Effects of sister chromatid cohesion proteins on *cut* gene expression during wing development in *Drosophila*

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Summary

The cohesin protein complex is a conserved structural component of chromosomes. Cohesin binds numerous sites along interphase chromosomes and is essential for sister chromatid cohesion and DNA repair. Here, we test the idea that cohesin also regulates gene expression. This idea arose from the finding that the *Drosophila* Nipped-B protein, a functional homolog of the yeast Scc2 factor that loads cohesin onto chromosomes, facilitates the transcriptional activation of certain genes by enhancers located many kilobases away from their promoters. We find that cohesin binds between a remote wing margin enhancer and the promoter at the *cut* locus in cultured cells, and that reducing the dosage of the Smc1 cohesin subunit increases *cut* expression in the developing wing margin. We also find that *cut* expression is increased by a unique *pds5* gene mutation that reduces the binding of cohesin to chromosomes. On the basis of these results, we posit that cohesin inhibits long-range activation of the *Drosophila cut* gene, and that Nipped-B facilitates activation by regulating cohesin-chromosome binding. Such effects of cohesin on gene expression could be responsible for many of the developmental deficits that occur in Cornelia de Lange syndrome, which is caused by mutations in the human homolog of Nipped-B.

Key words: Eco1/deco, HEAT Repeat, Insulator, Scc3/stromalin, Smc1, Spo76

Introduction

Current evidence indicates that most transcriptional activator proteins recruit the basal transcriptional machinery and increase its binding to a promoter (Ptashne and Gann, 2001). When an activator binds DNA within a kilobase or so of the promoter, looping of the chromatin between the activator and the promoter suffices to support activation. There are several cases in higher organisms, however, in which activators bind to sequences located many kilobases away from the promoters they activate. In these cases, the local concentration of the activator relative to the promoter it activates is not higher than its concentration relative to many other promoters (Rippe, 2001). This suggests that mechanisms other than diffusion-driven chromatin looping support long-range activation. Indeed, several sequences that facilitate specific long-range interactions have been identified in *Drosophila*, mostly in the antennapedia and bithorax homeotic gene complexes (Hendrickson and Sakonju, 1995; Hopmann et al., 1995; Lin et al., 2003; Calhoun et al., 2002; Calhoun and Levine, 2003; Lin et al., 2004; Orihara et al., 1999; Qian et al., 1992; Ronshaugen and Levine, 2004; Zhou and Levine, 1999).

In addition to gene-specific sequences, there are likely to be general factors that act to support long-range activation in many genes (reviewed by Dorsett, 1999). One origin of this idea is that insulator sequences, such as the one in the *Drosophila gypsy* transposon, block diverse enhancers in many genes. Insulators only block when between an enhancer and a promoter, and thus it has been postulated that they interfere with general factors that function between many enhancers and promoters to facilitate enhancer-promoter communication (Dorsett, 1999).

To identify general facilitators of enhancer-promoter communication, genetic screens were conducted to isolate factors that support activation of the *cut* gene by a wing margin-specific enhancer located 85 kbp upstream of the promoter (Morcillo et al., 1996; Morcillo et al., 1997; Rollins et al., 1999). The region between this enhancer and the promoter contains many enhancers that activate *cut* in specific tissues during embryogenesis and larval development (Jack and DeLotto, 1995). In addition to tissue-specific activators that bind to the wing margin enhancer, these screens identified two proteins, Chip and Nipped-B, that are expressed in virtually all cells, and facilitate the expression of diverse genes. Chip interacts with many DNA-binding proteins, and likely supports the cooperative binding of proteins to enhancers and to sites between enhancers and promoters (Morcillo et al., 1997; Torigoi et al., 2000; Gause et al., 2001).

Nipped-B functions by a different mechanism. Unlike other *cut* regulators, Nipped-B is more limiting for *cut* expression when enhancer-promoter communication is partially compromised by a weak *gypsy* insulator than it is when the

enhancer is partially inactivated by a small deletion, leading to the idea that Nipped-B specifically facilitates enhancer-promoter communication (Rollins et al., 1999). Nipped-B homologs in *Saccharomyces cerevisiae*, *S. pombe* and *Xenopus* (Scc2, Mis4 and Xscc2), known collectively as adherins, load the cohesin protein complex onto chromosomes (Ciosk et al., 2000; Tomonaga et al., 2000; Gillespie and Hirano, 2004; Takahashi et al., 2004) (reviewed by Dorsett, 2004). Nipped-B is required for sister chromatid cohesion, and thus is a functional adherin (Rollins et al., 2004). The fact that Nipped-B is an adherin raises the critical question, addressed here, of whether or not cohesin plays a role in enhancer-promoter communication. In all metazoans examined, cohesin loading starts in late anaphase, and it is not removed from the chromosome arms until prophase. Cohesin, therefore, is a structural component of chromosomes during interphase, when gene expression occurs.

Cohesin consists of two Smc proteins, Smc1 and Smc3, and two accessory subunits, Rad21 (Mcd1/Scc1) and Stromalin (Scc3/SA) (Fig. 1) (Chan et al., 2003; Losada et al., 1998; Losada et al., 2000; Sumara et al., 2000; Tomonaga et al., 2000; Toth et al., 1999; Vass et al., 2003). Cohesin forms a ring-like structure (Anderson et al., 2002; Gruber et al., 2003; Haering et al., 2002; Losada et al., 2000; Weitzer et al., 2003). One idea is that adherins, such as Nipped-B, temporarily open the ring and allow it to encircle the chromosome (Arumugam et al., 2003). It is proposed that cohesin encircles both sister chromatids after DNA replication to establish cohesion. Cohesin binds every 10 kbp or so along the chromosome arms in yeast (Blat and Kleckner, 1999; Glynn et al., 2004; Laloraya et al., 2000; Lengronne et al., 2004; Tanaka et al., 1999). If it binds at a similar density in metazoans, it could potentially affect the expression of many genes.

