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Two Putative Acetyltransferases, San and Deco, Are Required for Establishing Sister Chromatid Cohesion in *Drosophila*

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Summary

Background: Sister chromatid cohesion is needed for proper alignment and segregation of chromosomes during cell division. Chromatids are linked by the multiprotein cohesin complex, which binds to DNA during G_1 and then establishes cohesion during S phase DNA replication. However, many aspects of the mechanisms that establish and maintain cohesion during mitosis remain unclear.

Results: We found that mutations in two evolutionarily conserved *Drosophila* genes, *san* (s*eparation* an*xiety*) and *deco* (D*rosophila* eco*1*), disrupt centromeric sister chromatid cohesion very early in division. This failure of sister chromatid cohesion does not require separase and is correlated with a failure of the cohesin component Scc1 to accumulate in centromeric regions. It thus appears that these mutations interfere with the establishment of centromeric sister chromatid cohesion. Secondary consequences of these mutations include activation of the spindle checkpoint, causing metaphase delay or arrest. Some cells eventually escape the block but incur many errors in anaphase chromosome segregation. Both *san* and *deco* are predicted to encode acetyltransferases, which transfer acetyl groups either to internal lysine residues or to the N terminus of other proteins. The San protein is itself acetylated, and it associates with the Nat1 and Ard1 subunits of the NatA acetyltransferase.

Conclusions: At least two diverse acetyltransferases play vital roles in regulating sister chromatid cohesion during *Drosophila* mitosis.

Introduction

Newly replicated sister chromatids must remain attached to each other until anaphase onset. Chromatids that separate too early are subject to failures in connections to the spindle [1, 2], to premature migration to the poles [3], or to misalignment at the metaphase plate [4]. All of these abnormal events disrupt chromosome segregation, producing genetically imbalanced aneuploid daughter cells that suffer a variety of detrimental consequences, such as increased progression along pathways leading to cancer [5, 6].

Cohesin, the protein complex that attaches sister chromatids to each other, is thought to function as an intermolecular DNA crosslinker. Cohesin contains a core of Scc1 (Rad21), Scc3, Smc1, and Smc3 proteins (reviewed in [7]), but other important proteins such as Pds5 are more loosely associated with this complex [8]. The establishment of cohesion takes place in several stages. First, cohesin is loaded onto DNA at specific sites along the chromosomes prior to S phase [9], assisted by Scc2/4 and chromatin remodeling complexes [10]. Heterochromatin proteins help deposit cohesin at a particularly high concentration at the centromeres [11]. Cohesion can, of course, only be established subsequently, after DNA has been replicated into sister chromatids. The establishment of cohesion is tightly coordinated with progression of the replication fork in S phase and requires specialized replication factors such as Trf4/DNA polymerase σ [12]. Of particular relevance here, a protein called Eco1p/Ctf7 is necessary for the establishment of cohesion during S phase; this protein is not, however, required for the earlier step in which cohesin is loaded onto the chromosomes [13–15]. Eco1p acts in vitro as an acetyltransferase that can acetylate itself as well as the Scc1 and Scc3 cohesin subunits, although the significance of this enzymatic activity to sister chromatid cohesion in vivo is uncertain [16]. The existence of a functional link between cohesion and DNA replication is further supported by the findings that Eco1p associates physically with components of replication factor C complexes [17] and that the *S. pombe* homolog of Eco1p, Eso1p, is present in the genome as a fusion with DNA polymerase η [15, 18].

For sister chromatids to separate at anaphase, the cohesin bonds holding them together must first be removed. In higher eukaryotes, the majority of cohesin delocalizes from the chromosomes during prophase without being cleaved [19]. Phosphorylation of cohesin subunits by Polo-like and Aurora kinases is thought to trigger this dissociation of cohesin from the chromosomes [20]. The remaining cohesin, which persists mostly at the centromere, is cleaved at anaphase onset through the action of a proteolytic cascade involving the anaphase-promoting complex/cyclosome (APC/C; reviewed in [21]). When chromosomes are properly aligned on the spindle in a bipolar fashion, the APC/C becomes activated and degrades securin, liberating the protease separase, which then cleaves the Scc1 subunit of cohesin. In this way, the cohesin bond holding the sister centromeres together is dissolved at anaphase onset, allowing the chromatids to be pulled toward opposite spindle poles.

During the course of large-scale genetic screens for mitotic mutations in *Drosophila,* we found mutations in two novel genes that abolished sister chromatid cohesion. In most cells, the resultant misalignment of chromosomes activates the spindle checkpoint, causing metaphase arrest. In a subset of cells, anaphase eventu-

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Table 1. Frequency of Mitotic Defects in *san* and *deco* Mutant Brains

	Number of Brains	Number of Fields	Mitotic Index (MI)	%Met	%Ana	%Ab	%Oc	%PSCS
Wild-type	5	313	1.04	88	12	0	0.3	0.5
san^{1}	7	243	1.63	83	17	76	51	78
san^{1}*/Df*	4	136	0.45	94	6	100	86	98
san^{2}	8	197	0.13	100	0	na	92	95
san^{1}*/san*^{2}	2	192	0.48	91	9	100	68	82
deco^{1}	3	148	2.07	76	24	96	46	81
deco^{1}*/Df*	3	175	0.73	86	14	100	75	96
deco^{2}	2	101	1.33	70	30	23	29	36
deco^{2}*/Df*	3	231	0.75	74	26	31	34	26
deco^{1}*/deco*^{2}	2	118	0.80	78	22	19	31	32

Parameters in untreated brains: MI, mitotic index (number of cells in mitosis divided by number of fields); %Met, percentage of mitotic cells in prometaphase/metaphase; %Ana, percentage of mitotic cells in anaphase/telophase; %Ab, percentage of anaphases exhibiting abnormalities, e.g., lagging chromosomes, unequal or disorganized anaphases; %Oc, percentage of mitotic cells exhibiting overcondensed chromosomes (including those with PSCS); %PSCS, percentage of prometaphase/metaphase cells with precocious sister chromatid separation, as scored in brains treated with colchicine/hypotonic solutions (wt, n = 535; *san*^{1}, n = 472; *san*^{1}*/Df*, n = 22; *san*^{2}, n = 65; *deco*^{1}, n = 235; *deco*^{1}*/Df*, n = 137; *deco*^{2}, n = 248; *deco*^{2}*/Df*, n = 95; *deco*^{1}*/deco*^{2}, n = 237). Na, not applicable.

ally occurs, but chromosome segregation is highly irregular. In cells mutant for either of these two genes, the Scc1 protein is delocalized from the centromeres of the detached chromatids, reflecting an instability of the cohesin complex on the chromosomes. To our surprise, both of these genes proved to encode putative acetyltransferases. One of the genes, *deco* (D*rosophila* eco*1p*), is the apparent fly homolog of yeast *eco1*. The product of the second gene, which we call *san* (s*eparation* an*xiety*), contains an unrelated N-acetyltransferase domain. The San protein itself is associated in a complex with the fly Nat1 and Ard1 N-acetyltransferase proteins. Our results thus suggest the existence of a network of acetyltransferases required for the establishment and/or maintenance of sister chromatid cohesion in metazoans.

Results

Mutations in *san* and *deco* Disrupt Sister Chromatid Cohesion and Mitotic Progression

We identified two mutant alleles of *san* and two mutant alleles of *deco* from genetic screens for mitotic mutants, amongst collections of mutagenized chromosomes causing late larval and pupal lethality in homozygotes ([22]; see Experimental Procedures). The rationale for these screens is that maternal stores of many proteins important for mitosis are sufficient for development to larval and pupal stages, but not for metamorphosis to the adult [23].

