronczki et al., 2003; Nasmyth and Haering, 2009). REC8 replaces RAD21/SCC1 in most meiotic cohesin and is essential for cohesin's major meiotic functions including accurate chromosome segregation and synapsis (Parisi et al., 1999; Lee et al., 2003; Xu et al., 2005). RAD21/SCC1 ectopical expression failed to rescue the REC8 mutant phenotype in budding or fission yeast. The importance of REC8 is underlined by its widespread conservation, the phenotypic similarity of *rec8* mutants in diverse model eukaryotes, and the requirement of Separase-mediated REC8 cleavage for homolog segregation at anaphase I and sister chromatid segregation in anaphase II in many eukaryotes (Klein et al., 1999; Buonomo et al., 2000; Siomos et al., 2001; Kitajima et al., 2003; Manheim and McKim, 2003; Petronczki et al., 2003; Kudo et al., 2009; Nasmyth and Haering, 2009).

However, REC8 is not universal since no REC8 homolog has been identified in *Drosophila*. In *Drosophila* female meiosis, although C(2)M has been identified as a kleisin meiosis protein, it is required only for recombination and SC formation, but not for cohesion (Heidmann et al., 2004; Khetani and Bickel, 2007). In addition, defect of *c(2)m* in females cause relatively mild nondisjunction in meiosis II segregation (ranging from 13.4% to 29.3%), but accurate chromosome segregation during male meiosis does not depend on C(2)M function (Manheim and McKim, 2003). Moreover, female germ-line expression of a mutated C(2)M transgene in which putative Separase cleavage sites were disrupted did not result in meiotic segregation defects (Heidmann et al., 2004). Finally, localization of C(2)M protein in whole-mount preparations indicates that,

like C(3)G protein, C(2)M is restricted to the subset of cells within each germ-line cyst that build synaptonemal complex (Manheim and McKim, 2003). In sum, these data suggest that C(2)M is not the REC8 homolog in *Drosophila*.

However, *Drosophila* has three unique meiotic cohesion proteins, SOLO, ORD, and SUN, playing similar roles as REC8. They are essential for cohesion and proper chromatid segregation in both sexes and for recombination and SC stability in females. SOLO, ORD, and SUN colocalize with cohesin subunit SMC1 and are required for its localization (Krishnan; Goldstein, 1980; Bickel et al., 1996; Bickel et al., 1997; Balicky et al., 2002; Webber et al., 2004; Khetani and Bickel, 2007; Yan et al., 2010; Yan and McKee, 2013).

SOLO

SOLO is a meiotic protein required for centromere cohesion, and cohesin complex localization in *Drosophila* meiosis. SOLO localizes to centromeres and chromosome arms in females from pre-meiotic cells to metaphase II, after which stage sister chromatid cohesin get dissolved. It physically interacts with and recruits SMC1 to chromosomes. In addition, localization of SOLO and SMC1 on meiotic centromeres after metaphase I depends on the cohesin protector Shugoshin family protein MEI-S332. In female meiosis, SOLO is required for synapsis, recombination and homolog bias in addition to its roles in cohesion and centromere cohesion (Yan et al., 2010; Yan and McKee, 2013). Although SOLO lacks statistically significant similarity with any known cohesin proteins, it works similarly to REC8 in every aspect, from its localization

patterns, its effect on cohesion, to its roles in synapsis and recombination (Parisi et al., 1999; Lee et al., 2003; Xu et al., 2005; Yan et al., 2010; Yan and McKee, 2013). Sequence alignment of SOLO homologs based on sequenced *Drosophila* genomes identified its C terminus as a conserved domain. SOLO C-terminal conserved domain may have significant functional importance since truncation of SOLO 21 residues from its C terminus via a nonsense mutation (L1010) abolishes its function (Yan et al., 2010). A further sequence alignment of SOLO C terminus with RAD21/REC8 suggests that all but one of the 13 most conserved residues in REC8 C termini alignment are conserved among *Drosophila* SOLO orthologs. This is unlikely to be coincidental as the average overall pairwise identity of the *D. melanogaster, D. pseudoobscura* and *D. virilis* homologs is less than 30%. Moreover, the residues of yeast SCC1 that make close contacts with SMC1 in an SCC1-SMC1 co-crystal structure and required for SCC1-SMC1 interaction (F528 and L532) corresponds to two SOLO C-terminal conserved residues (Y*1007 and L*1011, these two residues are marked with * to differentiate from the other SOLO C terminus conserved residues**;** we considered Y*1007 as the counterpart of F528 since Tyrosine exhibits similar structure as Phenylalanine) (Haering et al., 2002; Gruber et al., 2003; Haering et al., 2004) (**Figure 1-3**).

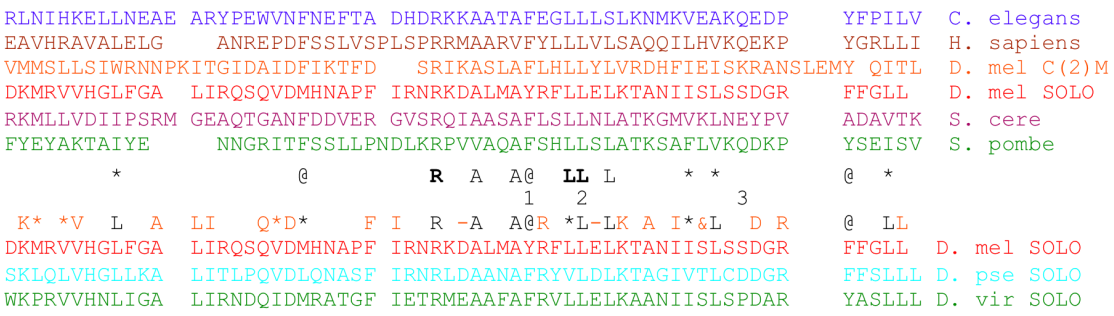


Figure 1-3 Manual alignment of C-terminus of Dmel SOLO with REC8 orthologs and two *Drosophila* SOLO orthologs

Manual alignment of C-terminus of Dmel SOLO with REC8 orthologs (upper panel) and two *Drosophila* SOLO orthologs (lower panel). Residues conserved in at least 5 of the REC8 orthologs are indicated in black. Note that all but one of these are also conserved in the *Drosophila* SOLO orthologs. Residues conserved in SOLO orthologs but not in REC8 orthologs are in orange. @ Y,F; * L,I,V,M; - D,E,Q; & S,T. 1, 2 and 3 indicate the three SCC1 residues demonstrated to be essential for SMC1 binding (Haering et al., 2004). Note that 1 and 2 (Y*1007 and L*1011 in Dmel SOLO) are conserved in all REC8 orthologs and all SOLO orthologs, whereas 3 is weakly conserved in both groups. L1010 (dark grey) in Dmel SOLO is mutated to a stop codon in *solo*$^{Z2\text{-}3534}$. REC8 alignment is from (Schleiffer et al., 2003).

Separase, a cysteine protease cleaving cohesin

During cell division, chromatids are tightly regulated for pairing and segregation through cohesion. In mitosis, sister chromatids are associated through cohesion from DNA replication in S phase to late metaphase, when they go through bi-orientation to opposite spindle poles. After metaphase, cohesion has to be resolved to release sister chromatids. A cysteine protease Separase is activated in the metaphase-anaphase transition by the degradation of its inhibitor Securin by Anaphase Promoting Complex/ cyclosome (APC/C). Activated Separase cleaves cohesin subunit SCC1 to release sister chromatids (Ciosk et al., 1998; Uhlmann et al., 1999). As a result, released sister chromatids go through bi-orientation to opposite spindle poles.

In meiosis, sister chromatids first go through mono-orientation in meiosis I then bi-orientation in meiosis II. Centromeric cohesin is protected in meiosis I by members from the Shugoshin family (Kitajima et al., 2006; Liu et al., 2013). Thus, only arm cohesion gets largely lost during prophase and prometaphase while centromeric cohesion is preserved until metaphase II. APC/C activated Separase cleaves cohesin subunit SCC1 to release sister chromatids (Hagting et al., 2002; Wirth et al., 2006).

***Drosophila* SSE, THR, and PIM work in a complex**

Drosophila Separase (SSE) is an evolutionarily diverged member of the Separase family. *Drosophila* SSE is only about one-third the size of other Separase with a diverged endoprotease domain. However, genetic analyses prove that SSE is required for sister chromatid segregation during mitosis. SSE works in a complex with the

securin homolog Pimples (PIM) and an activating subunit, Three Rows (THR). The entire protein complex as a whole is required for Separase activity and sister chromatids segregation (Jager et al., 2001). It is speculated that the ancient separase gene might have broken into two smaller genes during *Drosophila* evolution (Leismann et al., 2000). Accordingly, THR might correspond to the nonconserved N-terminal and SSE to the conserved C-terminal endoprotease domain of the other Separase proteins.

Chapter 2 - FUNCTIONAL STUDIES OF SOLO AND ITS INTERACTORS

INTRODUCTION

Cohesin complex is composed of four subunits including Structural Maintenance of Chromosomes 1 (SMC1), Structural Maintenance of Chromosomes 3 (SMC3), a kleisin subunit, which can be either the mitotic SCC1/RAD21 protein or its meiosis-specific paralog REC8, and a SCC3/SA-family subunit. SMC1 and SMC3 are long intramolecular coiled-coil proteins that form extended hairpin structures with the N- and C-terminal globular ATPase domains at one end and a globular hinge domain at the other. SMC1 and SMC3 bind at their hinge domains and to opposite ends of the kleisin subunit SCC1/RAD21/REC8 at their ATPase domains, forming a tripartite ring that embraces pairs of sister chromatids. The SA subunit binds to the kleisin subunit and contributes to cohesin chromosome binding (Klein et al., 1999; Watanabe and Nurse, 1999; Haering et al., 2002; Gruber et al., 2003; Schleiffer et al., 2003; Nasmyth and Haering, 2009).

Usually, meiosis utilizes a unique subunit REC8 in the cohesin complex. REC8 is required for accurate chromosome segregation, centromere cohesion, as well as synapsis during meiosis (Parisi et al., 1999; Lee et al., 2003; Xu et al., 2005). However, no REC8 paralog has been identified in *Drosophila* meiosis although it is conserved in many species from yeast to human (Parisi et al., 1999; Kitajima et al., 2003; Xu et al., 2005). One of the signatures of the SCC1/RAD21/REC8 protein is that they are members of the kleisin superfamily with Winged Helix Domains (WHDs). In *Drosophila* female meiosis, although C(2)M has been identified as a meiosis protein with kleisin motif, it is required only for recombination and SC formation, not for cohesion

(Heidmann et al., 2004; Khetani and Bickel, 2007). In addition, defect of *c(2)m* in females cause relatively mild nondisjunction in meiosis II segregation (ranging from 13.4% to 29.3%), and accurate chromosome segregation during male meiosis does not depend on C(2)M function (Manheim and McKim, 2003). Moreover, female germ-line expression of a mutated C(2)M transgene in which putative Separase cleavage sites were disrupted did not result in meiotic segregation defects (Heidmann et al., 2004). Finally, localization of C(2)M protein in whole-mount preparations indicates that, like C(3)G protein, C(2)M is restricted to the subset of cells within each germ-line cyst that build synaptonemal complex (Manheim and McKim, 2003). In sum, these data suggest that C(2)M is not the REC8 homolog in *Drosophila*.

Compared to C(2)M, SOLO works like a cohesin protein in every aspect. It localizes to chromosomes from premeiotic cells to metaphase II, after which stage sister chromatid cohesin is lost. In addition, SOLO associates with SMC1 and is required for synapsis, recombination, and cohesion (Yan et al., 2010; Yan and McKee, 2013). SOLO, although lacking kleisin motif, is believed to have a functionally important C terminus since $solo^{Z2\text{-}3534}$, which creates a stop codon at residue L1010, leads to total loss of function. This C terminus shares twelve of the thirteen most conserved amino acids in REC8 and its homologs. Moreover, the residues (F528 and L532) of yeast SCC1 corresponding to two of SOLO conserved C-terminal residues (Y*1007 and L*1011) make close contacts with SMC1 in an SCC1-SMC1 co-crystal structure, and are required for the SMC1-SCC1 interaction (Haering et al., 2004).