Determining if the effects of Nipped-B on gene expression are mediated through cohesin is pertinent to Cornelia de Lange syndrome (CdLS, OMIM #122470), which is caused by heterozygous loss-of-function mutations in the human homolog of *Nipped-B*, *Nipped-B-Like* (*NIPBL*, GenBank Accession Number NM_133433) (Krantz et al., 2004; Tonkin et al., 2004). CdLS results in numerous birth defects, including slow physical and mental growth, upper limb deformities, gastroesophageal and cardiac abnormalities. These developmental deficits likely reflect changes in gene expression similar to those caused by heterozygous *Nipped-B* mutations.

Here, we examine binding of cohesin to the *cut* gene, and the effects that the Pds5 sister chromatid cohesion factor has on *cut* expression and cohesin binding to chromosomes. Our results are consistent with the idea that cohesin inhibits the activation of *cut* by the wing margin enhancer.

Materials and methods

Effects of mutations on *ct*^{K} expression

Males with lethal mutations were crossed to *cm ct*^{K} females and the *ct*^{K} mutant phenotype was quantified by counting wing margin nicks in at least 30 male progeny (Rollins et al., 2004). Crosses were performed at 25°C, except for those with *pds5* mutants, which were performed at 27°C. Statistical tests were performed using Statview software (SAS Institute). Scott Page and R. Scott Hawley (Stowers Institute, Kansas City, MO) provided the *smc1*^{exc46} mutation. The *san* and *deco* mutations were provided by Byron Williams and Michael Goldberg (Cornell University, Ithaca, NY), and the *Sse* mutations by Stefan Heidmann (University of Beyreuth, Beyreuth, Germany).

Anti-Smc1 and anti-Stromalin antibodies

His$_6$-tagged N-terminal fragments of Smc1 and Stromalin were expressed in bacteria using the pMCSG7 vector (Stols et al., 2002), and purified under denaturing conditions using Qiagen NTA beads. Denatured protein was precipitated using 30% polyethylene glycol, suspended in phosphate-buffered saline (PBS) and then sent to the Pocono Rabbit Farm and Laboratory (Canadensis, PA) for immunization of a rabbit (Smc1) and a guinea pig (Stromalin). Inserts for protein expression were generated by PCR from cDNA clones (Stapleton et al., 2002) (Open Biosystems). Primers for Smc1 were 5′-TACTTCCAATCCAATGCCATGACCGAAGAGGACGACGAT-3′ and 5′-TTATCCACTTCCAATGCTAGATTTTGGCCAGGTCTCG-GGT-3′, which amplify sequences encoding amino acids 1 to 303. The primers for Stromalin were 5′-TACTTCCAATCCAATGCCATGGA-TGATCCGCCGCCGGAC-3′ and 5′-TTATCCACTTCCAATGCTA-CATATTCTCTTTCAATTCGGA-3′, which amplify sequences encoding amino acid residues 1 to 300.

Immunostaining of polytene chromosomes

Salivary glands were fixed for 30 seconds in 2% formaldehyde, then in 45% acetic acid and 2% formaldehyde for 3 minutes (Lis et al., 2000), before storage in 67% glycerol and 33% PBS at –20°C. Anti-stromalin serum was used at 1:100, anti-SMC1 at 1:100, donkey anti-guinea pig Cy3 serum (Jackson ImmunoResearch) at 1:200, and donkey anti-rabbit FITC serum (Sigma) at 1:200. Pre-immune serum was used at the same dilution as the primary antibodies. Primary antibody staining was done for 3 hours at room temperature or overnight at 4°C. Secondary antibody staining was performed for one hour at room temperature. Epifluorescence microscopy was performed with a Nikon microscope equipped with a digital camera and Northern Eclipse software. Micrographs were adjusted using Adobe Photoshop software.

Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed as described by others (Orlando et al., 1997; Schwartz et al., 2005), with modifications. Kc cells were cultured in Schneider's media to 6×10^{6} cells/ml and fixed with 1% formaldehyde for 10 minutes. Fixation was stopped with 0.125 M glycine (pH 7.0). Fixed cells were washed once in 200 ml PBS, once in buffer A [10 mM Hepes (pH 7.9), 10 mM EDTA, 0.5 mM EGTA, 0.25% Triton X-100] and once in buffer B [10 mM Hepes (pH 7.9), 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.01% Triton X-100]. The cells were sonicated in the presence of glass beads (400 to 600 microns) in buffer [10 mM Hepes (pH 7.9), 1 mM EDTA, 0.5 mM EGTA]. Sarkosyl was added to 0.5%, followed by centrifugation in an Eppendorf microfuge at top speed for 10 minutes at 4°C. The supernatant was stored at –80°C.

For immunoprecipitation, a 200 μl chromatin aliquot containing 100 μg of DNA was adjusted to a total volume of 500 μl in immunoprecipitation buffer [10 mM Hepes (pH 7.9), 140 mM NaCl, 1 mM EDTA (pH 8.0), 1% (v/v) Triton X-100, 0.1% (w/v) sodium deoxycholate, 0.2% (w/v) SDS], and pre-cleared by incubation with 30 μl of protein A agarose beads (Pierce) for 3 hours at 4°C, followed by centrifugation to remove the beads. Each aliquot was incubated with 20 μl of the appropriate pre-immune or immune serum overnight at 4°C. Immunocomplexes were bound to 30 μl of protein A beads at 4°C for 4 hours. Beads were collected by centrifugation for 15 seconds at top speed in an Eppendorf microfuge, washed 5 times with 1 ml of immunoprecipitation buffer, once with 1 ml of LiCl buffer [250 mM LiCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5% NP-40, 0.5% sodium deoxycholate], and twice with 1 ml of TE at 4°C. Beads were suspended in 100 μl of TE, and crosslinks reversed by RNase A and proteinase K treatment (Orlando et al., 1997). The

samples were extracted once with phenol-chloroform and twice with chloroform. DNA was precipitated with 0.3 M sodium acetate (pH 5.3) and ethanol, with 30 μg of glycogen carrier. Precipitates were washed with 70% ethanol, dissolved in 200 μl of TE, and stored at –20°C.