Because the phenotype associated with the *san*^{1} mutation, but not the *san*^{2} mutation, became more severe when gene dosage was lowered by combination with a deficiency for the locus, we regard *san*^{1} as a strong hypomorphic allele and *san*^{2} as a null mutation (Table 1). This conclusion is supported by Western blot data (see below), which demonstrated that trace San protein is present in *san*^{1} animals but is completely absent in *san*^{2}. The same genetic criterion indicates that *deco*^{1} and *deco*^{2} are both hypomorphic mutations. The *deco*^{2} mutation is much weaker than *deco*^{1}, but defects in sister chromatid cohesion are still clearly apparent even in *deco*^{2} homozygotes (Table 1).

The effects of *san* and *deco* mutations on mitosis were initially examined in larval brain squashes using standard orcein staining techniques. Prometaphase cells could be identified by the stereotypical arrangement of chromosomes prior to their alignment at the metaphase plate [23]. At this stage, each chromosome normally consists of two sister chromatids weakly associated along their arms but tightly associated at their centromeres (Figure 1A). Sister chromatids normally do not separate from each other until anaphase onset. In most *san* and *deco* mutant cells, however, the centromeric connections between sister chromatids are already abolished by prometaphase (Figures 1B and 1C). To better visualize and quantify this phenotype of precocious (or premature) sister chromatid separation (PSCS; [24]), we also observed brains treated with a hypotonic solution that eliminates the interactions between chromatid arms while leaving normal centromere attachments intact (Figure 1E; see also [23]). The vast majority of *san* and *deco* mutant cells treated in this way clearly exhibited PSCS (Figures 1F and 1G; Table 1), verifying the findings in untreated cells.

Many cells in both colchicine-treated and untreated *san* and *deco* mutant brains contained overcondensed sister chromatids exhibiting PSCS with no evidence of anaphase movement (Figures 1D and 1H; Table 1). The highly compacted chromatin in these cells suggests a mitotic delay or arrest: if a wild-type cell is arrested in prometaphase by treatment with microtubule poisons such as colchicine, the chromosomes continue to condense and eventually, after prolonged incubation, overcondense in the absence of division [25]. The absence of anaphases in the brains of animals carrying the null *san*^{2} mutation and the elevated mitotic index (the number of mitotic cells per microscopic field) seen in animals homozygous for either the *san*^{1} or *deco*^{1} mutations provide further support for the existence of mitotic delay/arrest (Table 1). This latter point is complicated by the finding that certain combinations of stronger mutant alleles surprisingly result in extremely low mitotic indices. The paradox is likely explained by the extensive apoptotic cell death observed in these genotypes (data

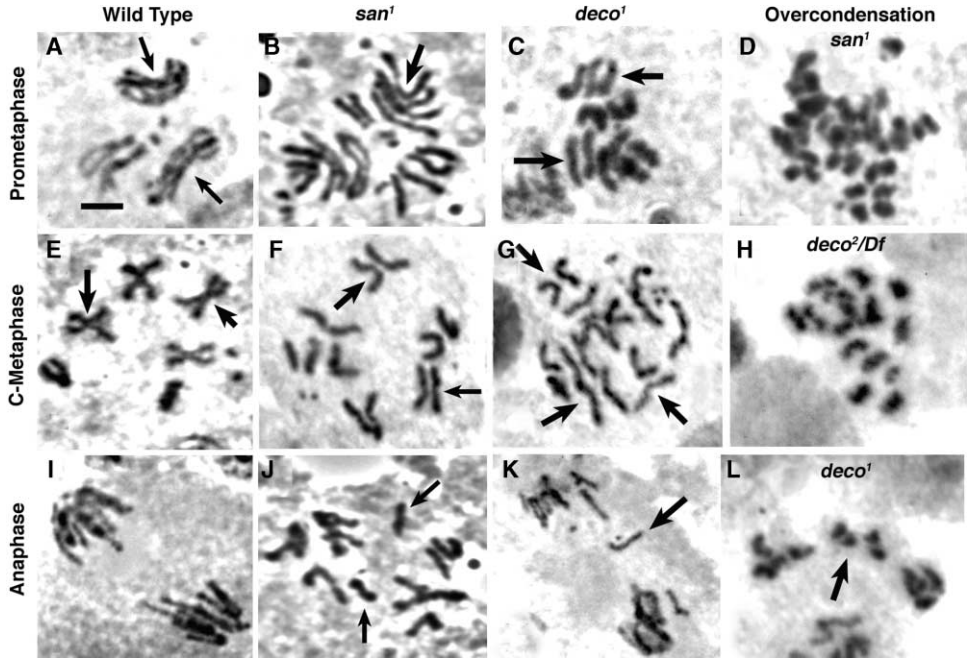

Figure 1. Mitotic Defects in *san* and *deco* Mutants

Larval brain neuroblast chromosomes were stained with orcein. (A–D) Untreated cells at prometaphase. (E–H) Colchicine-treated cells in c-metaphase. In wild-type, sister chromatids are attached at the centromeres (arrows; [A and E]). Sister chromatids separate prematurely at their centromeres in *san* and *deco* homozygotes (arrows; [B, C, F, and G]). In mutant anaphases (J–L), but not in wild-type anaphases (I), some sister chromatids migrate properly to the poles while others lag behind (arrows). (D, H, and L) Mitotic figures containing overcondensed chromatids caused by mitotic arrest/delay in *san* and *deco* mutants. Bar, 5 μm.

not shown), which would decrease the mitotic index even if many cells are arrested in mitosis.

In larvae of all mutant genotypes excepting san^2 homozygotes, some anaphases were still observed, many containing overcondensed chromatids consistent with mitotic delay. In almost all of these anaphases, chromatid movements were highly aberrant (Figures 1J–1L; Table 1). Most of these mutant anaphases contained one or more lagging chromatids that failed to migrate toward the poles. In other cases, chromatids were directed in many different orientations and not always toward the poles. Finally, chromatin bridges were often evident at telophase (data not shown). The aberrant segregation of chromatids during anaphase leads to high levels of aneuploidy visible in san^1 and *deco* mutant metaphases (Figures 1B, 1G, and 1H; see also Figure 2 below). Rare polyploid cells (e.g., Figure 1G) can be explained by defects in cytokinesis or by reversion to interphase followed by another round of mitosis, as proposed by Gatti and Baker [26]. None of these defects are likely to result from problems in the spindle apparatus: centrosomes and spindle microtubules are morphologically normal in *san* mutants (see supplemental data).

Given the defects in sister chromatid cohesion observed in *san* and *deco* mutants, we were surprised to find that many chromosomes were still able to congress to the metaphase plate (Figure 2; also see supplemental data). This congression is, however, not entirely normal. First, small chromatin entities were situated away from the metaphase plate in a large fraction of mutant cells (30%–40% versus 2% in wild-type). These outward lying chromatin structures always contained centromeres, as they associated with the centromere-specific, CENP-A H3-like protein CID (Figure 2). Their small size suggests that many of these entities are intact fourth chromosomes. Second, chromosomes were less tightly arrayed at the metaphase plate in *san* and *deco* mutants than in wild-type. Sister centromeres were correctly oriented toward the poles but were further apart than normal (wild-type: 0.5 ± 0.3 mm, $n = 111$; san^1: 1.2 ± 0.8 mm, $n = 104$; $deco^1$: 1.4 ± 1.1 mm, $n = 87$; Student's t test, $p << 0.001$ for san^1 versus wild-type or for $deco^1$ versus wild-type; Figure 2). These chromosomal configurations at the metaphase plate suggest that centromeres are separated and precociously pulled toward the poles in mutant metaphases but that residual cohesion along the arms prevents complete dissociation of the larger chromosomes.