Based on the SOLO and REC8 sequence alignment, we mutated a series of SOLO C-

terminal conserved residues and found that they are required for accurate chromosome segregation, SOLO chromosome localization, and centromere cohesion. These data suggest that SOLO might have a functionally similar C terminus as that of REC8. To further support the hypothesis that SOLO might be a REC8 paralog, yeast two-hybrid was carried out to study the direct interactions between SOLO and cohesin proteins. Another signature of the REC8 family cohesin subunits is that it get cleaved by cysteine protease Separase. In this study, yeast two-hybrid was performed to detect the physical interaction between SOLO and the *Drosophila* Separase homolog SSE *in vivo*.

MATERIALS AND METHODS

Construction of SOLO mutant fusion clones and generation of transgenic flies

Six SOLO mutant constructs with N terminal fluorescence protein Venus were cloned. They were UASp:: Venus-SOLO^{R1000E}, UASp:: Venus-SOLO^{Y*1007R}, UASp:: Venus-SOLO^{L1010R}, UASp:: Venus-SOLO^{L*1011R}, UASp:: Venus-SOLO$^{Y*1007R\&L*1011R}$, UASp:: Venus-SOLO$^{L1010R\&L*1011R}$. QuikChange II site-directed mutagenesis kit (Agilent Technologies) was used to make the SOLO C terminal residue mutations. Vector of pENTRY^{TM}/D-TOPO (Invitrogen) fused with SOLO was used as the template for the mutants. The following primers were used for mutagenesis:

SOLO ^{R1000E}

Forward primer 5'-ATATGCACAATGCACCATTCATCCGAAATGAAAAGGATGCGTTGATGG-3'

Reverse primer 5'-CCATCAACGCATCCTTTTCATTTCGGATGAATGGTGCATTGTGCATAT-3'

SOLO^{Y*1007R}

Forward primer 5'-AAATCGCAAGGATGCGTTGATGGCACGCCGCTTTTTACTCGAGCT-3'

Reverse primer 5'-AGCTCGAGTAAAAAGCGGCGTGCCATCAACGCATCCTTGCGATTT-3'

SOLO^{L1010R}

Forward primer 5’-GATGCGTTGATGGCATATCGCTTTCGCCTCGAGCTTAAGACTGCAAA-3’

Reverse primer 5’-TTTGCAGTCTTAAGCTCGAGGCGAAAGCGATATGCCATCAACGCATC-3’

SOLO^{L*1011R}

Forward primer 5’-GATGGCATATCGCTTTTTACGCGAGCTTAAGACTGCAAAT-3’

Reverse primer 5’- ATTTGCAGTCTTAAGCTCGCGTAAAAAGCGATATGCCATC-3’

SOLO$^{Y*1007R\& L*1011R}$

Forward primer 5’-TCGCAAGGATGCGTTGATGGCACGCCGCTTTTTACGCGAGCTTAAGACTGC-3’

Reverse primer 5’-GCAGTCTTAAGCTCGCGTAAAAAGCGGCGTGCCATCAACGCATCCTTGCGA-3’

SOLO$^{L1010R\&L*1011R}$

Forward primer 5'-GCAAGGATGCGTTGATGGCATATCGCTTTCGCCGCGAGCTTAAGACTGCAA-3'

Reverse primer 5'-TTGCAGTCTTAAGCTCGCGGCGAAAGCGATATGCCATCAACGCATCCTTGC-3'

The PCR products were treated with DpnI to digest the original template plasmid. The newly amplified constructs with SOLO mutations were recombined with pPVW vector

(Invitrogen, Gateway® LR Clonase® II Enzyme mix, *Drosophila* Genomics Resource Center), making the germline transformation vectors P[w^{+mc}, UASp:: Venus-$SOLO^{R1000E}$], P[w^{+mc}, UASp:: Venus-$SOLO^{Y*1007R}$], P[w^{+mc}, UASp:: Venus-$SOLO^{L1010R}$], P[w^{+mc}, UASp:: Venus-$SOLO^{L*1011R}$], P[w^{+mc}, UASp:: Venus-$SOLO^{Y*1007R\&\ L*1011R}$], and P[$w^{+mc}$, UASp:: Venus-$SOLO^{L1010R\&L*1011R}$]. All constructions contain Venus, UAS sequences for GAL4 transcriptional activation and *mini-white* for detecting transformants. All constructs were midi-precipitated, microinjected and transformed into w^{1118} flies (BestGene, Inc.). Transformants were mapped and crossed for rescue experiments.

Fly stocks and culture methods

The *solo*$^{Z2\text{-}0198}$ mutation line was from the Zuker-2 (Z2) collection with EMS-mutagenized second chromosomes (Koundakjian et al., 2004). *Df(2L)A267* is from Bloomington Stock Center at the University of Indiana. For the nondisjunction assays, tested males were crossed singly with three virgin females in shell vials; tested virgin females were crossed singly with two male flies. All flies were maintained on standard cornmeal molasses medium at 25 °C. Parents were discarded on day 10 and progenies were counted until day 20.

Transgene rescue experiments

Male flies with genotype of +/B^{s}Yy+; *Df(2L)A267*, cn/*solo*$^{Z2\text{-}0198}$, cn; [UASp:: Venus-

SOLO^{L1000E}]/ [nanos:: GAL4-VP16] and sibling controls without [nanos:: GAL4-VP16] were used to rescue the *solo* null mutant phenotype. All the other five mutant lines were crossed and tested with similar strategies.

Female virgin flies with genotype +/w; *Df(2L)A267*, cn/*solo*$^{Z2\text{-}0198}$, cn; [UASp:: Venus-SOLO^{L1000E}]/ [nanos:: GAL4-VP16] and sibling controls without [nanos:: GAL4-VP16] were used to rescue the *solo* null mutant phenotype. All the other five mutant lines were crossed and tested with similar strategy.

Sex chromosome nondisjunction assays

Male flies with genotype of +/B^{s}Yy+; *Df(2L)A267*, cn/ *solo*$^{Z2\text{-}0198}$, cn; [UASp: Venus-SOLO^{L1000E}]/ [nanos:: GAL4-VP16] and sibling controls without [nanos:: GAL4-VP16] were crossed singly to 3 y w female virgins with normal X chromosome for sex chromosome nondisjunction assay in 25°C. The progenies from this cross are: B^{+}♀, B^{s}♂, B^{s}♀, and B^{+}♂. NDJ%=(XY+O)/N. All the other five mutant lines were crossed and tested with similar strategies.

Female virgin flies with genotype of +/w; *Df(2L)A267*, cn/*solo*$^{Z2\text{-}0198}$, cn; [UASp:: Venus-SOLO^{L1000E}]/ [nanos:: GAL4-VP16] and sibling controls without [nanos:: GAL4-VP16] were crossed singly to 2 X^Y,y^{B} In(1)EN males for sex chromosome nondisjunction assay in 25°C. The progenies from this cross are: B ♀, B^{+}♂, B^{+}♀, and B♂. NDJ%=2(B^{+}♀ + B♂)/ (N+ B^{+}♀ + B♂). All the other five mutant lines were crossed and tested with similar strategies.

Immunofluorescence staining

Testes and ovaries immunostaining and DAPI staining were performed as describe before (Page and Hawley, 2003; Thomas et al., 2005; Yan and McKee, 2013). Spermatocyte developmental stages were distinguished by cell sizes, DNA territories, and cell numbers per cyst according to the criteria described before (Cenci et al., 1994). Primary CID antibody was used at 1:1000 (Active Motif, anti-rabbit). The secondary Alexa Fluor 555 donkey anti rabbit IgG (H+L) (Molecular Probes) was used at 1: 2000. UASp:: Venus-SOLO and UASp:: Venus-SOLO mutants driven by nanos:: GAL4-VP16 were detected by FITC channel (Van Doren et al., 1998).

Microscopy and image processing

All images were collected with a microscope (Axioplan; Carl Zeiss, Inc.) equipped with a 100W mercury lamp (HBO; Carl Zeiss, Inc.), Plan Neofluar 100Å~/1.30 NA oil immersion lenses (Carl Zeiss, Inc.), and a high resolution charge-coupled device camera (Roper Industries) at room temperature. Z-series images acquired by the Plan Neofluar 100Å~/1.30 NA oil immersion lens will be projected onto a single view using MetaMorph software (MDS Analytical Technologies). Maximum or sum projections of deconvolved Z series will be obtained using the software MetaMorph. Images were processed with Photoshop CS6 (Adobe) and Image J (National Institutes of Health).

Plasmid constructions for two-hybrid assays

The SMC1, SMC3a, SMC3b, SA, RAD21, SSE, SSE C497S and SOLO cDNAs were polymerase chain reaction (PCR) amplified from either constructs or cDNA reverse transcripted from mRNA (Invitrogen). Translational fusions between full-length SMC1, SMC3a, SMC3b, SA, SSE, SSE C497S cDNAs and the GAL4 activation domain (AD) were respectively constructed in the pGAD-C1 vector; translational fusions between full-length RAD21, and SOLO cDNA and the DNA-binding domain (BD) were constructed in the pGBDU-C1 vector (James et al., 1996). Plasmids were transformed into *S. cerevisiae* strain PJ69-4A (MATa trp1-901 leu2-3, 112 ura 3-52 his3-200 GAL4Δgal80ΔLYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ) using the high-efficiency lithium acetate transformation procedure described by (Gietz, 1995). Transformed cells were plated and kept in selective media in 30 °C. HIS3 gene controlled by GAL4 promoter was used as the primary marker for protein-protein interactions.

SOLO truncations without N termini SOLOΔNT (aa138-1031), and without C termini SOLOΔCT (aa1-971) were PCR amplified, cloned into the pGBUD-C1 vector, and transformed into *S. cerevisiae* strain PJ69-4A.

RESULTS

SOLO C terminus conserved residues are required for accurate chromosome segregation in both sexes

C terminus sequence alignment of SOLO orthologs in ten *Drosophila* species identified its C terminus as a conserved domain. Compared with the thirteen most conserved residues in REC8 C termini alignment, twelve of them exist and are also conserved in SOLO C terminus. Among them, two C-terminal residues (Y*1007 and L*1011) correspond to the yeast SCC1 C terminal residues (F528 and L532) making close contacts with SMC1 based on an SCC1-SMC1 co-crystal structure, and they are required for the SMC1-SCC1 interaction (Haering et al., 2002; Gruber et al., 2003; Haering et al., 2004).

In order to test the functional importance of Y*1007, L*1011 and their neighboring conserved residues, site-directed mutagenesis (Strategene) was carried out to construct SOLO mutants. Six SOLO mutants ($SOLO^{R1000E}$, $SOLO^{Y*1007R}$, $SOLO^{L1010R}$, $SOLO^{L*1011R}$, $SOLO^{Y*1007R\&L*1011R}$, and $SOLO^{L1010R\&L*1011R}$, residues labeled with * correspond to residues essential for interaction to SMC1 in yeast SCC1) were tagged with fluorescent protein Venus, respectively. SOLO C terminus mutant constructs were microinjected for transgene flies and screened by BestGene Inc. In order to test if these mutations interfere with accurate chromosome segregation, the six lines of SOLO C terminus residue mutants were expressed to rescue the *solo* null mutant phenotypes. Flies with $SOLO^{R1000E}$ or $SOLO^{Y*1007R}$ mutations rescue the nondisjunction phenotype in males, respectively, but mutants $SOLO^{L1010R}$, $SOLO^{L*1011R}$, $SOLO^{Y*1007R\&L*1011R}$, and

$SOLO^{L1010R\&L*1011R}$ failed to rescue the *solo* null nondisjunction completely (20.9%, 23.0%, 46.8%, and 33.0% nondisjunction rates, respectively). It is worth noting that mutation of residues Y*1007R and L*1011R together cause a high nondisjunction rate of 46.8%, which is as high as *solo* null mutant of 46.7% (**Table 2-1 a**). In females, all mutations affect accurate chromosome segregation, especially $SOLO^{L1010R}$, $SOLO^{L*1011R}$, $SOLO^{Y*1007R\&L*1011R}$, and $SOLO^{L1010R\&L*1011R}$ (58.3%, 52.9%, 57.1%, and 53.7% nondisjunction rates, respectively). These mutations failed to rescue the *solo* null mutant, which has a nondisjunction rate of around 60.6% (**Table 2-1 b**). In addition, compared with males, SOLO C terminus mutant female flies encounter more severe defects in chromosome segregation.