The amount of *cut* regulatory region DNA recovered in the immunoprecipitates was determined by PCR using amplicons (see Table S1 in the supplementary material) ranging from 150 to 236 bp in size, spaced at intervals of 1 kbp, starting 0.6 kbp upstream of the wing margin enhancer and extending 2.8 kbp downstream of the transcription start site. PCR was conducted for 30 cycles with 1 μl of the sample as template. PCR products were quantified after gel electrophoresis using an Alpha Innotech FluorChem imager. For each amplicon, the amount of product obtained from the immune serum precipitation was divided by the amount obtained with pre-immune precipitation to calculate enrichment. Most amplicons were amplified two to three times and the amounts averaged.

P element excision alleles of *pds5*

P{EPgy2}CG17509^{EY06473} is an insertion in the first exon of the *Drosophila pds5* homolog. Excisions of P{EPgy2}CG17509^{EY06473} were generated using transposase (Δ2-3) and selecting for flies that lost the P element mini-*white* eye color marker. These were screened by PCR using a P-specific primer and a second primer either 5′ or 3′ to the insertion site. The *pds5*^{e3} excision deleted sequence 5′ to the insertion site, whereas *pds5*^{e6} deleted sequences 3′ to the insertion site.

Neuroblast squashes

Squashes of *pds5* mutant third instar neuroblasts were performed after colchicine treatment (Gatti et al., 1994). Homozygous mutant larvae were selected using mouthpart pigmentation from *y w*; *pds5*^{e3}/CyO, Df(2R)*Kr*$^{-}$ Tp(1;3)*y*$^{+}$ and *y w*; *pds5*^{e6}/CyO, Df(2R)*Kr*$^{-}$ Tp(1;3)*y*$^{+}$ cultures.

Northern blots and 5′ RACE

RNA was isolated using Trizol (Gibco BRL) and northern blots were performed using radioactively labeled single-stranded RNA probes (Dorsett et al., 1989). *pds5* probe vectors were prepared by cloning PCR products of exon 9 from genomic DNA into the *Bam*HI and *Eco*RI sites of pGEM-1 (Promega). The primers were 5′-ATT-AGATCTCGTCTTTTCGGCTCATTTCTTCAC-3′ and 5′-ATTAGA-ATTCGCGGTAGTTTCTCTTGGGCAC-3′.

5′ RACE of *pds5*^{e6} transcripts was performed using BD SMART™ RACE cDNA amplification and the products were cloned into a TOPO TA pCRII vector (Invitrogen). Clones were sequenced by Retrogen (San Diego, CA), and a consensus sequence was generated using CodonCode Aligner software (CodonCode). Random hexamer primers were used for reverse transcription, and the *pds5* gene-specific primer was 5′-CTGAAGACTTGGGTGATTGAGCAGGAAG-3′.

PCR analysis of *pds5* mutations

Genomic DNA was prepared from *pds5*^{e3} and *pds5*^{e6} larvae using squishing buffer [10 mM Tris-HCl (pH 8.2), 1 mM EDTA, 25 mM NaCl and 200 μg/ml Proteinase K] (Gloor et al., 1993), and subjected to PCR using several primer pairs to determine the extent of deletions caused by P excision. Primer sequences can be found in Table S2 in the supplementary material.

Results

Cohesin inhibits *cut* expression

Previous RNAi experiments showed that slightly reducing the Stromalin (Scc3) and Rad21 (Mcd1/Scc1) subunits of cohesin increased expression of the *cut* gene in the developing wing margin (Rollins et al., 2004). To determine if the Smc1 cohesin subunit plays a similar role, we tested a null allele of the *smc1* gene generated by excision of a viable P transposon insertion near the transcription start site (Scott Page, Bhani Singh, and R. Scott Hawley, manuscript in preparation). The *smc1*^{exc46} allele is recessive lethal and chromosome squashes show precocious sister chromatid separation (Scott Page, Bhani Singh, and R. Scott Hawley, personal communication).

The effects of *smc1*^{exc46} on the mutant phenotype displayed by the *ct*^{K} *gypsy* transposon insertion allele of *cut* were used to determine changes in *cut* expression (Fig. 1). The *ct*^{K} *gypsy* insulator partially blocks activation of *cut* by the wing margin enhancer, causing a scalloped wing phenotype (Fig. 1) sensitive to the dosage of factors that regulate *cut* (Gause et al., 2001; Rollins et al., 2004). A decrease in *cut* expression increases nicks in the wing margin, and an increase in expression leads to fewer nicks. The wing-nicking assay is a highly specific and sensitive measure of the activation of *cut* by the wing margin enhancer, in the developing margin cells of the wing discs during the 24-hour period centered around pupariation (Dorsett, 1993; Jack et al., 1991).

In repeated experiments, the heterozygous *smc1*^{exc46} mutation reduced the number of *ct*^{K} wing margin nicks relative to the number observed with the heterozygous parental chromosome (Fig. 1). The difference was significant (P<0.0001). We conclude that, similar to the effects of reducing the Stromalin (Scc3) and Rad21 (Mcd1/Scc1) cohesin subunits, reducing the levels of the Smc1 subunit increases *cut* expression. Because all three cohesin subunits have a similar effect, we conclude that the cohesin complex inhibits *cut* expression.