The Spindle Checkpoint Is Activated in *san* and *deco* Mutants

PSCS in *san* and *deco* mutants could be due to a failure of the spindle checkpoint, causing cells to progress prematurely into anaphase. To check this possibility, we assayed levels of cyclin B protein. Because cyclin B is normally degraded at the metaphase/anaphase transition, its presence is a sensitive indicator of APC/C activity [27]. For example, cyclin B levels remain high in wild-type brain cells arrested in prometaphase with colchicine (Figures 3A and 3B; see also [28]), but similarly treated *Drosophila* mutants that bypass the spindle checkpoint have drastically lowered cyclin B levels and exhibit PSCS (Figures

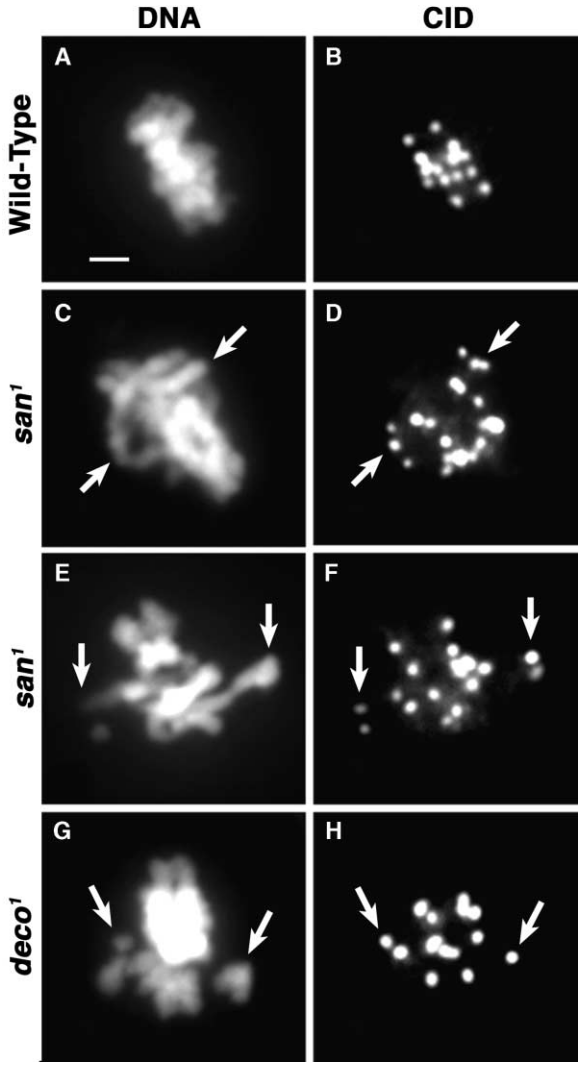

Figure 2. Unattached Centromeres in *san* and *deco* Metaphases

(A and B) In wild-type metaphases, centromeres are paired closely, as represented by CID staining.

(C–H) In *san* and *deco* metaphases, sister centromeres are always further apart and directed to opposite spindle poles. Some chromosomes are situated off the metaphase plate, with their centromeres directed toward the nearest pole (arrows). Sister centromere attachment is thus compromised in these mutants at metaphase, but many sister chromatids still appear to be associated with each other by arm cohesion. The mutant metaphases depicted here have more (C–F) or fewer (G and H) than 16 CID-staining loci and are thus aneuploid. Bar, 5 μm. Color images showing the merged colocalization of CID and DNA may be found in the supplemental data.

3E and 3F; [22, 29]). In contrast, when *san* and *deco* mutant brains were treated with colchicine, cyclin B levels were elevated even in cells exhibiting PSCS (Figures 3I, 3J, 3M, and 3N).

We also determined levels of the Bub3 checkpoint protein at the kinetochore as an alternative method for ascertaining the mitotic state of mutant cells. In wild-type, Bub3 is found at high levels at the kinetochores of prometaphase and metaphase chromosomes (Figures 3C and 3D), but its levels fall precipitously at anaphase onset, with only traces remaining at the kinetochore (data not shown and [30]). In colchicine-treated mitotic mutants with defective checkpoints, Bub3 levels drop to low, anaphase-like levels in cells with PSCS (Figures 3G and 3H, and [22]). In *san* and *deco* mutants treated in the same manner, however, Bub3 levels remain high at the kinetochore, even on detached sister chromatids (Figures 3K, 3L, 3O, and 3P). Similar results were obtained for the localization of Bub1 (data not shown; [31]).

To summarize, mutations in *san* and *deco* do not alter checkpoint signaling. Our results indicate that PSCS in *san* and *deco* mutants is not due to a bypassed spindle checkpoint, but is more likely caused by defects in cohesion per se. We presume that PSCS in fact activates the spindle checkpoint in mutant cells, explaining the mitotic delay/arrest discussed above.

Mutations in *san* and *deco* Affect the Distribution of Cohesion Proteins

To investigate the cause of disrupted sister chromatid cohesion, we examined the localization of several cohesion-related proteins in *san* and *deco* mutants. Scc1/Rad21, a subunit of the cohesin complex, is found in the area of cohesion between sister centromeres in wild-type metaphases and is normally cleaved by separase when anaphase occurs [32]. Scc1 is enriched in centromeric heterochromatin and weakly distributed along chromosome arms in wild-type cells arrested in prometaphase by colchicine (Figures 4A and 4B). In *san* and *deco* mutant prometaphases displaying precocious sister chromatid separation, Scc1 does not accumulate on the centromeres of the individualized chromatids, although trace Scc1 staining is still observed on the chromosome arms (Figures 4E, 4F, 4I, 4J, 4M, and 4N). The sister chromatid separation seen in the mutants is thus coincident with the degradation or mislocalization of Scc1/Rad21 specifically at the centromeres. Interestingly, Scc1 localization to the nucleus during interphase in *san* and *deco* mutants is identical to that observed in wild-type (see supplemental data). This finding suggests that cohesin is loaded properly onto the chromosomes but that it cannot be maintained at the centromeres through prometaphase. The supplemental data also shows that neither Scc1 nor the cohesin subunit stromalin (SA; [33]) is destabilized in *san* or *deco* mutants.

Mei-S332 is another centromeric protein required for sister chromatid cohesion during meiosis and mitosis [34]. Mei-S332 normally localizes to the centromeres of mitotic chromosomes at prometaphase (Figures 4C and 4D) and then is dispersed or degraded when sister chromatids separate at anaphase [35]. In *san* and *deco* mutants, Mei-S332 protein still accumulated normally at centromeres both in colchicine-treated cells and in prometaphase/metaphase untreated cells, even when sister chromatids had precociously separated (Figures 4G and 4H). In approximately half of the anaphase figures, Mei-S332 was no longer at the centromeres as in wild-type. Surprisingly, however, in the remaining 50% of san^1 and $deco^1$ anaphases, Mei-S332 remained at the centromeres, sometimes on all of the chromatids (Figures 4K and 4L), but in other cells only on the chromatids lagging at the position of the metaphase plate (Figure 4O and 4P). Thus, the normal mechanisms controlling Mei-S332 delocalization at the metaphase/anaphase transition are disrupted in *san* and *deco* mutants.