Table 2-1 Sex chromosome nondisjunction in SOLO C terminus residue mutants

(a) Sex chromosome nondisjunction assay in males; **(b)** Sex chromosome nondisjunction in females.

a

Paternal genotypes	X	Y	XY	O	N	%NDJ
solo/+	1326	1265	1	15	2607	0.61
solo/*Df*	314	191	76	366	947	46.7
solo/*Df*; Venus-SOLO^{R1000E}/nanos::GAL4-VP16	305	274	0	7	586	1.19
solo/*Df*; Venus-SOLO^{Y*1007R}/nanos::GAL4-VP16	436	272	0	25	733	3.41
solo/*Df*; Venus-SOLO^{L1010R}/nanos::GAL4-VP16	197	156	3	90	446	20.9
solo/*Df*; Venus-SOLO^{L*1011R}/nanos::GAL4-VP16	125	103	0	68	296	23.0
solo/*Df*; Venus-SOLO$^{Y*1007R\&L*1011R}$/nanos::GAL4-VP16	95	80	22	132	329	46.8
solo/*Df*; Venus-SOLO$^{L1010R\&L*1011R}$/nanos::GAL4-VP16	72	48	2	57	179	33.0

b

Paternal genotypes	B♀	B^{+}♂	B^{+}♀	B♂	N	%NDJ
solo/+	646	793	13	10	1462	3.10
solo/*Df*	42	44	31	35	152	60.6
solo/*Df*; Venus-SOLO^{R1000E}/nanos::GAL4-VP16	95	124	11	6	236	13.4
solo/*Df*; Venus-SOLO^{Y*1007R}/nanos::GAL4-VP16	69	89	15	15	188	27.5
solo/*Df*; Venus-SOLO^{L1010R}/nanos::GAL4-VP16	39	44	26	32	142	58.3
solo/*Df*; Venus-SOLO^{L*1011R}/nanos::GAL4-VP16	29	28	18	14	90	52.9
solo/*Df*; Venus-SOLO$^{Y*1007R\&L*1011R}$/nanos::GAL4-VP16	9	15	8	8	40	57.1
solo/*Df*; Venus-SOLO$^{L1010R\&L*1011R}$/nanos::GAL4-VP16	35	34	23	17	109	53.7

solo mutant: *solo*$^{Z2\text{-}0198}$; *Df*: *Df(2L)A267*

N: total number of progeny scored. The percentage of X-Y NDJ was calculated as 100x(XY +O)/N in the assay of males and 100x(2B♀+2B^{+}♂)/(N+ B♀+B^{+}♂) in the assay of females

SOLO C terminus conserved residues are required for its chromosome localization

In order to test if SOLO C terminus conserved residue mutations interfere its chromosome localization, transgene flies with SOLO C terminus mutations were activated by nanos:: GAL4-VP16 to rescue *solo* null mutant phenotypes. Testes and ovaries DAPI staining was performed and Venus signals were observed to detect the localization of SOLO mutants. Compared with wildtype Venus-SOLO which localizes to centromeres from spermatogonia to metaphase II in male meiosis, and localizes to both centromeres and chromosome arms in female meiosis, $SOLO^{R1000E}$, $SOLO^{Y*1007R}$, $SOLO^{L*1011R}$, $SOLO^{L1010R\&L*1011R}$ are able to localize to testes tips and germariums at early meiotic stages. These SOLO mutants signals disappear at different developmental stages (**Table 2-2**). Mutant $SOLO^{L1010R}$ cause localization failures even from the beginning of meiosis in females while it has very weak Venus signals at early prophase in males. The signals disappear early in late prophase. Mutation with both Y*1007R and L*1011R together failed to localize to either spermatocytes or germariums even from the beginning (**Table 2-2, Figure 2-1**). These results are consistent with the chromosome segregation data and proved that residues Y*1007, L1010, and L*1011 contribute significantly to SOLO localization and function.

Table 2-2 SOLO mutants localization failure stages in spermatocytes

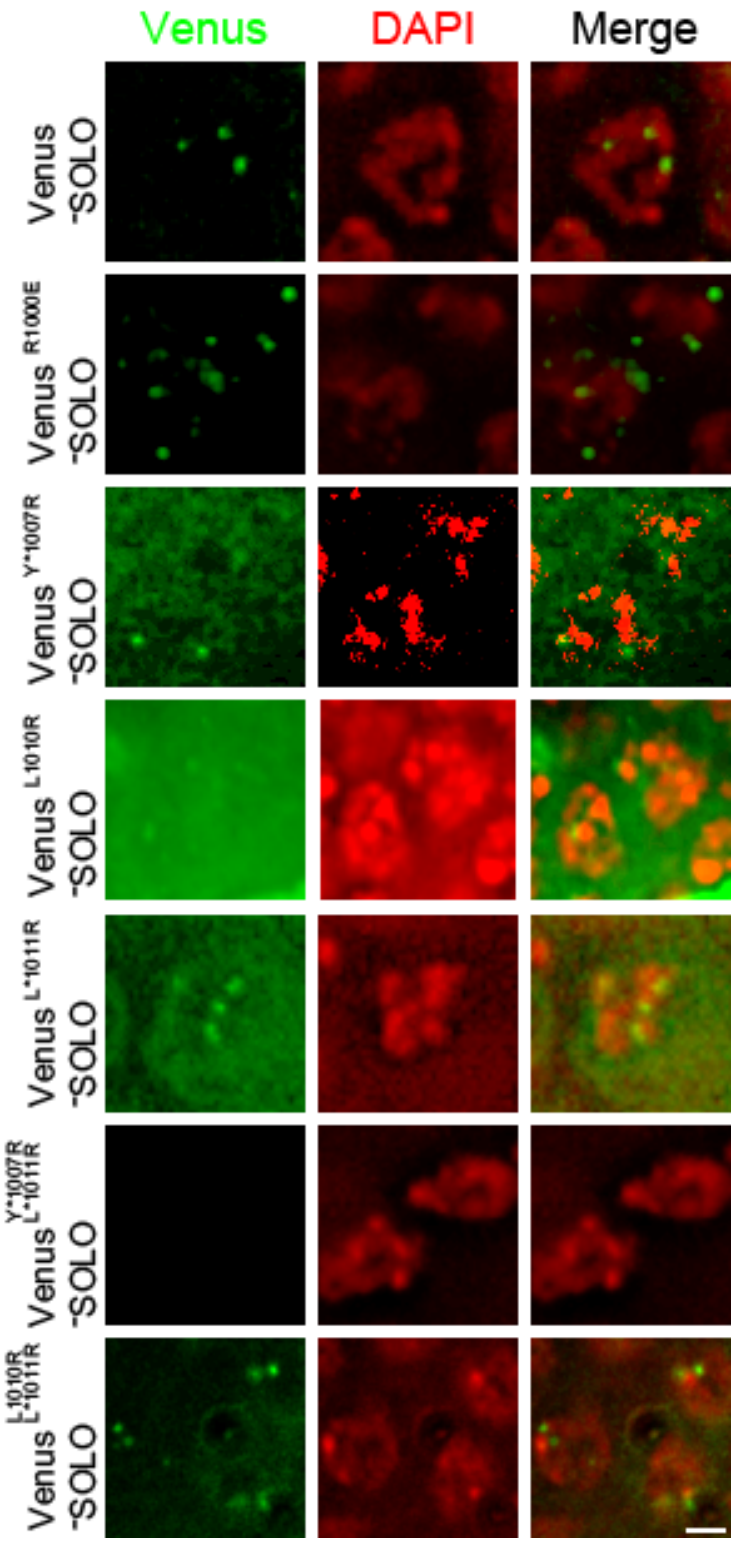
SOLO mutants	Localization in early prophase cells	Earliest stage without detectable Venus signals
Venus-SOLO	Yes	-
$SOLO^{R1000E}$	Yes	Metaphase II
$SOLO^{Y*1007R}$	Yes	Metaphase II
$SOLO^{L1010R}$	Yes	Late prophase
$SOLO^{L*1011R}$	Yes	Anaphase I
$SOLO^{Y*1007R\&L*1011R}$	No	Spermatogonia
$SOLO^{L1010R\&L*1011R}$	Yes	Metaphase I

Spermatocytes from transgenic males stained with DAPI and imaged for the fluorescence of Venus-SOLO and Venus-SOLO C terminus mutants. Spermatocytes were detected for SOLO chromosome localization from spermatogonia to metaphase II cells. The earliest stages for detectable Venus-SOLO mutants localization failure were recorded.

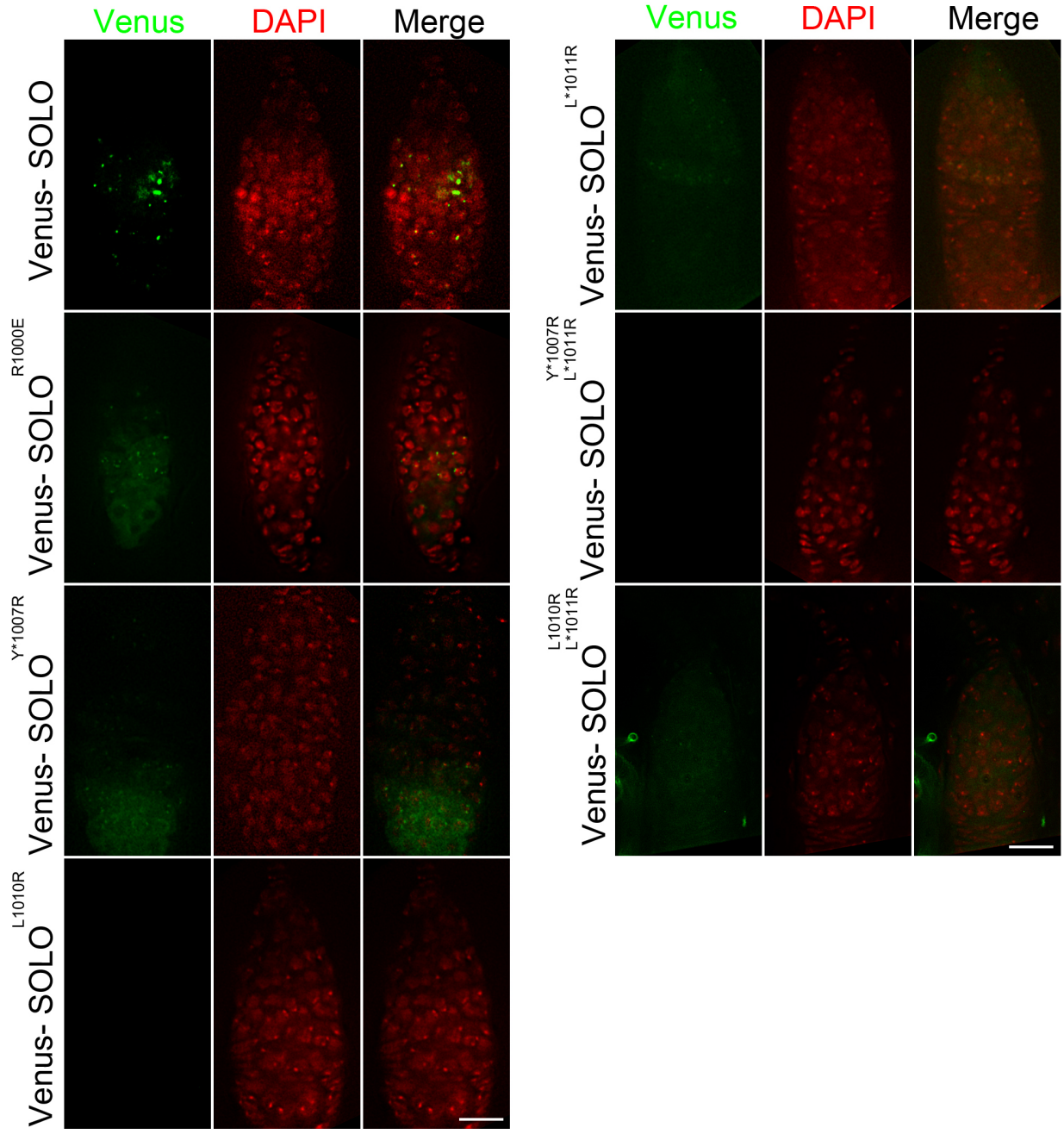
Figure 2-1 SOLO C terminus conserved residues are required for its localization

(a) Spermatocytes from transgenic males stained with DAPI and imaged for the fluorescence of Venus-SOLO and Venus-SOLO C terminus mutants. Each panel shows one or two nucleus at early prophase. Size bar represents 2 μm. **(b)** Germarium from transgenic females stained with DAPI and imaged for the fluorescence of Venus-SOLO and Venus-SOLO C terminus mutants. Each panel shows one germaria at stage 1. Size bar represents 10 μm.