We also tested mutations in genes that modulate cohesin activity for effects on *cut* expression. The *separation anxiety* (*san*) and *deco* (*eco* – FlyBase) genes encode putative

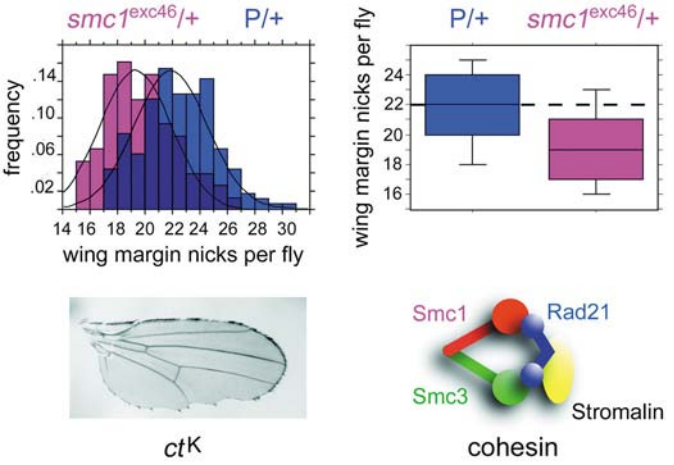

Fig. 1. An *smc1* null mutation increases expression of the *ct*^{K} allele of *cut*. (Lower left) A *ct*^{K} wing with margin nicks. (Lower right) Schematic of the cohesin complex. (Upper left) The distribution of wing nicks in *ct*^{K} males heterozygous for the viable P insertion used to generate *smc1*^{exc46} (blue), and in *ct*^{K} males heterozygous for *smc1*^{exc46} (red). The curved lines show normal distributions calculated from the histograms. (Upper right) Box plots of the wing nick distribution for both genotypes; horizontal lines for each box represent the tenth, twenty-fifth, fiftieth, seventy-fifth and ninetieth percentiles. The difference between the two distributions is significant using the Bonferroni/Dunn test (P<0.0001). The *smc1*^{exc46} mutation gives fewer nicks, indicating greater *cut* expression.

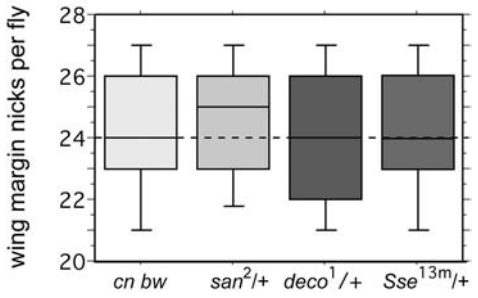

Fig. 2. Mutations in the *san*, *deco* and *Sse* genes do not affect ct^K expression. Shown are box plots of the wing nick distributions obtained for ct^K males heterozygous for the indicated mutations. Parental chromosomes for the san^2, $deco^1$ and Sse^{13m} mutations are not available, so a *cn bw* stock was used as a control. None of the distributions differ from each other in Bonferroni/Dunn tests. Similar results were obtained with san^1 and $deco^2$ mutations (not shown).

acetyltransferase proteins that are required for sister chromatid cohesion and the association of cohesin with centromeric regions (Williams et al., 2003). *Separase* (*Sse*) encodes a protease that cleaves cohesin to permit sister chromatid separation (Jäger et al., 2001). Mutations in these genes did not have significant effects on the ct^K mutant phenotype (Fig. 2). It is possible that heterozygosity for these mutations did not sufficiently alter cohesin activity to change *cut* expression. Alternatively, these proteins may affect cohesin only at the centromere.

Cohesin binds to the *cut* locus

If cohesin directly regulates *cut*, we would expect it to bind to the *cut* locus. We immunostained salivary gland polytene chromosomes with anti-Smc1 and anti-Stromalin (Scc3) to determine whether cohesin binds to *cut*. In wild type, Stromalin and Smc1 co-localize on polytene chromosomes as expected (Fig. 3A). Distinct regions of cohesin staining were seen in both bands and interbands (Fig. 3B). Pre-immune serum for both antisera did not stain above background, and the secondary antibodies did not show cross-species reactivity. We conclude that cohesin binds many sites along all chromosome arms.

We observed staining in some chromosomal puffs, suggesting that cohesin associates with transcribed loci. To test this, we determined whether cohesin binds to heat-shock puffs. After 20 minutes of heat shock at 37°C, cohesin staining was observed in the 93D puff, but not in the others (not shown). Thus, cohesin localization does not correlate with transcription.

The examination of several nuclei showed that the chromosome band containing the *cut* locus (7B3-4) consistently displayed cohesin staining (Fig. 6B). This band contains 150 kbp of DNA, and four genes other than *cut*, in the regulatory region between the wing margin enhancer and the *cut* promoter (Drysdale et al., 2005). At least three of these genes are testis specific (Andrews et al., 2000). Salivary glands do not express *cut*, but because cohesin is a constitutive chromosomal component, and probably binds to *cut* in most cells, these data are consistent with the view that the effects of cohesin on *cut* expression in the wing margin are direct.