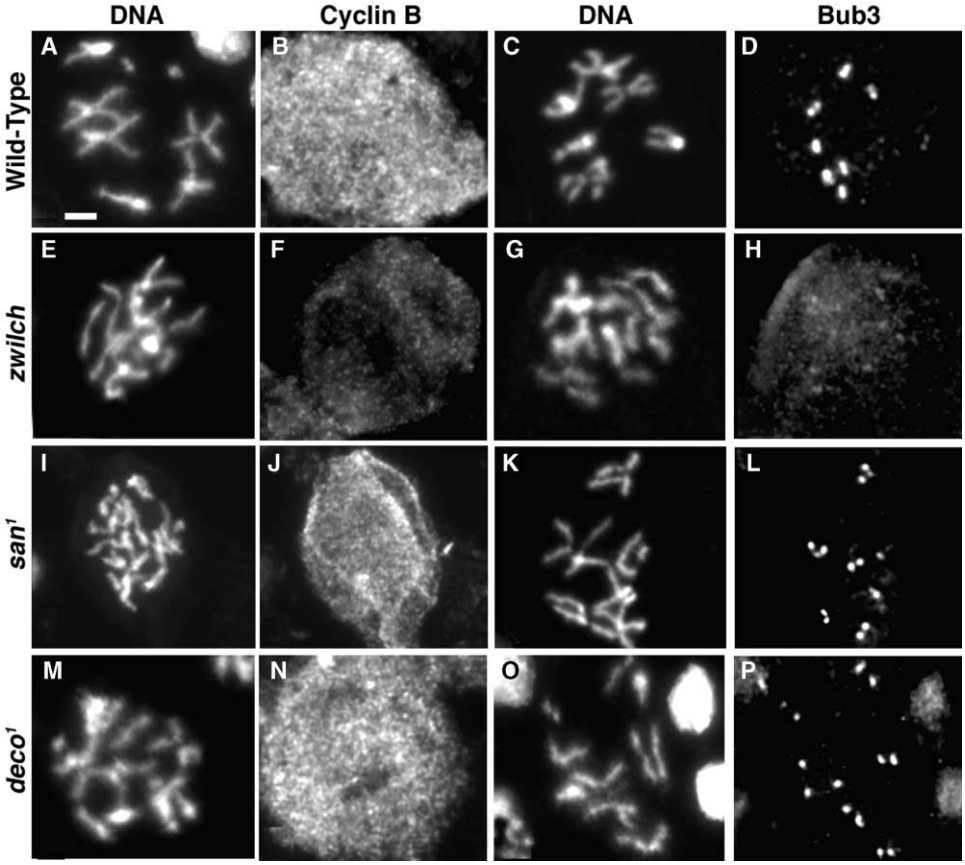

Figure 3. Activation of the Spindle Checkpoint in *san* and *deco* Mutants

Colchicine-treated cells were stained for cyclin B and Bub3 proteins to determine whether the spindle checkpoint is active, as indicated by high levels of cyclin B distributed throughout the cell and Bub3 at the kinetochore (A–D). In *zwilch* mutants (E–H), the spindle checkpoint is bypassed, resulting in sister chromatid separation and reduced, anaphase-like levels of cyclin B and Bub3 (see also [16]). However, in *san* and *deco* mutants (I–P), the spindle checkpoint remains activated even in the presence of sister chromatid separation, as witnessed by high levels of cyclin B (I, J, M, and N) and Bub3 (K, L, O, and P). Bar, 5 μm. Color images showing the merged colocalization of cyclin B or Bub3 with DNA may be found in the supplemental data.

Sister Chromatid Separation in *san* and *deco* Mutants Is Independent of Separase

To determine where *san* and *deco* function in the chromatid cohesion pathway, we made double mutant strains that also carry mutations in *sse*, which encodes the *Drosophila* separase. Sister centromeres do not separate from each other during mitosis in *sse* mutants (Figures 5A and 5B and [23, 36]). In both san^1 *sse* and in $deco^1$ *sse* double mutants, sister chromatids separate prematurely as they do in san^1 or in $deco^1$ mutants alone (Figures 5C–5F). San and Deco thus act upstream of separase in ensuring sister chromatid cohesion. In other words, if sister chromatids are never properly attached, then separase function is irrelevant for sister chromatid separation.

The *san* Gene Encodes a Novel, Conserved, Putative N-Acetyltransferase

We identified *san* as the gene CG12352 using a variety of approaches; of particular note, treatment of *Drosophila* tissue culture cells with CG12352 double-stranded RNA (dsRNA) disrupts sister chromatid cohesion (see supplemental data). The hypomorphic san^1 allele is caused by the insertion of a *hobo* transposable element into the first intron of CG12352, while the null san^2 allele is the result of a P element insertion into the 5′-UTR of the gene.

BLAST searches with the predicted 184 amino acid San protein identified homologs of unknown function in mosquitoes (80% identity), mice and humans (75%), nematodes (52%), and in plants such as cotton and *Arabidopsis* (49%) [37]. Searches of the Conserved Domain Database (NCBI) and PROSITE revealed that San posesses a domain (amino acids 74–129) conserved among protein acetyltransferases; within this domain are two subdomains (amino acids 74–94 and 117–129) that are responsible for acetyl CoA binding [37, 38]. While the classification of San as a specific type of acetyltransferase cannot be absolutely discerned from sequence analysis, the homology strongly suggests that San is a peptide N-acetyltransferase. Proteins of this type transfer acetyl groups from acetyl-CoA to the N terminus of target proteins [37, 38].

The San Protein Is Associated with Other Acetyltransferases and Is Itself Acetylated

Polyclonal antibodies were raised in rabbits against a GST-San fusion protein. On Western blots, the antibodies recognize the original fusion protein as well as un-

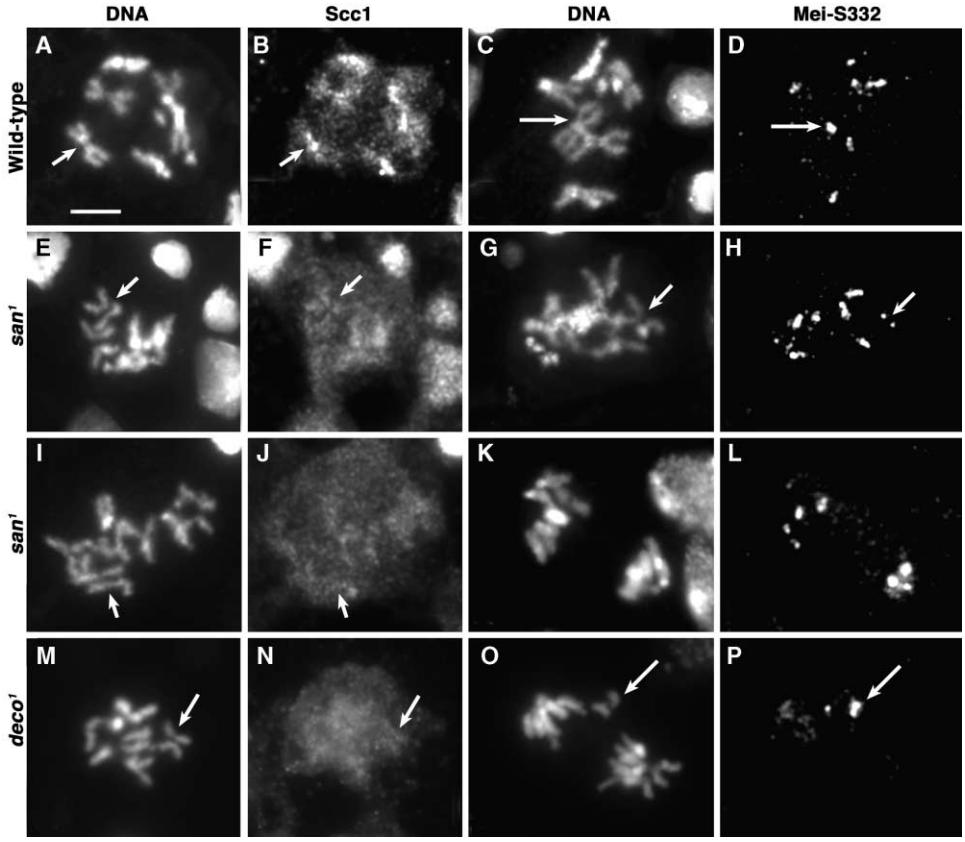

Figure 4. Scc1, but Not Mei-S332, Is Absent from the Centromeric Regions of *san* and *deco* Mutants