a


Venus
DAPI
Merge
Venus -SOLO
Venus R1000E -SOLO
Venus Y*1007R -SOLO
Venus L1010R -SOLO
Venus L*1011R -SOLO
Venus Y*1007R L*1011R -SOLO
Venus L1010R L*1011R -SOLO

b


Venus
DAPI
Merge
Venus- SOLO
Venus- SOLO R1000E
Venus- SOLO Y*1007R
Venus- SOLO L1010R
Venus- SOLO L*1011R
Venus- SOLO Y*1007R L*1011R
Venus- SOLO L1010R L*1011R

SOLO C terminus conserved residues are essential for centromere cohesion

In order to further study if the SOLO C terminus mutations have any effect on centromere cohesion, immunofluorescence staining against centromere identifier (CID), a centromere-specific histone H3–like protein that enables simultaneously visualization of all centromeres, was used to monitor centromere behaviors in spermatocytes (Ahmad and Henikoff, 2001; Blower and Karpen, 2001). CID signal spots were counted at different meiotic stages. Since *Drosophila* has 4 pairs of chromosomes, and the forth chromosome is small and usually hard to detect, in wildtype spermatocytes, an average of 7 to 8 spots should be detected since late prophase in meiosis I. Spermatocytes from each SOLO C terminus mutant flies were scored and grouped based on the cell stages and CID spots numbers (**Table 2-3**). No significant abnormal CID signals have been detected in early prophase stages from S1 to S4 in SOLO mutants. However, from stage S5, SOLO mutants start to exhibit increased CID signal numbers in different levels (**Figure 2-2 and Table 2-3**). Consistent with the data from nondisjunction assays and the cytological staining, mutation of Y*1007R and L*1011R together disrupts SOLO function to a similar level as *solo* null mutant in which 100% cells scored exhibited more than 8 CID spots since late prophase (Yan et al., 2010) (**Figure 2-2, Table 2-3**). Typical cells with equal to or more than 8 CID spots were shown from both Venus-SOLO and Venus-SOLO$^{Y*1007R\&L*1011R}$ **(Figure 2-2)**. These data, together with the former results, suggest that SOLO C terminus conserved residues, especially Y*1007 and L*1011 play significant roles in aspects of regulating accurate chromosome segregation, chromosome localization, and centromere cohesion.

Table 2-3 Quantification of CID spots in SOLO C terminus residue mutant spermatocytes at different stages

a Percentage of cells with more than 8 CID spots in SOLO C terminal residue mutant spermatocytes at different stages

	Control	*solo*	SOLO^{R1} 000E	SOLO^{Y*} 1007R	SOLO^{L1} 010R	SOLO^{L*} 1011R	SOLO^{Y*} 1007R&L*1011R	SOLO^{L1} 010R&L*1011R
Late prophase I	16% (31)	97% (37)	5% (20)	22% (54)	86% (14)	73% (18)	100% (26)	90% (29)
Prometaphase I	0 (4)	75% (16)	0 (8)	11% (9)	90% (10)	27% (11)	100% (3)	100% (11)
Metaphase I	8% (12)	100% (7)	0 (21)	29% (14)	82% (11)	36% (22)	100% (13)	100% (19)

The percentages of spermatocytes with more than eight spots are shown. The number of nuclei scored is in parentheses.

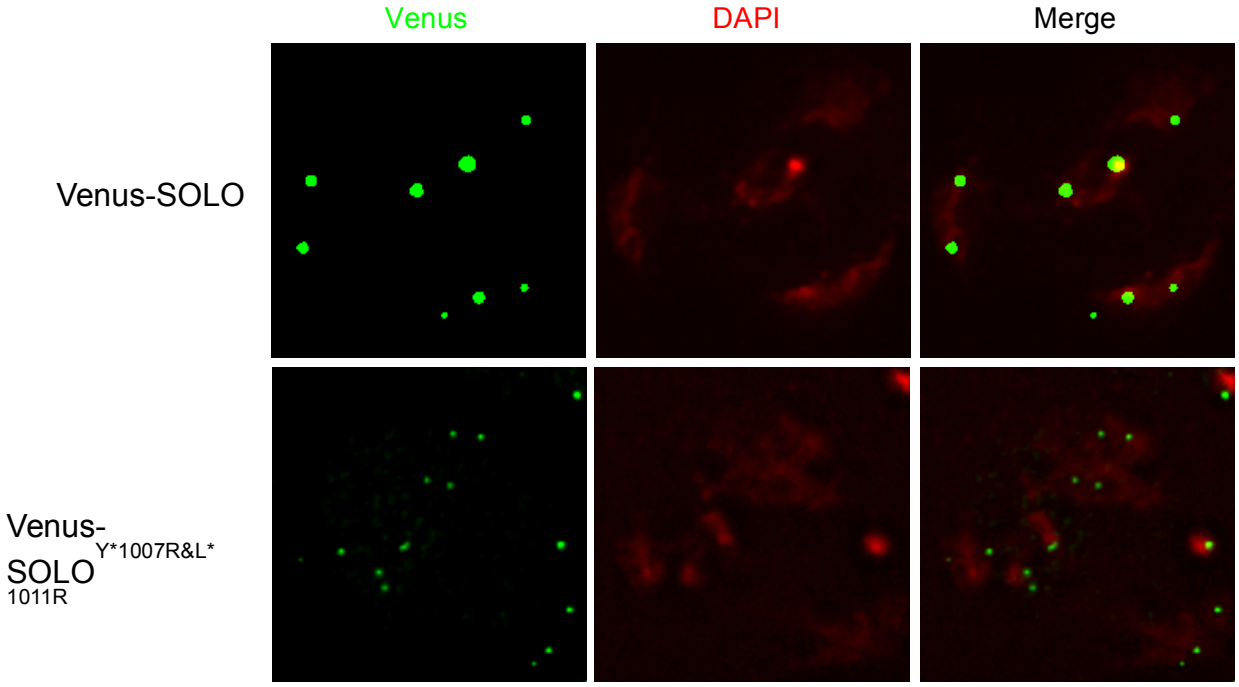
b Average CID spots in SOLO C terminal residue mutant spermatocytes at different stages

	Control	*solo*	SOLO^{R1} 000E	SOLO^{Y*} 1007R	SOLO^{L1} 010R	SOLO^{L*} 1011R	SOLO^{Y*} 1007R&L*1011R	SOLO^{L1} 010R&L*1011R
Late prophase I	7.5	11.5	7.0	7.7	11.9	10.7	11.5	10.8
Prometaphase I	7.5	10.3	6.8	7.4	10.8	8.2	13.7	11.0
Metaphase I	7.8	12.9	8.0	8.4	11.1	8.4	10.9	11.9

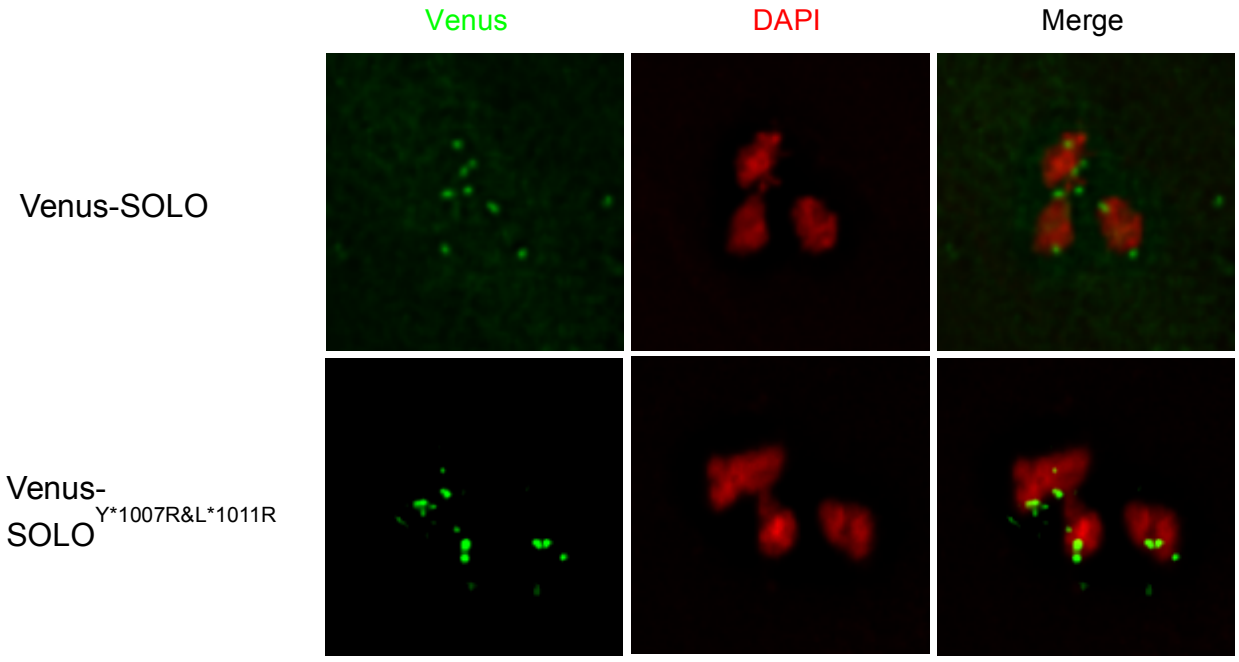
Figure 2-2 Centromere cohesion is disrupted in SOLO$^{Y*1007R\&L*1011R}$ mutant

Testes from Venus-SOLO and SOLO$^{Y*1007R\&L*1011R}$ mutant were used to rescue the *solo* null phenotype. Spermatocytes were stained with DAPI to visualize DNA, and with anti-CID antibody to identify centromeres. Sum projections of 3D-deconvolved Z series stacks were performed to obtain CID signals. Spermatocytes from stages of **(a)** late prophase spermatocytes; **(b)** prometaphase cells; **(c)** metaphase cells were shown.

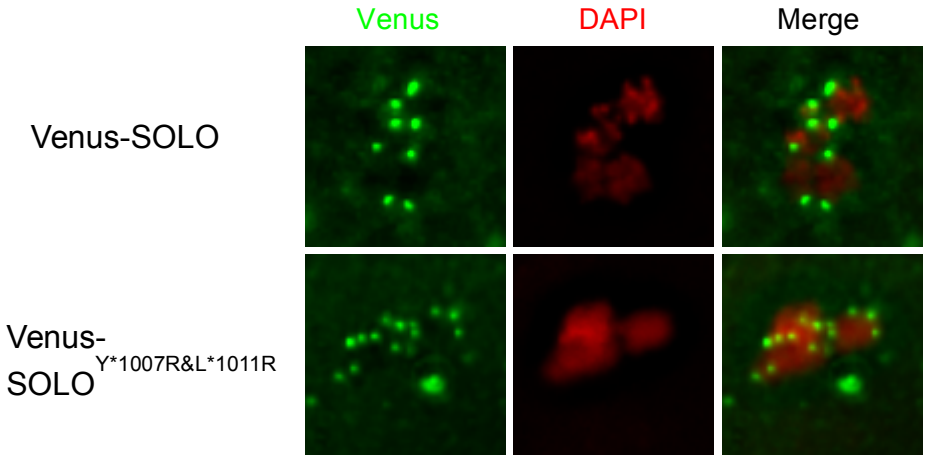
a



b


Venus
DAPI
Merge
Venus-SOLO
Venus-SOLO Y*1007R&L*1011R

c


Venus
DAPI
Merge
Venus-SOLO
Venus-SOLO Y*1007R&L*1011R

SOLO N-terminal RGG domain is required for accurate chromosome segregation

The first three exons of solo gene are shared with vasa; they encode the same RGG rich N-terminal domain. This RGG domain is not a classical cohesin motif. RGG domains mainly work in the following ways based on previous studies: 1) RNA binding and translational regulation (Blackwell and Ceman, 2011); 2) transcriptional activation (Alex and Lee, 2005); 3) subcellular localization (Passos et al., 2006). In order to explore the function of SOLO N-terminal RGG domain, we made a Venus tagged SOLO mutant transgene fly without N-terminal 1-137 amino acids containing the RGG repeats. Flies with Venus-SOLOΔNT transgene activated by nanos:: GAL4-VP16 driver to rescue *solo* null mutant phenotypes were used to test the role of SOLO N-terminal RGG domain in chromosome segregation. Both male and female flies were used for nondisjunction assays. SOLOΔNT males have a 34.4% nondisjunction rate, and females have a 45.9% nondisjunction rate (**Table 2-4**). These data proved that SOLO N terminus RGG repeats rich domain is essential for accurate chromosome segregation.