Further support is provided by chromatin immunoprecipitation experiments (Fig. 4), which show that cohesin binds to the regulatory region of *cut* in *Drosophila* cultured Kc cells of embryonic origin. We examined an 85-kbp

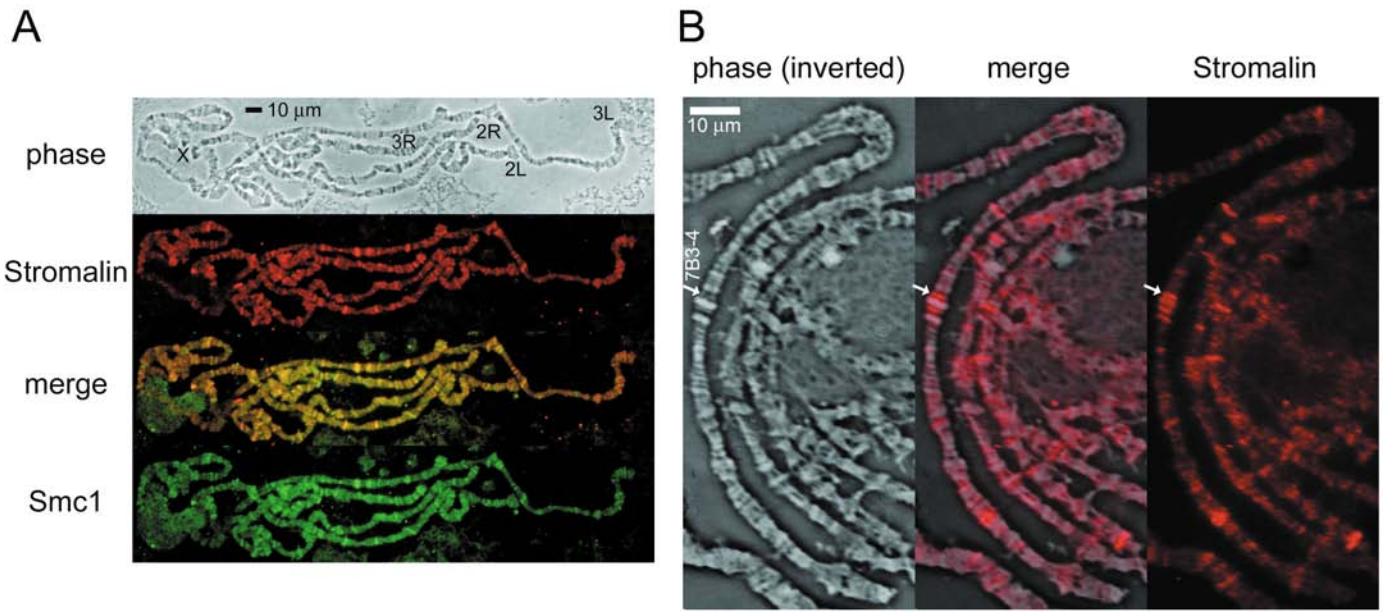

Fig. 3. Cohesin associates with the *cut* locus in salivary gland chromosomes. (A) Wild-type (Oregon R) polytene chromosomes double immunostained for the Smc1 (green) and Stromalin (red) cohesin subunits. The merged image shows that both subunits bind the same sites. Identities of the chromosome arms are indicated in the phase-contrast micrograph. The staining pattern is reproducible in several spreads. Neither pre-immune serum showed staining, and the secondary antibodies did not show cross-species reactivity. (B) Higher magnification of the anti-Stromalin staining (red), showing cohesin association with the 7B3-4 region (arrows) containing *cut*. The phase-contrast micrograph is a photographic negative to make it easier to see cohesin staining in the merge.

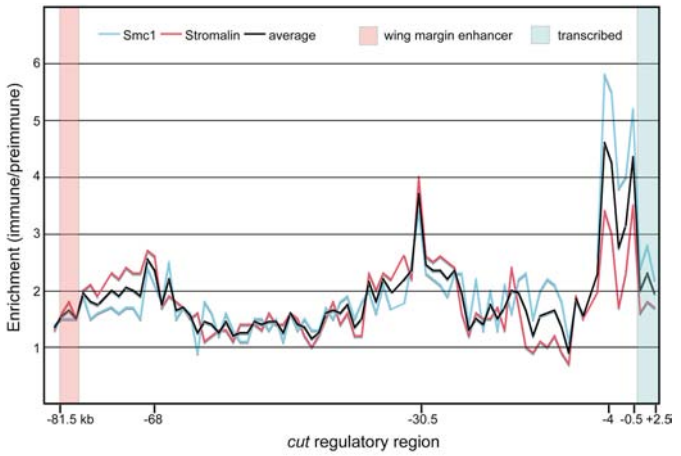

Fig. 4. Cohesin binds multiple sites in the *cut* regulatory region in Kc cells. Chromatin immunoprecipitation was performed with pre-immune and immune serum for Smc1 and Stromalin, and PCR amplicons spaced 1 kbp apart starting 0.6 kbp upstream of the wing margin enhancer (salmon-colored bar) extending into the transcribed region (blue-green bar) 2.8 kbp downstream of the transcription start site. Enrichment of each amplicon is plotted as the ratio of the amount of PCR product obtained with the immune serum relative to the amount obtained with the pre-immune serum. Most points represent the average of two or three measurements. Enrichment by Smc1 immune serum is plotted in blue, enrichment by Stromalin serum in red, and the black line is the average of the Smc1 and Stromalin values. This reveals cohesin-binding sites at 0.5, 4, 30.5, and 68 kbp upstream of the promoter. These peaks are recognized by an increase in the immune to pre-immune ratio relative to the baseline, which as expected, is close to 1.

region encompassing the wing margin enhancer and the promoter, which revealed four cohesin-binding sites. The amounts of *cut* DNA precipitated by the immune and pre-immune sera were determined by PCR at 1 kb intervals, and enrichment of each amplicon was measured by the ratio of the amount of immune PCR product to amount of pre-immune PCR product. As expected, the baseline approached an immune to pre-immune ratio of 1, and peaks of cohesin binding were recognized by increases in the ratio over the baseline. Two binding sites were centered 0.5- and 4-kbp upstream of the promoter, one was centered about 30.5-kbp upstream of the promoter, and another small broad peak was 68-kbp upstream of the promoter. The same sites were seen with both antisera, and neither pre-immune serum showed enrichment of any sequences. Thus, in addition to the non-dividing polytene salivary cells, cohesin also binds *cut* in predominantly diploid dividing cells of embryonic origin. Based on the assumption that cohesin is a constitutive chromosomal component, and the finding that it binds to the *cut* locus in two very different cell types, we posit that it also binds *cut* in the developing wing margin cells and that the effects of cohesin on *cut* expression in the developing wing are direct.