In wild-type metaphases, Scc1 localizes to the centromeric domain of attached sister chromatids (arrows, [A and B]). In *san* and *deco* mutants, Scc1 is no longer associated with the centromeres of separated sister chromatids (arrows; [E, F, I, J, M, and N]). Mei-S332, which during metaphase normally occupies the area between attached sister centromeres (arrows, [C and D]), remains at centromeres even when they are separated in *san* mutants (arrows, [G and H]) and in *deco* mutants (data not shown). During anaphase in wild-type, Mei-S332 protein disappears from the centromeres ([34] and data not shown). In many *san* and *deco* mutant anaphases, however, Mei-S332 protein abnormally persists at the centromeres (K and L) or is specifically associated with lagging chromatids (arrows, [O and P]). Bar, 5 μm. Color images showing the merged colocalization of Scc1 or Mei-S332 with DNA may be found in the supplemental data.

fused San protein produced in baculovirus and in *E. coli* (data not shown). Affinity-purified anti-San antibody recognizes a single band of approximately 18 kDa in extracts of wild-type *Drosophila* larvae; this is the size of the protein predicted from the cDNA sequence. In san^1 mutant larvae, however, the level of this protein is approximately 10-fold lower, while no San protein is apparent in san^2 mutant larvae (Figures 6A and 6B). These findings support the genetic data presented above suggesting that san^1 is a strong hypomorphic allele, while san^2 is a null mutation.

The intracellular localization of the San protein was studied using the affinity-purified antibody as an indirect immunofluoresence probe (Figures 6C and 6D). During interphase in wild-type neuroblasts, San localizes to the cytoplasm. During the entry into mitosis, San becomes distributed throughout the entire cell in a punctate pattern. Cells stain uniformly for San from metaphase through telophase. These signals are greatly diminished in the brains of san^1 homozygotes (Figures 6E and 6F) and disappear in san^2 mutant larvae (data not shown).

To examine the possibility that San acts in a complex with other proteins, we probed Western blots of total embryo extracts fractionated by gel exclusion chromatography (Figure 7A). San protein eluted from the column in peak fractions with an apparent total molecular weight of between 150 and 250 kDa. Given that the San polypeptide is only 18 kDa, this finding strongly suggests that San associates with other proteins in a multiprotein complex. No San was apparent in fractions corresponding to its monomeric size.

To identify other components of this complex, we performed immunoaffinity chromatography using a column of affinity-purified anti-San antibody covalently coupled to protein A-Agarose beads. This anti-San column was used to purify San and its associated proteins from embryo extracts (Figure 7B). After extensive washing in 100 mM KCl, proteins were successively eluted from the column, first with 1 M KCl, then with 1.5 M $MgCl_2$, and finally with low pH (see Experimental Procedures). Many proteins were seen in the 1 M KCl eluate; most of these seemed to bind nonspecifically to IgG or the protein A-Agarose beads because they also appeared in eluates from a preimmune IgG control column (data not shown). In the subsequent 1.5 M $MgCl_2$ elution, two predominant protein bands were observed that

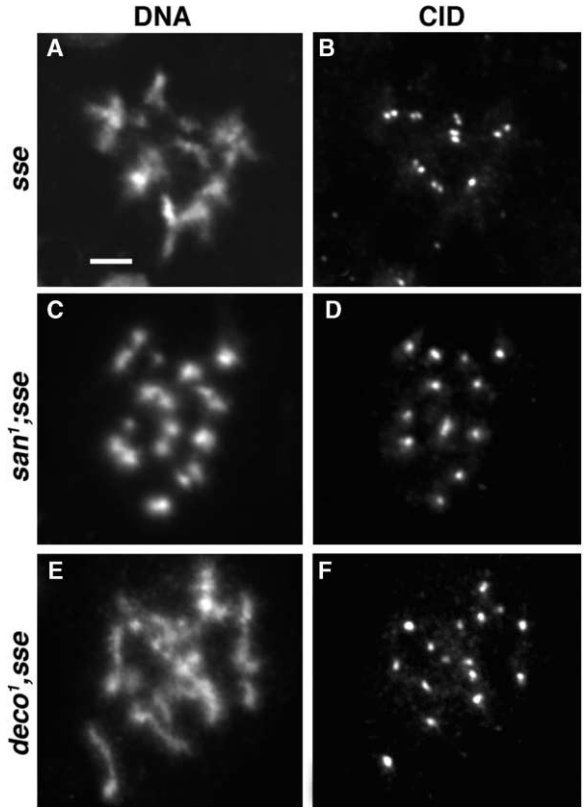

Figure 5. Separase Is Not Necessary for PSCS in *san* and *deco* Mutants

Cells were treated with colchicine and stained to visualize DNA and CID. (A and B) In the *sse* mutant, sister chromatids remained attached and centromeres (CID) were visible as tightly paired dots. (C–F) In *san;sse* and *deco,sse* double mutants, sister chromatids were separated as seen in *san* and *deco* mutations alone (see Figures 1, 3, and 4 above.) In these cases, single (unattached) chromatids are each associated with one spot of CID staining. Bar, 5 μm.

were not present in the control. These proteins, migrating at roughly 25 and 100 kDa on SDS-PAGE (Figure 7B), were analyzed by matrix-assisted laser desorption/ionization mass spectroscopy (MALDI). The 25 kDa band was identified as the product of the gene CG11989, which encodes the fly homolog of *S. cerevisiae* Ard1 (dArd1). The 100 kDa band was identified as the product of the gene CG12202, which encodes the fly homolog of *S. cerevisiae* Nat1 (dNat1). Ard1 is the catalytic subunit of one of the major N-acetyltransferases in *S. cerevisiae*, while Nat1 is an additional subunit present in this same N-acetyltransferase complex [37, 39]. On Western blots, the anti-San antibody did not reveal any cross-reactivity with either of these two proteins (Figure 6A). In the final low pH elution, San itself was eluted, as seen on Western blots and by MALDI analysis (data not shown.)

Further analysis of the MALDI spectra revealed that San is itself likely to be internally acetylated at least at lysine 47, since the peptide containing residues 35–47 differs by +42 Da from the predicted value, a molecular weight difference that can be ascribed to acetylation [40]. We could not determine if the N terminus of San was acetylated because the predicted N-terminal tryptic peptide (three amino acids) was too small for MALDI analysis.

The *deco* Gene Encodes the *Drosophila* Homolog of Eco1p/Ctf7p

The *deco*^{1} mutation was identified in a large-scale screen for ethylmethanesulfonate (EMS)-induced mutations on the *Drosophila* third chromosome that caused aberrations in larval brain mitoses. We looked for such defects in over 1500 mutant stocks preselected by the criterion that they die during late larval or pupal stages. We have identified *deco* as the gene CG8598. The strong *deco*^{1} allele contains a nonsense mutation that shortens the Deco protein products. The weakly hypomorphic *deco*^{2} allele (see Table 1) is caused by a P element insertion into the 5′-UTR of CG8598 (for details, see supplemental data).