Table 2-4 Sex chromosome nondisjunction in SOLO N terminus RGG domain deletion mutant

(a) Sex chromosome nondisjunction assay in males; **(b)** Sex chromosome nondisjunction in females.

a

Paternal genotypes	X	Y	XY	O	N	%NDJ
solo/+	144	116	0	0	260	0
solo/*Df*	62	34	10	87	193	50.3
solo/*Df*; Venus-SOLOΔNT /nanos::GAL4-VP16	106	111	16	98	331	34.4

b

Paternal genotypes	B♀	B^+♂	B^+♀	B♂	N	%NDJ
solo/+	96	76	0	1	173	1.15
solo/*Df*	10	6	6	10	32	66.7
solo/*Df*; Venus-SOLOΔNT /nanos::GAL4-VP16	37	29	14	14	94	45.9

solo mutant: $solo^{Z2\text{-}0198}$; *Df*: *Df(2L)A267*

N: total number of progeny scored. The percentage of X-Y NDJ was calculated as 100x(XY +O)/N in the assay for males and 100x(2B♀+2B^+♂)/(N+ B♀+B^+♂) in the assay for females

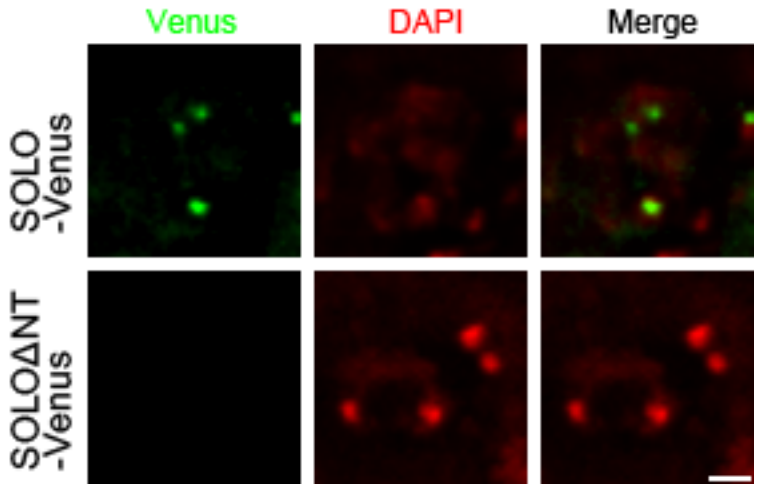
RGG domain is required for SOLO chromosome localization

In order to test if SOLO N terminal RGG domain contribute to its chromosome localization, transgene flies without the first 137 amino acids of SOLOΔNT were constructed and activated by nanos:: GAL4-VP16 to rescue *solo* null mutant. Testes and ovaries DAPI staining was performed and Venus signals were observed to detect the localization of SOLOΔNT. The mutant SOLO failed to localize to spermatocytes and germarium even from the beginning of meiosis (**Figure 2-3**). These results proved that SOLO N terminus RGG domain contribute to its chromosome localization.

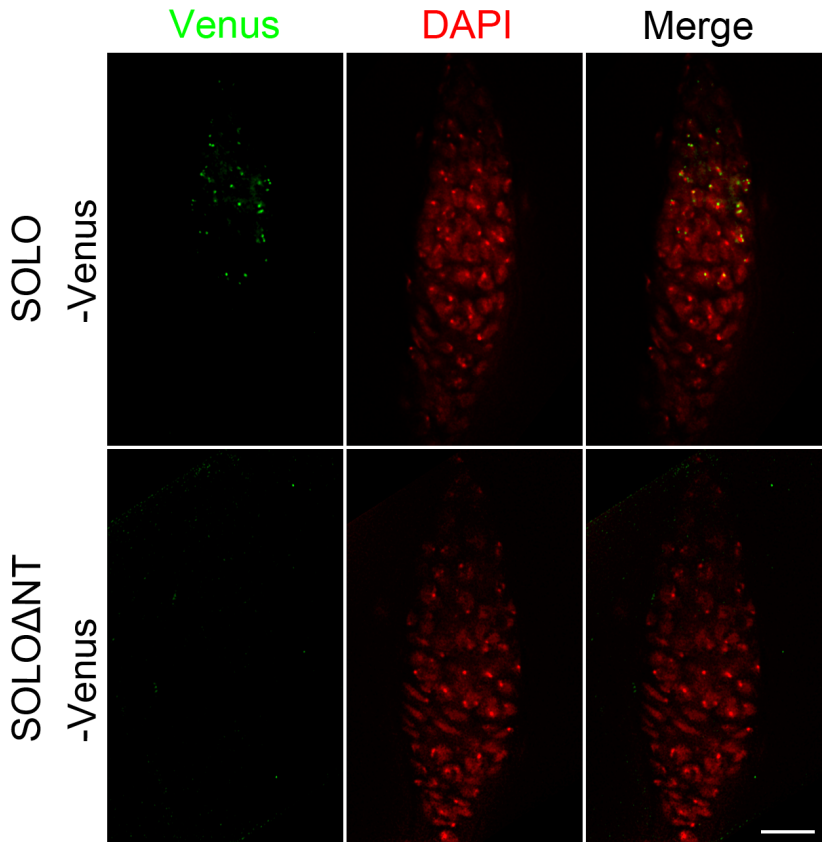
Figure 2-3 N-terminal RGG domain is required for SOLO localization

(a) Spermatocytes from transgenic males stained with DAPI and imaged for the fluorescence of SOLO-Venus and SOLO ΔNT-Venus. Each panel shows one nucleus at early prophase. Size bar represents 2 μm. **(b)** Germarium from transgenic females stained with DAPI and imaged for the fluorescence of SOLO-Venus and SOLO ΔNT-Venus. Each panel shows one germaria at stage 1. Size bar represents 10 μm.

a


Venus
DAPI
Merge
SOLO -Venus
SOLOΔNT -Venus

b


Venus
DAPI
Merge
SOLO -Venus
SOLOΔNT -Venus

SOLO directly interacts with SMC1, SMC3a, and SA in yeast two-hybrid

The yeast two-hybrid system has been widely used to study protein-protein interactions under *in vivo* conditions (Young, 1998). Trans-activation of the GAL4-responsive HIS and lacZ reporter genes is used as the marker of physical interactions. In our study, SOLO and RAD21 full-length coding sequences were first respectively fused in-frame to the yeast GAL4 DNA binding domain (BD) as "bait" constructs. The full-length coding sequences of SMC1, SMC3a, SMC3b, and SA were fused in-frame with the GAL4 transcription activation domain (AD), respectively. It is worth noting that two SMC3 splicing isoforms, SMC3a (CAP-PA in Flybase) and SMC3b (CAP-PB in Flybase), have been predicted in *Drosophila* genome. SMC3b lacks the first 146 amino acids compared with SMC3a. In this study, we cloned both isoforms for the yeast two-hybrid experiments. Combinations of the following plasmids were co-transformed into yeast cells: AD: SMC1 + BD: RAD21, AD: SMC1 + BD: SOLO, AD: SMC3a + BD: RAD21, AD: SMC3a + BD: SOLO, AD: SMC3b + BD: RAD21, AD: SMC3b + BD: SOLO, AD: SA + BD: RAD21, and AD: SA + BD: SOLO.

Yeast growth on plates with selective medium lacking histidine indicates AD and BD fused protein-protein interaction. RAD21 as a mitotic cohesin kleisin subunit which connects SMC1 and SMC3 to form the ring complex, has been used as a positive control. Yeast carrying single fused protein construct and/ or empty vectors served as negative controls. No significant cell growth occurred on his- plates, except BD: RAD21, which survived with very low viability. Although sensitive and useful for weak interaction detection, HIS3 reporter gene leaky expression has been documented (James et al., 1996). Another possibility is RAD21, as a cohesin, might be involved in transcription

activation. Results from genome-wide assays in *Drosophila* developing wings and cultured cells support that, together with Polycomb proteins, RAD21 is involved in transcriptional regulation during development (Schaaf et al., 2013). Even though the autonomous expression of HIS3 reporter might have resulted in growth for yeast cells harboring BD: RAD21, tested yeast carrying plasmids encoding the AD: SMC1 and BD: RAD21, AD: SMC3a and BD: RAD21, AD: SMC3b and BD: RAD21, or AD: SA and BD: RAD21 exhibited much better growth on the his- plate than the AD: empty and BD: RAD21 yeast cells did, indicating that the growth of the yeast with full-length SMC1 and RAD21, SMC3a/ SMC3b and RAD21, and SA and RAD21 combinations are not due to leaky expression of HIS3 only (**Figure 2-4**).

Similarly, yeast cells carrying AD: SMC1 + BD: SOLO, AD: SMC3a + BD: SOLO, AD: SA + BD: SOLO have significant stronger surviving abilities compared with negative controls (**Figure 2-4**). These data suggest that SOLO interacts with SMC1, SMC3a, and SA proteins.

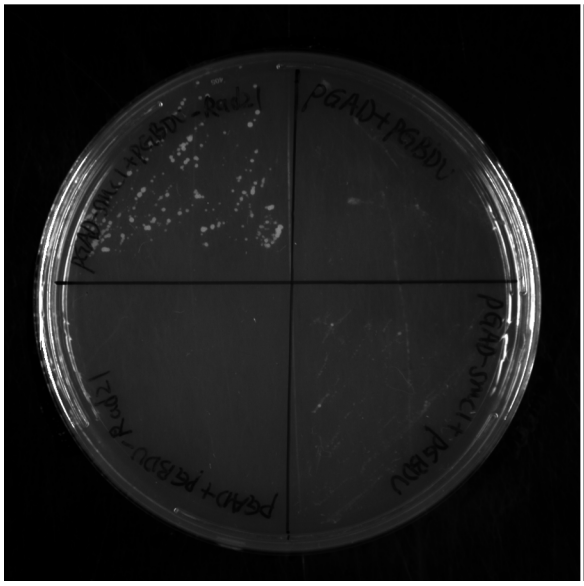
In addition, based on our results, RAD21 might have a stronger interaction affinity with SMC3b, while SOLO might prefer interacting with SMC3a (**Figure 2-4**). Thus, SMC3b might be a mitotic specialized isoform.

Figure 2-1 SOLO interacts with SMC1, SMC3a, and SA

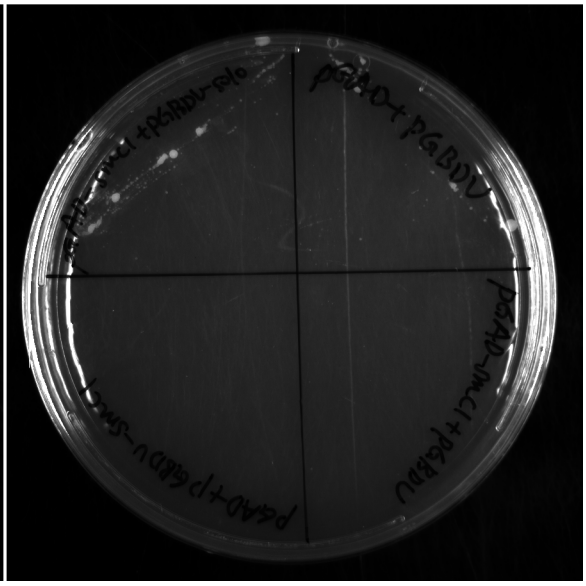
The upper left section of each plate shows the yeast cells harboring plasmids encoding the indicated GAL4AD fusions in combination with the indicated GAL4BD fusions. All the other three sections are negative controls.