Identification of the *Drosophila pds5* gene

We considered the possibility other factors recruited by cohesin could inhibit *cut* activation. In fungi, the Pds5 (Spo76) protein is required for sister chromatid cohesion (Hartman et al., 2000; Panizza et al., 2000; Tanaka et al., 2001). Pds5 associates with cohesin sites on chromosomes, and requires cohesin for association.

By sequence analysis, the CG17509 gene (Celniker et al., 2002) was identified as the likely *pds5* homolog (Fig. 5B). The P{EPgy2}CG17509^{EY06473} transposon insertion in the first exon is homozygous viable. It was mobilized to generate two recessive lethal mutations, *pds5*^{e3} and *pds5*^{e6}, that fail to complement each other, and a deletion of the region [Df(2R)BSC39]. Both homozygous mutants and the heteroallelic combination are lethal in late third instar to early pupal stages of development. Late third instar larvae of both mutants display small or missing imaginal

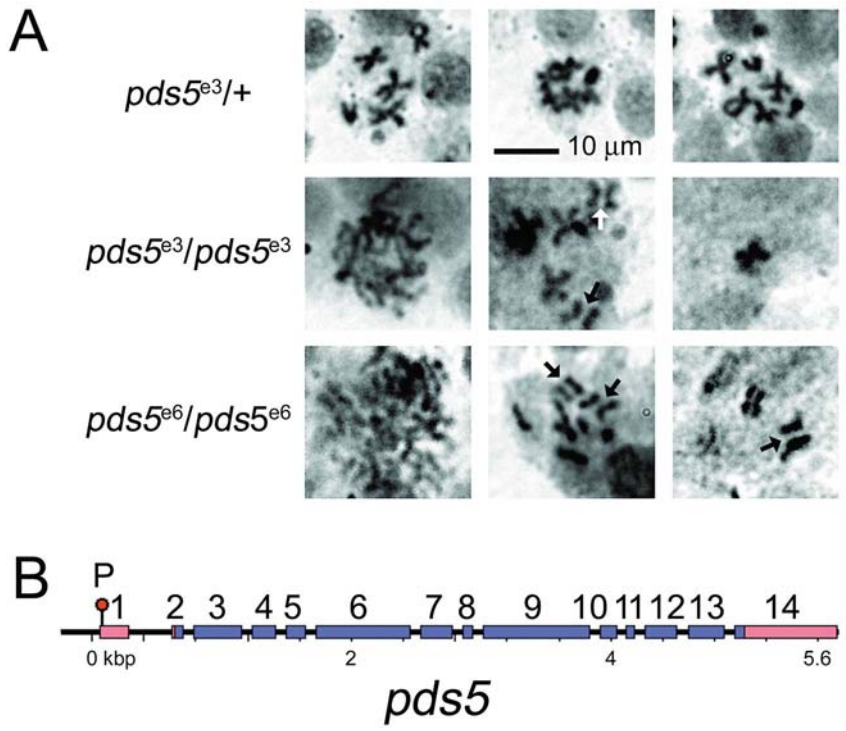

Fig. 5. *Drosophila pds5* mutations cause chromosome segregation and cohesion abnormalities. (A) The panels show sample third instar neuroblast metaphases for the indicated genotypes. For heterozygous *pds5*^{e3}/+ mutants, 117 metaphases were scored: 15.4% showed aneuploidy and 12.8% showed precocious sister chromatid separation (PSCS), similar to wild type (Rollins et al., 2004). Fifty-one metaphases were scored for homozygous *pds5*^{e3} mutants: all showed aneuploidy and 65% displayed PSCS (arrows). Thirty-two homozygous *pds5*^{e6} metaphases were scored: 87.5% showed aneuploidy and 93.8% showed PSCS. (B) Predicted structure of the *pds5* gene (CG17509). The site of the viable P transposon insertion (P{EPgy2}CG17509^{EY06473}) used to generate *pds5*^{e3} and *pds5*^{e6} is shown by the red circle. Exons are numbered boxes, with blue indicating the open reading frame.

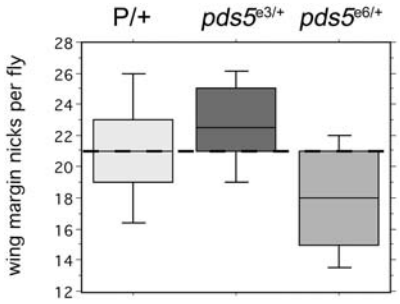

Fig. 6. The *pds5*^{e3} and *pds5*^{e6} mutations have different effects on *ct*^{K} expression. Shown are box plots of the distributions of wing nicks for *ct*^{K} males heterozygous for each *pds5* mutation or the parental P transposon insertion used to generate the *pds5* mutations. By the Bonferroni/Dunn test, the difference between the *pds5*^{e3} and *pds5*^{e6} distributions is significant (P<0.0001), as is the difference between the parental chromosome (P/+) and the *pds5*^{e6} distribution (P=0.0008). The difference between *pds5*^{e3} and the parental chromosome is not significant (P=0.0562).

discs, and the larval brains are approximately half the volume of wild type, consistent with a mitotic defect.