Flybase [41] catalogs two alternative transcripts of CG8598. The larger of these transcripts, CG8598-RA, encodes a polypeptide of 1052 amino acids that contains at its C terminus the entire polypeptide of 535 amino acids encoded by the shorter CG8598-RB transcript. Much of the region shared by these two polypeptides is homologous to Eco1p/Ctf7p of *S. cerevisiae* and to *S. pombe* Eso1p and also contains motifs present in acetyltransferases [16]. Specifically, the C-terminal 225 amino acids of both Deco proteins are 24% identical to the entire length of Eco1p, including the conserved zinc finger and acetyltransferase domains. Almost all of these C-terminal amino acids would be missing from the truncated *deco*^{1} gene product. BLAST searches with the remaining regions toward the N termini of the two CG8598 polypeptides detected no additional significant homologies.

The Deco protein sequence itself does not predict with any certainty the type of acetyltransferase activity it would possess, although Ivanov et al. [16] have demonstrated that its homolog Eco1p/Ctf7p in *S. cerevisiae* can acetylate lysines internal to itself and a number of cohesin subunits in vitro.

Discussion

Sister Chromatid Separation in *san* and *deco* Mutants

We have identified two different *Drosophila* genes, *san* and *deco,* whose function is critical for sister chromatid cohesion. The phenotypes associated with mutations in the two genes are nearly identical: sister chromatids are unconnected when condensed chromosomes can first be observed in prophase/prometaphase. We presume that the problems in cohesion begin even earlier, before the chromosomes are visibly condensed. In *S. cerevisiae*, Eco1p function is specifically required during S phase. The loss of cohesion in *eco1* mutants cannot be rescued by expression of Eco1p after replication, and the absence of Eco1p subsequent to S phase does not adversely affect the maintenance of sister chromatid cohesion [13, 14]. Similar conclusions have been drawn regarding Eso1p function in *S. pombe* [15]. Our working model is thus that the San and Deco proteins are both

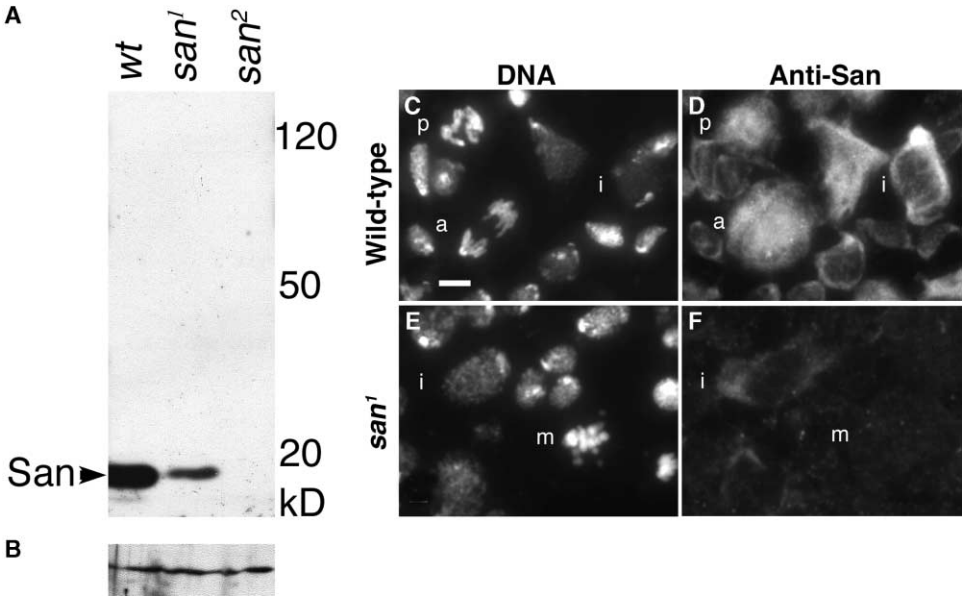

Figure 6. The San Protein

(A) Western blot of larval extracts probed with purified anti-San antibody. The San protein is detected at a size of 18 kDa in wild-type. In *san*^{1}, the levels of San are greatly reduced compared with wild-type. The production of San is abolished in *san*^{2} mutant larvae.

(B) The same blot was stripped and reprobed with anti-Zwilch [22] as a loading control.

(C–F) Intracellular localization of the San protein in larval brains. Wild-type cells (C and D) contain San protein in the cytoplasm in interphase cells (i) and are distributed throughout the cell during mitosis (p, prometaphase; a, anaphase). In *san*^{1} (E-F), levels of San staining are greatly reduced in both interphase (i) and metaphase (m) cells. Bar, 5 μm.

necessary for the establishment of sister chromatid cohesion in S phase. This hypothesis is strongly supported by our finding that sister chromatid separation in *san* and *deco* mutants does not require separase (Figure 5), implying that sister chromatids are never properly connected in the mutants. Our observation that Scc1 localizes normally to the nucleus during interphase in *san* and *deco* mutants (see supplemental data) further suggests that similar to yeast Eco1p, San and Deco are unlikely to be involved in the loading of cohesin onto chromosomes prior to S phase.

Our results indicate that mutations in *san* and *deco* primarily disrupt sister chromatid cohesion at the centromeres but have little or no effect on associations along sister chromatid arms. For example, in Figure 2 it can be seen that the small chromosomes in the mutants are pulled precociously toward the poles, while linkage along the arms still allows the larger chromosomes to congress to a recognizable metaphase plate even though their sister centromeres are separated and drawn toward the poles. Similar kinds of metaphase figures are seen the larval brains of animals carrying certain *polo* alleles [42]. Since Polo is required for dissolution of cohesin from chromosomal arms during prophase [43], such chromosomal configurations may generally represent cases in which sister centromeres are detached while associations along the arms remain. The very small, mostly heterochromatic fourth chromosomes are likely to be deficient for such arm associations. Associations along the arms may also explain why unconnected sister chromatids from the same pair remain closely adjacent to each other in karyotype preparations from *san* and *deco* mutants (Figure 1) in spite of the defects in centromeric sister chromatid cohesion.

What is the molecular basis for the discrimination between the sister chromatid linkages at the centromeres and those along the chromosome arms? Several recent studies indicate that in metazoan organisms, the dissolution of cohesin is regulated differently at these locations. The majority of cohesin is removed from the chromosomes during prophase/prometaphase in the so-called "prophase dissolution pathway" [19]. The remaining pool of cohesin is found mainly, though not exclusively, at the centromeres and is cleaved at anaphase onset by the anaphase-promoting complex/cyclosome (APC/C). We have demonstrated that the cohesin subunit Scc1 is delocalized from the centromere in *san* and *deco* mutants but that trace amounts remain on the arms (Figure 4). Trace amounts of cohesin could be responsible for the associations between chromatid arms in mutant metaphases. If true, this would imply that the San and Deco proteins discriminate, directly or indirectly, between cohesin on the centromeres and the arms. However, Morrison et al. [44] have proposed an alternative model positing that linkages between sister chromatids other than cohesins, such as the intercatenation of DNA by Topoisomerase II, may be important for connections along the arms. In support of this argument, cohesin deficient *Drosophila* and chicken Dt40 cells still exhibit associations between sister chromatids [4, 45].