a

AD: SMC1 + BD: RAD21

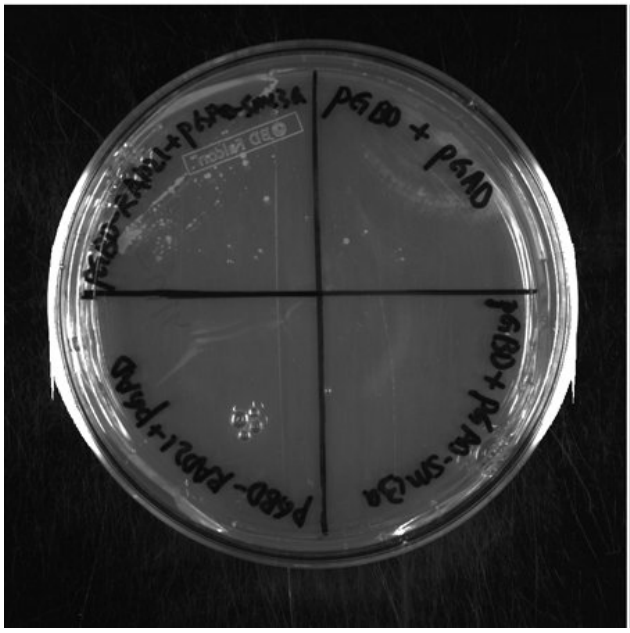


AD: SMC1 + BD: SOLO

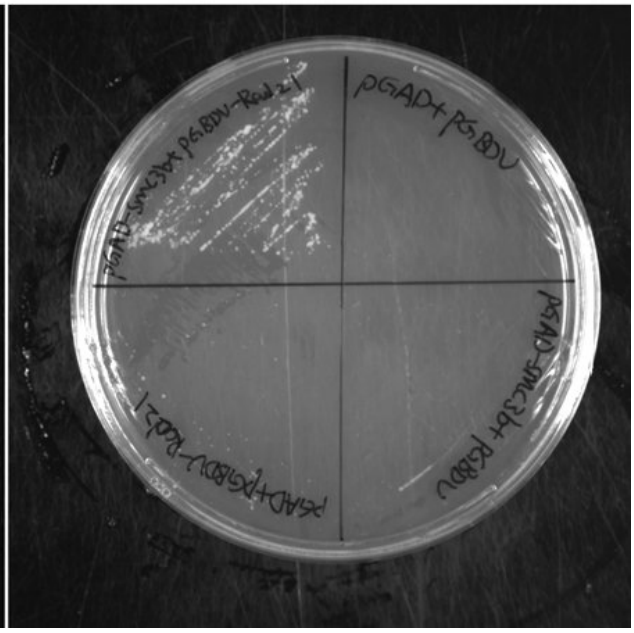


b

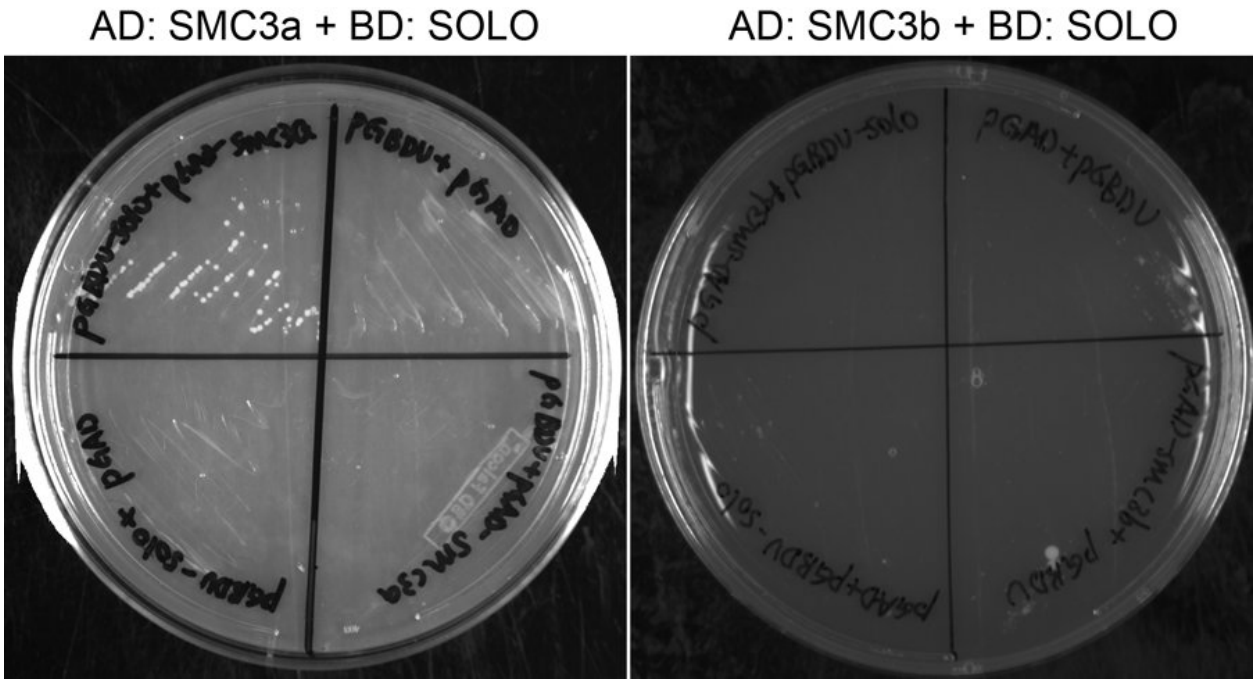
AD: SMC3a + BD: RAD21



AD: SMC3b + BD: RAD21



b’



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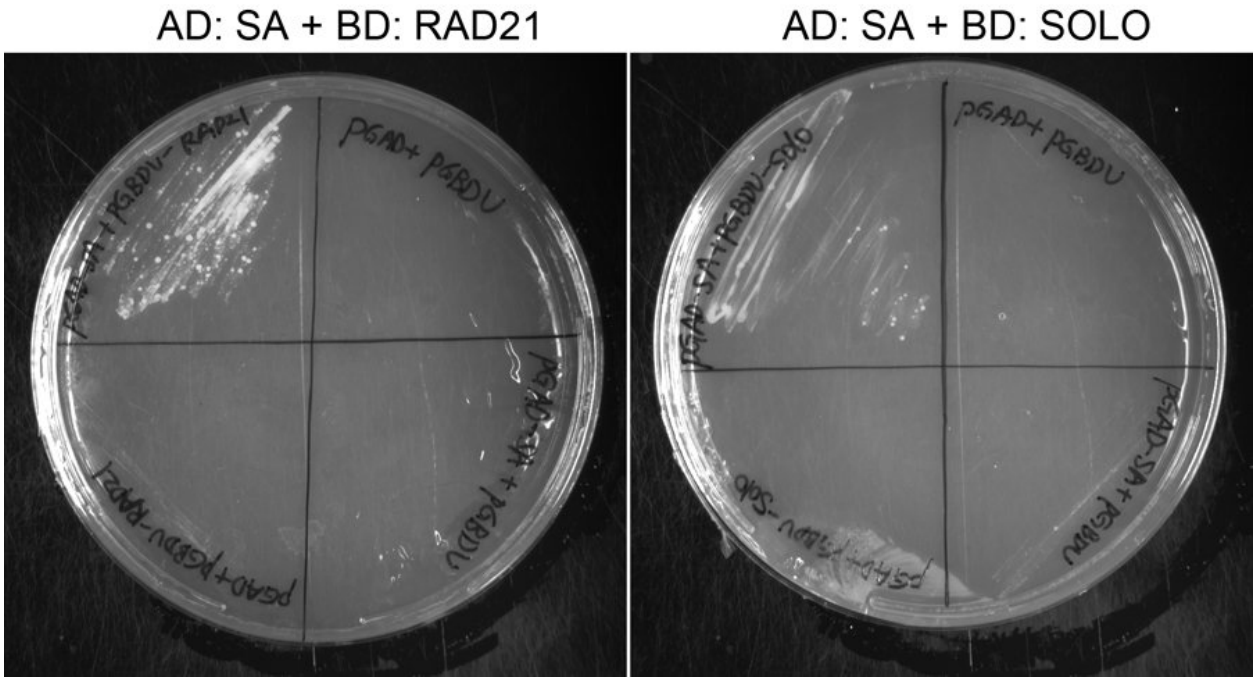


SOLO C terminus interacts with SMC1 and N terminus interacts with SMC3

To form a closed ring, RAD21 N terminus interacts with SMC3 while its C terminus interacts with SMC1. In order to detect the manner SOLO interacts with SMC1 and SMC3, domain truncations of SOLO were constructed. The N terminus of SOLO (aa 1-137) was deleted and SOLO △NT (aa 138-1031) was fused with GAL4-BD (BD: SOLO△NT). Similarly, SOLO C-terminal domain (aa 972-1030) was deleted and SOLO △CT (aa 1-971) was fused with GAL4-BD (BD: SOLO△CT). Yeast cells were transformed with plasmid combinations of AD: SMC1 + BD: SOLO△NT, AD: SMC1 + BD: SOLO△CT, AD: SMC3a + BD: SOLO△NT, AD: SMC3a +BD: SOLO△CT, AD: SMC3b + BD: SOLO△NT, AD: SMC3b + BD: SOLO△CT.

BD: SOLO △NT, but not BD: SOLO △CT, interacts with AD: SMC 1. These findings suggest that SOLO interacts with SMC1 with its C-terminal domain (**Figure 2-5 a**).

On contrary, BD: SOLO △CT interacts with AD: SMC 3a although the interactions seem to be weaker compared to the full-length protein. On the other hand, BD: SOLO △NT failed to interact with AD: SMC3a. These taken together suggest that SOLO interact with SMC3a with its N-terminal domain (**Figure 2-5 b**).

Consistent with earlier results that SMC3b interacts with RAD21 but not SOLO, SMC3b failed to interact with either SOLO N terminus or C terminus truncated proteins in this test (**Figure 2-5 c**).

To sum up, results from yeast two-hybrid support that SOLO interacts with SMC1 by its C terminus, and with SMC3a by its N terminus. In addition, SOLO might work with

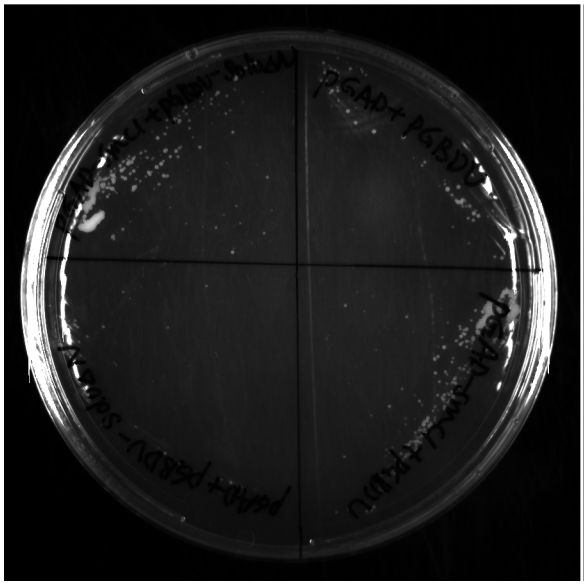
SMC3a as meiosis cohesin proteins while RAD21 might work with both SMC3a and SMC3b in mitotic cells.

Figure 2-2 SOLO interacts with SMC3a with its N terminus while it interacts with SMC1 with its C terminus

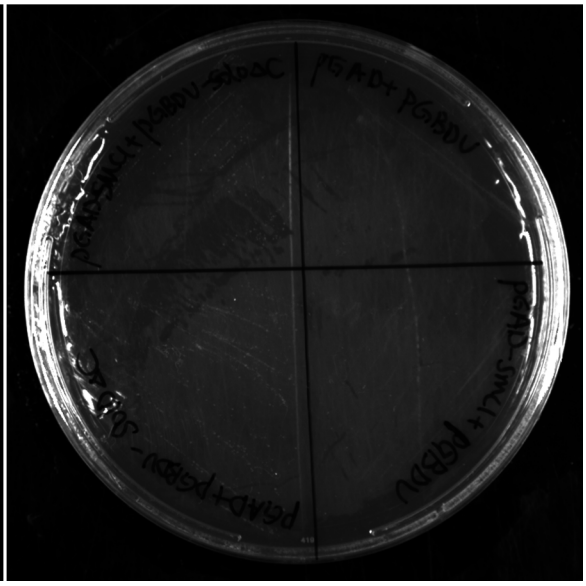
The upper left section of each plate shows the yeast cells harboring plasmids encoding the indicated GAL4AD fusions in combination with the indicated GAL4BD fusions. All the other three sections are negative controls.