We examined neuroblast metaphase nuclei from mutant third instar larvae for cohesion defects (Fig. 5A). Despite examining more than 30 metaphases from each, we were virtually unable to find a normal metaphase in the *pds5* mutants. Nearly all displayed aneuploidy (Fig. 5A, 100% and 87.5% for *pds5*^{e3} and *pds5*^{e6}, respectively), and most displayed precocious sister chromatid separation (Fig. 5A, 65% and 93.8% for *pds5*^{e3} and *pds5*^{e6}, respectively). By contrast, 15.4% of the *pds5*^{e3}/+ heterozygote metaphases showed aneuploidy and 12.8% showed sister chromatid separation, similar to the frequencies observed with wild-type neuroblasts (Rollins et al., 2004). We conclude that the *pds5*^{e3} and *pds5*^{e6} mutations affect chromosome segregation and sister chromatid cohesion, and that CG17509 encodes a functional Pds5 homolog.

The *pds5*^{e3} and *pds5*^{e6} mutations differ in their effect on *cut* expression

We tested the effect of the *pds5* mutations on the *ct*^{K} phenotype relative to the viable P element insertion used to generate them. Unexpectedly, the two mutations had different effects (Fig. 6). The *pds5*^{e3} mutation slightly increased the number of wing margin nicks, indicating that it decreased *cut* expression, whereas *pds5*^{e6} increased *cut* expression. Although we consistently observed a small increase in wing margin nicks with *pds5*^{e3}, the wing nicking was not significantly different from that seen with the parental chromosome (P=0.0562). The decreased nicking associated with the *pds5*^{e6} allele, however, was significantly different from that of the parental chromosome (P=0.0008), and *pds5*^{e3} (P<0.0001). We conclude that the *pds5*^{e6} mutation dominantly increases *cut* expression, and that the *pds5*^{e3} mutation may cause a small decrease.

pds5^{e6} produces an altered transcript

We examined *pds5* expression in the two *pds5* mutants to determine why they have different effects on *cut* expression. Northern blots revealed a *pds5* transcript of the expected size (4.6 kb, Fig. 7A) in embryos prior to zygotic gene expression (Fig. 7A, lane 1), indicating that it is maternal. The transcript was present at 10- to 25-fold lower levels in larvae. To avoid detecting maternal *pds5* mRNA, we examined the transcripts

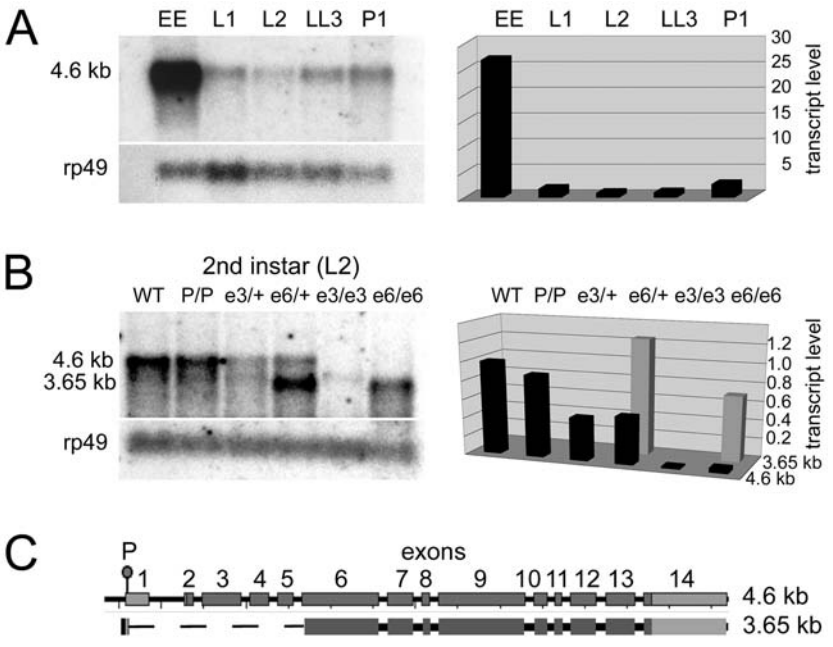

Fig. 7. The *pds5*^{e6} mutant produces a transcript lacking exons 1 through 5. (A) Northern blot of wild-type (Oregon R) total RNA from different developmental stages: EE, early embryo (0 to 30 minutes post egg-laying); L1, first instar larvae; L2, second instar larvae; LL3, late third instar larvae; P1, 0- to 1-day-old pupae. Each lane contained 5 μg of total RNA and the blot was probed sequentially for *pds5* (4.6 kb) and *rp49* transcripts. The bar graph shows phosphorimager quantification of the northern, with *pds5* transcript levels normalized to *rp49* levels, and the amount of *pds5* transcript present in second instar larvae was set to 1 unit. (B) Northern blot of total RNA from the indicated genotypes: WT, *y w*; P/P, homozygotes for the viable insertion (P{EPgy2}CG17509^{EY06473}) used to generate the *pds5* mutations; e3/+, *pds5*^{e3}/+; e6/+, *pds5*^{e6}/+; e3/e3, *pds5*^{e3} homozygotes; e6/e6, *pds5*^{e6} homozygotes. Each lane contained 5 μg of total RNA and the blot was probed for *pds5* exon 9. The bar graph to the right shows phosphorimager quantification with *pds5* transcript levels normalized to *rp49* levels and the amount of 4.6 kb transcript present in wild type (WT) set to 1 unit. Black bars indicate the levels of the 4.6 kb transcript, and gray bars indicate the level of 3.65 kb transcript in heterozygous and homozygous *pds5*^{e6} mutants. (C) Diagram of the *pds5* gene and the *pds5*^{e6} mutant 3.65 kb transcript determined by 5′ RACE. The 3.65 kb transcript begins 67 nucleotides upstream of the *pds5* start site predicted by EST sequences. The 3.65 kb transcript includes the first few nucleotides of exon 1 and 28 nucleotides of P element sequence fused to sequence near the start of *pds5* exon 6. The 5′ RACE sequence is shown in Fig. S1 in the supplementary material.

produced in *pds5*^{e3} and *pds5*^{e6} second instar larvae. We were unable to detect *pds5* transcripts in homozygous *pds5*^{e3} mutants (<2% of wild type), but saw a shorter transcript (3.65 kb) at levels similar to those of wild type in both heterozygous and homozygous *pds5*^{e6} mutants (Fig. 7B). A wild-type sized transcript was present in heterozygous *pds5*^{e6} mutants, but was undetectable in homozygotes. The viable parental P insertion line used to generate both lethal *pds5* alleles produced wild-type levels of a wild-type sized transcript (Fig. 7B).