We do not completely understand how *san* and *deco* mutations influence the distribution of the sister chromatid cohesion protein Mei-S332. The exact function of

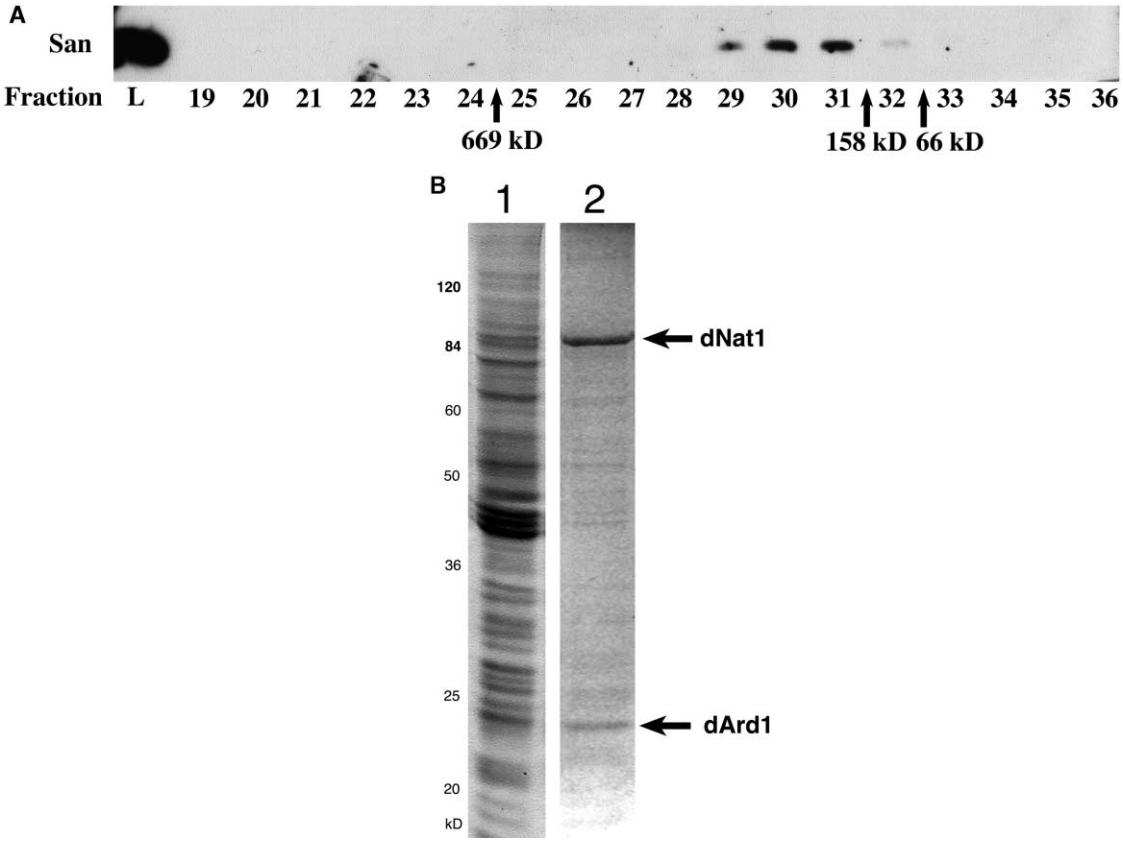

Figure 7. The San Protein Complex

(A) Western blot of total embryo extracts fractionated by Superose 6 gel exclusion sizing column chromatography (fractions 19–36) and an aliquot of the original total extract load (L), both probed with affinity-purified anti-San antibodies. San eluted from the column in peak fractions with an apparent molecular weight of 150–250 kDa. The void volume was at fraction 13, and the salt front in fraction 42. Standards: 669 kDa (thyroglobulin), 66 kDa (bovine serum albumin), and 158 kDa (aldolase).

(B) Coomassie-stained gel lanes containing starting total embryonic extract (lane 1), and the proteins eluting with 1.5 M $MgCl_2$ from an anti-San affinity column (lane 2). The protein bands were analyzed by MALDI (see Experimental Procedures). The 25 kDa band was identified as the product of the gene CG11989, which encodes the fly homolog of *S. cerevisiae* Ard1 (dArd1). The 100 kDa band was identified as the product of the gene CG12202, which encodes the fly homolog of *S. cerevisiae* Nat1 (dNat1).

this protein in promoting cohesion is not known, but it appears that Mei-S332 is required primarily for the maintenance of sister chromatid cohesion until anaphase rather than for the establishment of cohesion during S phase. In support of this idea, Mei-S332 normally associates with centromeres only during the interval between prometaphase and anaphase onset [34]. We found that Mei-S332 is targeted normally to centromeres in *san* and *deco* mutants, even on precociously separated chromatids (Figure 4). Thus, the centromeric binding of Mei-S332 is independent of *san* or *deco* activity. Because cohesin does not accumulate at centromeres in *san* or *deco* mutant prometaphase/metaphase cells, one surprising inference of this finding is that the association of Mei-S332 with centromeres may also be independent of centromeric cohesin. However, since trace amounts of cohesin remain on chromosomes in *san* and *deco* mutants, this possibility should be directly examined in cohesin-deficient cells [45].

The normal mechanisms controlling Mei-S332 delocalization from the centromere at the metaphase/anaphase transition are disrupted in *san* and *deco* mutants. We observed many mutant anaphases in which Mei-S332 remains at the centromeres of all chromosomes and other anaphases in which anti-Mei-S332 only stains the centromeres of lagging chromosomes. The varied behavior of this protein in *san* and *deco* mutant anaphases might be explained if the mutant cells can enter anaphase when cyclin B levels are higher than would allow anaphase to begin in wild-type cells, because of the defects in sister centromere cohesion.

Cell Cycle Progression in *san* and *deco* Mutants

Although chromosome alignment is hindered in *san* and *deco* mutants, most chromosomes congress to a recognizable metaphase plate. The overcondensation of chromatids in metaphase and anaphase figures from most mutant combinations, as well as the absence of anaphases in the null san^2 allele, indicate that cells subsequently become subject to a mitotic delay or arrest. We believe that activation of the spindle checkpoint is responsible for this defect in cell cycle progression; similar mitotic delays observed in cohesin-depleted vertebrate and *Drosophila* tissue culture cells have been

ascribed to defects in kinetochore function that invoke the spindle checkpoint [4, 45, 46]. Our findings that cyclin B and Bub3 levels at kinetochores remain high in *san* and *deco* mutant cells with precociously separated sister chromatids (Figure 3) provide direct evidence that the spindle checkpoint is indeed being maintained. These observations further show that PSCS in *san* and *deco* mutants is not caused by failure of the spindle checkpoint. Mutations in genes encoding spindle checkpoint components such as *bub1, bub3*, *zwilch, rod*, or *zw10* result in cells with separated chromatids that have low, rather than high, levels of cyclin B and of kinetochore-associated Bub3 [22, 30, 31, 47].

Despite the mitotic delay/arrest, some cells in the *deco* mutants as well as in the hypomorphic san^1 mutant were eventually able to enter anaphase. Segregation defects were prevalent in these cells, presumably due to the presence of unattached or missituated chromosomes prior to anaphase onset, and/or asynchrony in the events associated with the metaphase-to-anaphase transition.

San and Deco Are Putative Acetyltransferases

The San and Deco proteins possess sequence motifs that classify them as two distinct types of acetyltransferases. We presume that San and Deco have the acetyltransferase activities predicted by these homologies, but we do not yet have any direct evidence that either protein has such a biochemical function.