a

AD: SMC1 + BD: SOLOΔNT

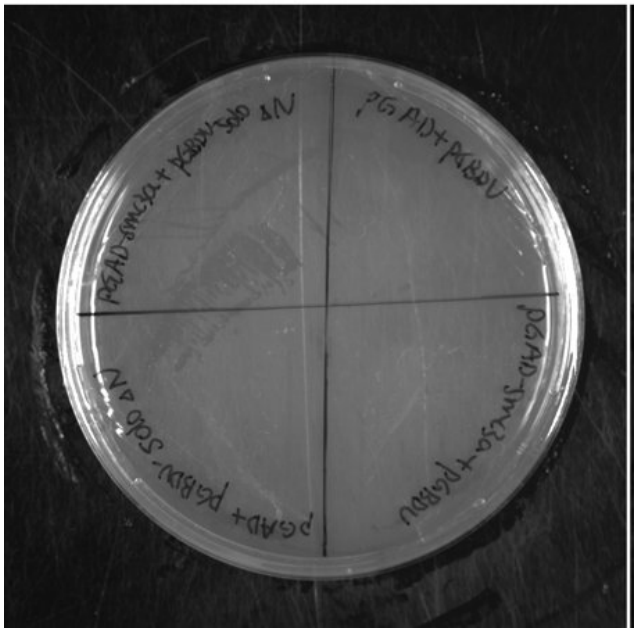


AD: SMC1 + BD: SOLO1ΔCT

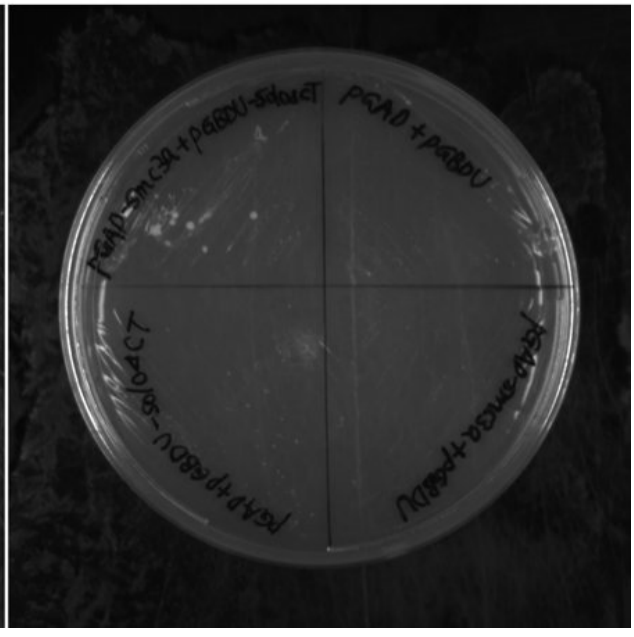


b

AD: SMC3a + BD: SOLOΔNT

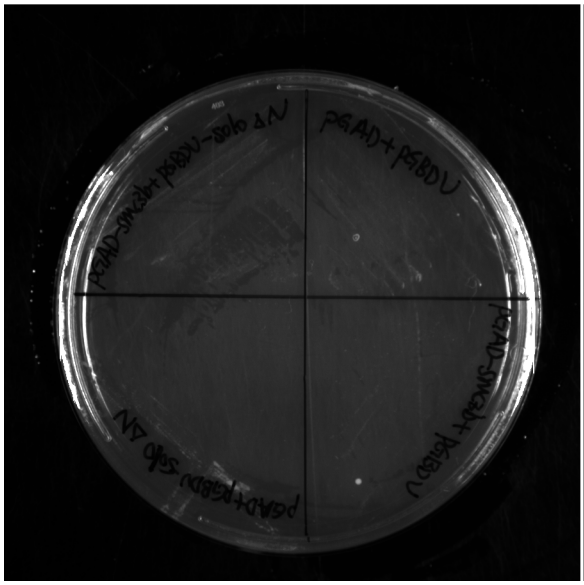


AD: SMC3a + BD: SOLOΔCT

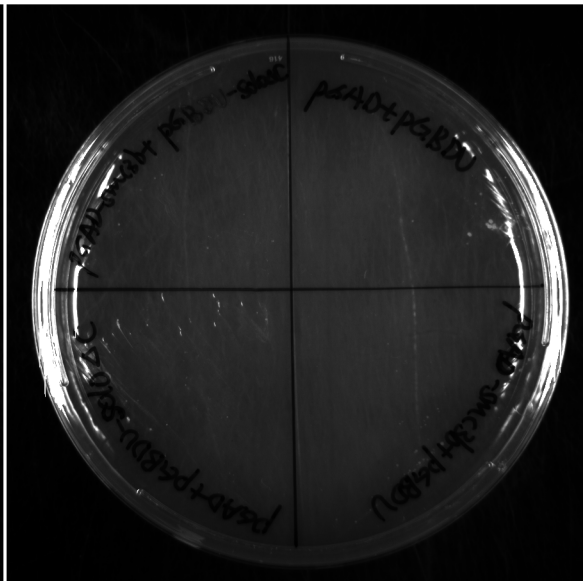


c

AD: SMC3b + BD: SOLOΔNT



AD: SMC3b + BD: SOLOΔCT



SOLO directly interacts with SSE

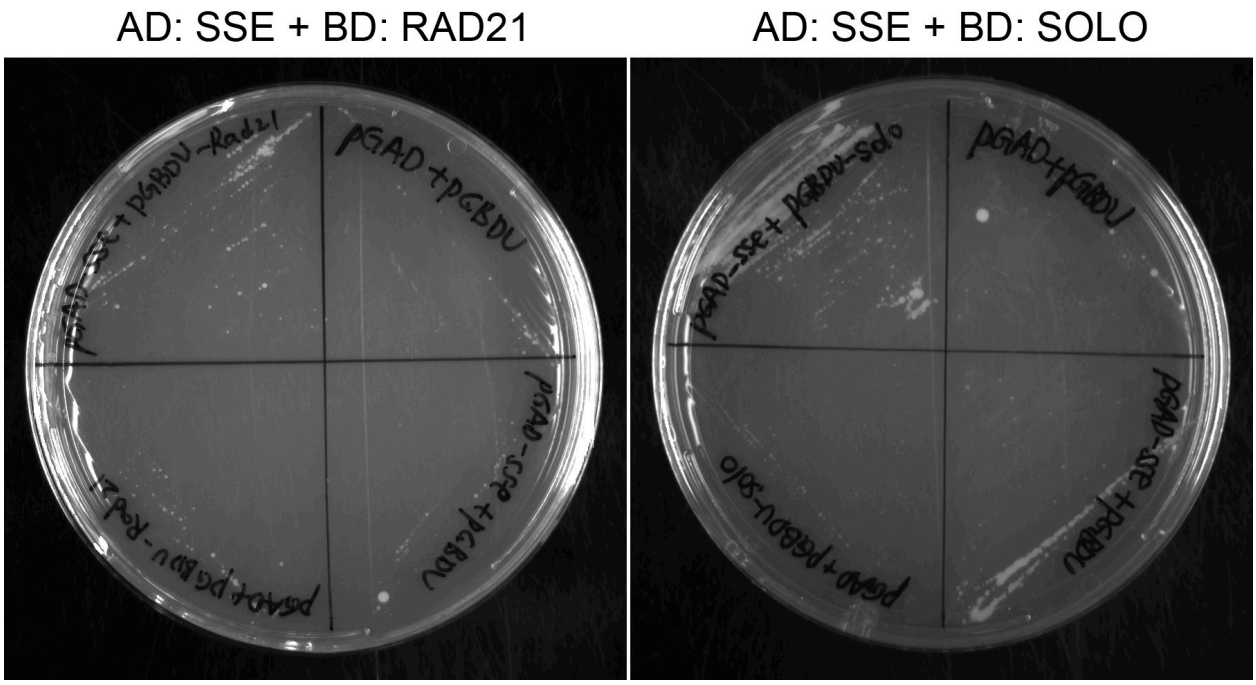
In our study, SOLO and RAD21 coding sequences were fused in-frame to the yeast GAL4 DNA binding domain (BD) respectively as “bait” constructs. The coding sequences of SSE and SSE C497S were fused in-frame with the GAL4 transcription activation domain (AD), respectively. Combinations of the following plasmids were co-transformed into yeast cells: AD: SSE + BD: RAD21, AD: SSE C497S + BD: RAD21, AD: SSE + BD: SOLO, AD: SSE C497S + BD: SOLO. Yeast growth on plates with selective medium lacking histidine indicates AD and BD fused protein-protein interaction. RAD21 as a classical mitotic cohesin was used as an SSE substrate positive control. Yeast carrying single fused protein construct and/ or empty vectors served as negative controls.

Similar to the cell growth in the AD: SSE+ BD: RAD21, cells harboring AD: SSE + BD: SOLO survived on the his^- plates. That indicates SOLO directly interacts with SSE. In addition, the protease dead SSE C497S enhanced the interactions with both RAD21 and SOLO based on our results (**Figure 2-6**).

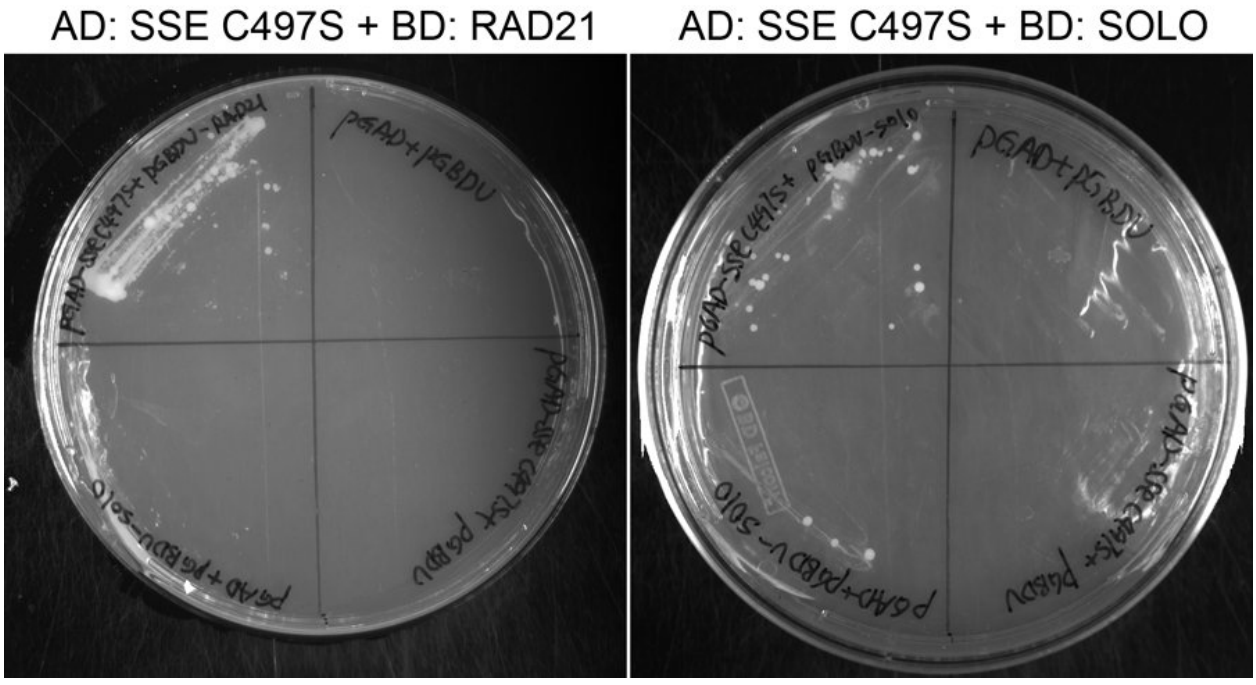
Figure 2-6 SOLO interacts with SSE and protease dead SSE C497S

The upper left section of each plate shows the yeast cells harboring plasmids encoding the indicated GAL4AD fusions in combination with the indicated GAL4BD fusions. All the other three sections are negative controls.

a


AD: SSE + BD: RAD21
AD: SSE + BD: SOLO

b


AD: SSE C497S + BD: RAD21
AD: SSE C497S + BD: SOLO

DISCUSSION

SOLO C-terminal conserved residues are required for accurate chromosome segregation, its chromosome localization, and centromere cohesion

Cohesin is a tripartite ring constituted by three proteins. In yeast and mammalians, SCC1 connects the ATPase heads of a V-shaped SMC1/3 heterodimer to complete the ring. C terminus SCC1 has been co-crystalized with SMC1 head domain and among the residues from the interface, residue F528, and L532 have been identified as essential for the interaction (Haering et al., 2004). In our study, sequence analysis of SOLO identified its C terminus as a relatively conserved domain. A further sequence alignment of SOLO C terminus with RAD21/REC8 suggests that all but one of the thirteen most-conserved residues in REC8 C termini alignment are conserved among *Drosophila* SOLO orthologs. This is unlikely to be coincidental as the average overall pairwise identity of the *D. melanogaster, D. pseudoobscura* and *D. virilis* homologs is less than 30%. Moreover, the residues (F528 and L532) of yeast SCC1 that correspond to two of those conserved C-terminal residues (Y*1007 and L*1011) make close contacts with SMC1 in a SCC1-SMC1 co-crystal structure, and are required for the SMC1-SCC1 interaction (Haering et al., 2002; Gruber et al., 2003; Haering et al., 2004). *Drosophila* with mutations of SOLO^{Y*1007R} and SOLO^{L*1011R} have nondisjunction rates of 3.41% and 23.0% in males while 27.5% and 52.9% in females, respectively. However, mutation of both residues lead to SOLO completely loss of function, with a nondisjunction rate of 46.8% in males and 57.1% in females, compared to *solo* null at 46.7% for males and 60.6% for females. In addition, SOLO$^{Y*1007R\&L*1011R}$ failed to localize to centromeres during meiosis in both sexes. Centromeres cohesion is disrupted in SOLO $^{Y*1007R\&L*1011R}$

mutant male flies. With four pairs of chromosomes, usually less than 8 centromere spots are detected in meiosis I cells before anaphase. However, mutation of SOLO$^{Y*1007R\&L*1011R}$ increased the centromere spots to more than 8 in all the cells scored. Taken together, SOLO C terminus residues Y*1007 and L*1011 play significant roles in accurate chromosome segregation, SOLO chromosome localization, and centromere cohesion. These data suggests that SOLO may work as a non-canonical paralog of SCC1/RAD21/REC8 in *Drosophila* meiosis.