The conserved domains in San strongly suggest that it is an N-acetyltransferase. N-acetyltransferases take acetyl groups from acetyl-CoA and attach them to the α-amino group at a target protein's N terminus. This acetylation may occur either on the first methionine or on the first amino acid that remains after cleavage of the initial methionine by methionine aminopeptidase (reviewed in [37, 40]). Polevoda and Sherman [37] have classified the N-acetyltransferases in yeast into three major groups with different substrate specificities: NatA, NatB, and NatC. The Nat5p group, which includes San, is a newly discovered class of N-acetyltransferases whose substrates have not yet been elucidated. Two-dimensional gel analysis using *nat5* mutants failed to reveal detectable acetylation changes in any *S. cerevisiae* proteins [37], suggesting that Nat5p substrates are extremely rare. Unlike mutations in *san*, which are lethal to flies, *S. cerevisiae* with null mutations in *nat5* are viable [37].

Size exclusion chromatography indicates that the 18 kDa San protein exists in a 150–250 kDa complex (Figure 7A). Our immunoaffinity chromatography results (Figure 7B) suggest that this complex may include the *Drosophila* homologs of yeast Nat1p and Ard1p. These two proteins (approximately 100 and 20 kDa, respectively) could account for most of the total mass of the complex. In yeast, Nat1p and Ard1p are tightly associated as the major subunits of the NatA enzyme involved in the N-acetylation of numerous proteins including histone H2 [39]. Ard1p is the catalytic subunit of NatA, but the acetyltransferase activity of NatA also requires the function of Nat1p. Recently, Jeong et al. [48] surprisingly found that Ard1p in mammalian cells has the ability to acetylate internal lysines of hypoxia-inducing factor 1 (HIF-1), indicating that the acetyltransferase activity either of Ard-1p alone or of the NatA holoenzyme may in rare cases not be restricted to the N termini of target proteins.

It is not clear why San would associate with another acetyltransferase. San might associate with certain Nat1-Ard1 complexes to target specific substrates, but this model does not explain why San would itself have an acetyltransferase domain. Alternatively, Nat1 may be a subunit of two separate complexes, one of which uses San as the catalytic subunit and the other of which contains the Ard1 catalytic subunit. In this case, Ard1 may not be a bona fide binding partner of San; instead, Ard1 may have interacted indirectly with the anti-San column through its affinity for the Nat1 bound to San. The association of San with Nat1 or Ard1 may explain why our attempts to demonstrate an acetyltransferase activity with San protein made in baculovirus systems have thus far been unsuccessful (data not shown). Further work is clearly needed to determine the specific acetyltransferase activity of the putative San complex and the role of San within this complex.

Whereas San itself is predicted to be an N-acetyltransferase, Deco is likely to be a protein that acetylates internal lysines, in a fashion similar to the action of histone acetyltransferases. *S. cerevisiae* Eco1p acetylates Scc1, Scc3, Pds5, as well as itself, at internal lysines in vitro [16]. Eco1p failed to acetylate histones in these assays [16]. Though it is tempting to speculate that the requirement for Eco1p or Deco in sister chromatid cohesion reflects the acetylation of cohesin components, this may not be true. In yeast, efforts to demonstrate the acetylation of Scc1 and Scc3 in vivo were unsuccessful, and mutations that altered the targeted lysine in Scc1 were without obvious effect on sister chromatid cohesion [16]. We have shown here that in *Drosophila*, mutations in *deco* (as well as in *san*) result in delocalization of Scc1 from its normal position at the centromere. This might reflect a failure of Deco and/or San to acetylate cohesin, but the effect could instead indirectly represent the lack of acetylation of some other target protein(s).

A tantalizing clue to the function of Deco is provided by the recent finding that Eco1p in yeast is associated with components of three different replication factor C (RFC) complexes [17]. This has suggested a model that Eco1p can "piggyback" on the RFC complexes as they move along the replication forks. In *Drosophila*, *Rfc4* mutants are associated with defective sister chromatid cohesion in some cells that might possibly be ascribed to problems in Deco function [49]. This hypothesis can explain how Eco1p/Deco is coupled to the DNA replication machinery when sister chromatid cohesion is established during S phase, but it does not predict the targets of Eco1p acetyltransferase activity that are located at the replication forks and are involved in cohesion. The RFC proteins themselves are unlikely to be these targets, as investigators have been unable to show that Eco1p acetylates any of the RFC components tested in vitro [17].

In summary, our results not only provide the first evidence that the function of the yeast Eco1p/Ctf7 acetyltransferase in sister chromatid cohesion is evolutionarily

conserved in metazoans, but they also demonstrate that a second putative acetyltransferase (San) is also required for sister chromatid cohesion during mitosis in *Drosophila*. Because both the San and Deco proteins are well conserved in evolution, it is likely that these findings will be generalizable to many eukaryotic species. The association of San with the Ard1 and Nat1 acetyltransferases, as well as the acetylation of the San protein itself, further point to the existence of a network of acetyltransferases needed to keep newly replicated sister chromatids attached until anaphase onset.

Experimental Procedures

Cytological Preparations

Mitosis in larval brains was examined in aceto-orcein stained preparations, and the mitotic index and other mitotic parameters were scored as previously described [22]. Cytological observations were performed on stocks in which *san* or *deco* mutants were balanced over *T(2;3)CyO;TM6b,Tubby* or over *TM6C* marked with *Stubble* (*Sb*) and *Tubby* (*Tb*). The presence of the *Tubby* (*Tb*) marker allowed the selection of Tb^+ larvae homozygous for the mutants of interest. Brains were fixed, squashed, and immunostained according to [50], using mouse anti-tubulin (Amersham Corp., Arlington Heights, IL), rabbit anti-ZW10 [24], rabbit anti-cyclin B (gift of Dr. Christian Lehner, University of Bayreuth, Germany), rabbit anti-centrosomin (gift of Dr. Thomas Kaufman, Indiana University, Bloomington, IN), chicken anti-Bub1 [31], guinea pig anti-Mei-S332 [27], chicken anti-CID [51], rabbit anti-SCC1 [32, 45], and rabbit anti-Bub3 (gift of Dr. Claudio Sunkel, Universidade do Porto, Portugal). Secondary antibodies (all from Jackson Laboratories, West Grove, PA) were TRITC (tetramethyl rhodamine isothiocyanate)-conjugated anti-rabbit IgG, TRITC anti-chicken IgY, Cy2-conjugated anti-rabbit IgG, Cy3-anti guinea pig IgG, TRITC anti-mouse IgG, and TRITC-anti-rat IgG, all at 0.1 mg/ml final concentration and incubated overnight at 4°C. DNA was stained with Hoechst 33248 at a final concentration of 0.5 μg/ml. Samples were examined by epifluoresence, and images were recorded as previously described [22].

Anti-San Antibodies and Immunoaffinity Chromatography

The BsaI-EcoRI fragment from a full-length *san* cDNA was cloned in frame into the expression vector pGEX2. This construct includes amino acids 30–184 (the C-terminal end) of San. GST-San protein was induced with IPTG. The insoluble GST-San protein was excised from gels as described [24] and used for antibody production in rabbits (CBRC, Veterinary College, Cornell University, Ithaca, NY). Antibodies were immunoaffinity purified against the GST-San protein using standard procedures [22].

We have previously described immunoaffinity chromatography and mass spectrometry procedures used to identify proteins interacting with *Drosophila* ZW10 protein [22]. The same protocols were employed here to find San interactors, except that 2 mg of affinity-purified anti-San IgG were crosslinked to 1 ml of Affigel-Protein A beads (Bio-Rad Laboratories; Hercules, CA) to construct the immunoaffinity column. A control column was made by coupling similarly purified preimmune serum from the same rabbit to the column matrix.

Supplemental Data

Supplemental Data including experimental procedures and figures are available at http://www.current-biology.com/cgi/content/full/13/23/2025/DC1/.

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