SOLO N-terminal domain is required for accurate chromosome segregation and its localization

SOLO N-terminal domain (1-137 aa) encoding its first three exons is shared with a transcription factor VASA. The RGG domain is not a classical motif in the cohesin proteins, however, in our study, deletion of SOLO N-terminal RGG domain disrupts its function in accurate chromosome segregation and chromosome localization in both sexes. But how the RGG domain performs these functions is still unclear. One possible explanation is SOLO RGG domain might have a similar role to that of VASA, whose N-terminal RGG domain works as a substrate of members from the Arginine Methyltransferase family. This post-translational modification has been reported to regulate protein subcellular localization (Passos et al., 2006). However, no abnormal chromosome segregation patterns have been identified from *Drosophila* Arginine Methyltransferase family members DART RNAi flies (data not shown). These results might result from low RNAi efficiencies of these flies. It is also possible that the 9 members of the DART family might have redundant functions so that depletion of one

protein does not lead to overall functional defects. Another possible function of SOLO N terminal RGG domain might be interacting with SMC3 to form a cohesin ring. Since SOLO behaves like REC8 in every aspect and has a comparable C terminus to that of SCC1/ REC8, it is expected to connect SMC3 with its N terminus to form the ring. The function of both the C terminus and N terminus of SOLO were tested further in this study.

SOLO interacts with SMC1 and SMC3 with its C and N termini, respectively

Our yeast two-hybrid results suggest that SOLO exhibited consistent moderate-to-strong interactions with SMC1, SMC3a, and SA. Further tests using SOLO truncations suggest that SOLO interacts with SMC1 with its C terminus while it interacts with SMC3a with its N terminus. These findings are consistent with a former study that SOLO physically interacts with SMC1 *in vivo* (Yan and McKee, 2013). These findings taken together support the idea that SOLO forms a bridge between SMC1 and SMC3 via interactions at its C and N termini.

An interesting point is that SOLO can interact with SMC3 without initially interacting with SMC1, something yeast SCC1 cannot do (Nasmyth and Haering, 2009). No homology between the N terminus of SOLO and α-kleisins has been detected, so it is quite possible that the molecular basis for SOLO's interaction with SMC3 is different from that of REC8.

SOLO directly interacts with SSE

Although SOLO has been revealed to directly interact with SMC1 and SMC3, more

evidence needs to be provided to prove SOLO is a cohesin subunit. One important signature of cohesin SCC1/RAD21/REC8 subunit is that they get cleaved by a cysteine protease Separase after metaphase II.

Our data from yeast two-hybrid identified direct interaction between SSE/ protease dead SSE C497S and SOLO in a way similar to RAD21. We have performed co-immunoprecipitation and got encouraging results indicating that SOLO and SSE might associate *in vivo* (data not shown). These data together with SOLO functional similarities with REC8 support that SOLO might interact with SSE as its substrate.

Chapter 3 - GENERAL CONCLUSION AND FUTURE DIRECTIONS

GENERAL CONCLUSION

Many studies have been carried out to study the process of cohesion in yeast. Most of the important principles revealed during cell division have been examined in higher eukaryotes like mouse and humans. However, *Drosophila* meiosis is a specialized case for two reasons. First, *Drosophila* utilizes similar mechanisms as other species in female meiosis, but in males, no recombination, synapsis, or chiasmata exist. Instead, a protein complex including meiosis protein SNM and MNM contribute to homolog pairing. In addition, in the cohesin complex which is key to the cohesion process during cell division, no classic meiosis specific REC8 subunit works in *Drosophila* meiosis. Our results together with former studies support that SOLO might be the REC8 homolog in *Drosophila* meiosis.

SOLO C-terminal conserved residues are required for accurate chromosome segregation, its chromosome localization, and centromere cohesion in meiosis

A series of SOLO C-terminal conserved residues including the corresponding SMC1 interacting residues in yeast SCC1 were mutated (Y*1007R&L*1011R in *Drosophila* SOLO and F528 and L532 in budding yeast SCC1). The SOLO C terminus mutants were used to rescue the *solo* null mutant phenotypes. The mutation of both Y*1007R&L*1011R together disrupted SOLO function to a level similar to *solo* null. Accurate chromosome segregation was disrupted, SOLO$^{Y*1007R\&L*1011R}$ failed to localize to testes and germarium, and centromere cohesion was completely disturbed. These data taken together suggest that SOLO C terminus might play a similar role to that of

SCC1/ RAD21/ REC8.

SOLO N terminus conserved residues are required for accurate chromosome segregation and its chromosome localization

SOLO shares a N terminus containing 3 RGG repeats with VASA, whose N-terminal RGG domain works as a substrate of members from the Arginine Methyltransferase family. This post-translational modification has been reported to regulate protein subcellular localization (Passos et al., 2006). However, the functional importance of the N terminal domain in SOLO is unclear. We constructed a truncated SOLO without the first 137 amino acids in the N terminus, which are shared with VASA. UASp:: SOLO△NT driven by UASp:: nanos-GAL4-VP16 was used to rescue *solo* null phenotypes. SOLO△NT failed to localize to testes or germaria. In addition, the accurate chromosome segregation was disrupted in the SOLO△NT expressing flies. These results indicate that SOLO N terminus is essential for its subcellular localization and accurate chromosome segregation.

SOLO interacts with cohesin SMC1, SMC3a, and SA

In order to test whether SOLO is a subunit of the cohesin complex, direct interactions between SOLO and SMC1, SMC3a, SMC3b as well as SA were detected by yeast two-hybrid. Based on our results, SOLO interacts with SMC1, SA and SMC3a, but not SMC3b, in a way similar to cohesin protein RAD21 except that RAD21 interacts with

both SMC3 proteins but might preferentially interact with SMC3b. Further tests were carried out by domain truncations of SOLO. Two SOLO mutations were constructed, SOLO△NT (aa 138-1031) and SOLO△CT (aa 1-971). Consistent with RAD21, SOLO △NT interacts with SMC1 but failed to interact SMC3a. On contrary, SOLO△CT interacts with SMC3a but failed to interact SMC1. These results taken together support that SOLO might bridge SMC1 and SMC3a to form a ring structure in *Drosophila* meiosis. In addition, SOLO might work with SMC3a in meiosis, while RAD21 might work with both SMC3a and SMC3b in mitotic cells.

SOLO physically associates with SSE and protease dead SSE C497S

One signature of cohesin REC8 subunit is that it get cleaved by Separase to release sister chromatids. We proposed that SOLO might be a *Drosophila* REC8 homolog, accordingly, it is expected to be a substrate of *Drosophila* Separase homolog SSE. In order to detect if SOLO and SSE associate, both yeast two-hybrid and co-immunoprecipitation were performed. From our results, SOLO interacts with both wildtype SSE and a protease dead SSE C497S in yeast two-hybrid. In addition, we got encouraging results from co-immunoprecipitation indicating SOLO might interact with wildtype SSE *in vivo* (data not shown). These data suggest that SOLO associates with SSE.

FUTURE DIRECTIONS

The data obtained during the course of research for this thesis will be important in understanding the cohesin protein components and working mechanisms in *Drosophila* meiosis. A conserved SOLO C terminus has been identified by sequence alignment among the ten species of *Drosophila*. Further sequence analysis identified twelve of the thirteen REC8 C terminus most conserved residues have counterparts at SOLO C terminus. Mutations of a series of SOLO C terminus residues including counterparts of the two residues interacting SMC1 in SCC1 block its function in accurate chromosome segregation, chromosome localization, and centromere cohesion. In addition, results from yeast two-hybrid suggest SOLO can directly interact with SMC1 and SMC3 with its C and N terminus, respectively. In addition, like RAD21, SOLO interacts with SA based on our yeast two-hybrid data. Similarly to other SCC1/RAD21/REC8 homologs, SOLO might be a Separase substrate. *Drosophila* SSE and protease dead SSE C497S interacts with SOLO in yeast two-hybrid. These data taken together suggest that SOLO is possibly an unusual member of the REC8 family in *Drosophila* meiosis.

One interesting issue to explore is to identify the SOLO containing cohesion complex by co-immunoprecipitation followed by MALDI Mass Spectrometry. Yeast two-hybrid can be used as another strategy to identify SOLO interacting proteins. A library of proteins including meiotic and mitotic cohesion proteins has been constructed into either pGAD-C1 or pGBDU-C1 vectors (**Table 3-1**). By these studies, a clearer idea of how SOLO work in meiosis cohesion is expected. Among the cohesion proteins, two proteins ORD and SUN behaves similarly to SOLO. It will be interesting to explore if the three proteins

Table 3-1 A cohesion protein library constructed for yeast two-hybrid

pGAD-	pGBDU
SMC1, SMC3a, SMC3b, SOLO, MEI-S332, C(2)M, PDS5, SAN, ORD, SSE, SSE C497S, DART1, DART5	RAD21, SOLO, SOLOΔNT, SOLOΔCT, $SOLO^{R1000E}$, $SOLO^{Y*1007R}$, $SOLO^{L1010R}$, $SOLO^{L*1011R}$, $SOLO^{Y*1007R\&L*1011R}$, $SOLO^{L1010R\&L*1011R}$, SAN, MEI-S332, SA, SSE, SSE C497S

work in a complex or through different pathways.

In addition, it will be meaningful to identify the SSE cleavage sites in SOLO. Up to now, three potential SSE cleavage sites have been identified in SOLO. Transgenic flies have been constructed to express a SOLO mutant with disrupted potential SSE sites (by reversing D/ExxR to RxxD/E). Chromosome segregation patterns will be examined for detecting the functional importance of these sites. It would also be interesting to rescue the mutant sites individually to compare their effect on chromosome segregation patterns in order to get the working cleavage sites.

Another interesting issue that we should investigate is the two SMC3 splicing isoforms, SMC3a and SMC3b. Based on the finding from our yeast two-hybrid assays, SMC3a might prefer interacting with SOLO in meiotic cells while SMC3b might interact with RAD21 with higher affinity although both SMC3 isoforms interact with RAD21. It has been reported that specialized cohesin components work in mitosis and meiosis. In mammalians, SMC1 alpha and SMC1 beta isoforms are differentiated for mitosis and meiosis (Revenkova et al., 2004). In our study, reverse transcript Polymerase Chain Reaction (RT-PCR) has been carried out to detect the expression difference of SMC3a and SMC3b in different tissues including ovaries and embryos. However, no SMC3b specific DNA sequence is available so that there is a limitation in SMC3b direct detection. We used primers targeting SMC3a specific DNA sequence compared with a pair of primers targeting the shared region of SMC3a and SMC3b. However, we failed to get consistent significantly different mRNA levels using primers targeting different regions. The resolution for RT-PCR is not high enough to differ the signals. More sensitive Realtime-PCR could be used in future for detecting the mRNA level difference

between SMC3a and SMC3s together by using primers targeting different DNA regions. In addition, cytological studies could be carried out to directly detect the localization patterns of these two isoforms.

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