The northern blots indicate that *pds5*^{e3} is a null allele, and PCR analysis of mutant genomic DNA revealed that sequences starting at the P insertion site and extending upstream of the transcription start site are missing (data not shown). Thus, a reduction in Pds5 dosage slightly decreases *cut* expression. We conclude, therefore, that wild-type Pds5 does not contribute to the inhibition of *cut* expression by cohesin, but may slightly decrease the inhibitory effect.

The presence of a new transcript in the *pds5*^{e6} mutant suggested that it could produce a mutant protein lacking an activity crucial for sister chromatid cohesion that somehow interferes with the inhibition of *cut* by cohesin. PCR analysis of *pds5*^{e6} genomic DNA revealed that the region from the P insertion site through exon 5 is missing. 5′ RACE analysis of the *pds5*^{e6} transcript shows that it starts 67 nucleotides upstream of the wild-type start site predicted by EST analysis (Fig. 7C). The *pds5*^{e6} transcript extends from the start site to the P insertion site. The next 17 nucleotides are from the end of the P insertion, followed by 12 nucleotides of internal P sequence fused to the *pds5* sequence 11 nucleotides downstream of the exon 6 5′ splice site (Fig. 7C). The exon 6 sequences present in the mutant transcript contain six in-frame AUG codons, two of which match a consensus (RNVATGR) for *Drosophila* translation initiation sites (Cavener and Ray, 1991). Thus the *pds5*^{e6} mutant transcript encodes a protein lacking the N terminus.

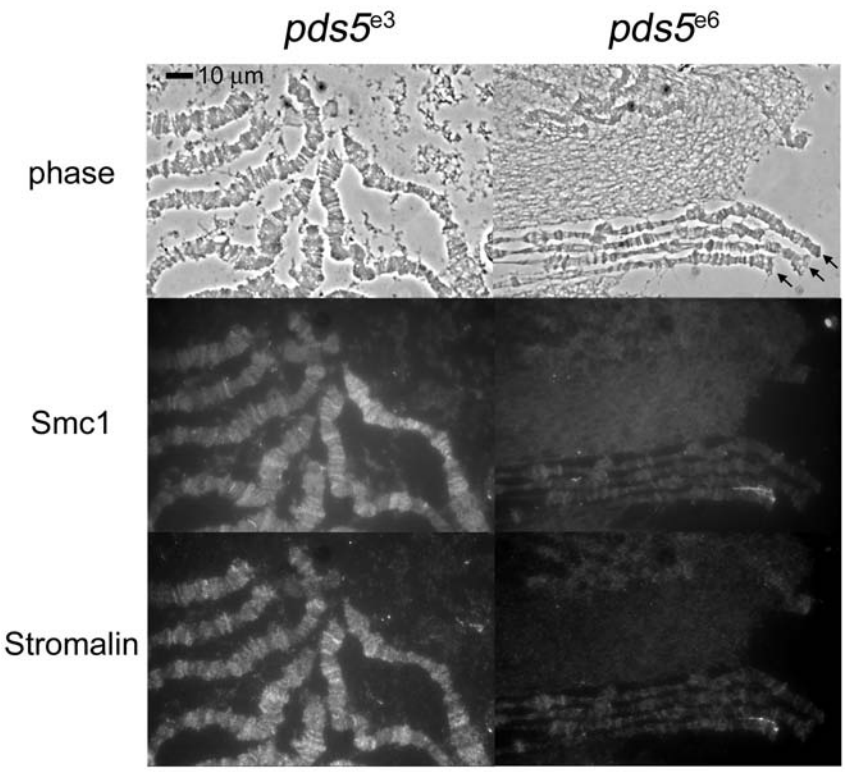

Fig. 8. The *pds5*^{e6} mutation reduces the binding of cohesin to salivary gland chromosomes. The panels show double immunostaining of wild-type and homozygous *pds5* mutant polytene chromosomes for Stromalin and Smc1. Chromosome morphology is altered in both mutants. The *pds5*^{e3} chromosomes are slightly thicker and less extended, and the *pds5*^{e6} chromosomes (arrows) are thinner. Chromosomes from both show banding and developmental puffs, indicating that they are transcribed. Immunostaining of chromosomes from size-matched mutants and wild type was performed at the same time with the same antibody dilution; digital micrographs were taken using identical exposures and were adjusted identically for reproduction. Examination of multiple nuclei from multiple glands showed that the staining intensity of the *pds5*^{e3} mutant is indistinguishable from that of wild type, whereas the *pds5*^{e6} mutants show strongly reduced staining, similar to background. Staining in *pds5*^{e3} mutants appears somewhat less discrete than in wild type.

pds5^{e6} reduces association of cohesin with chromosomes

The effects of the two *pds5* mutations on *cut* expression correlate with a difference in cohesin binding to chromosomes (Fig. 8). Although the salivary glands of the homozygous *pds5* mutants are substantially reduced, we obtained polytene chromosomes from both. Morphology was altered enough to make it difficult to identify specific loci. Nevertheless, individual chromosome tips could be identified, and developmental puffs, including the puff at 2B, were present in both mutants, indicating that the chromosomes are transcribed. In size-matched third instars, the *pds5*^{e3} mutant chromosomes were thicker than wild type, and the *pds5*^{e6} mutant chromosomes were thinner (Fig. 7). The *pds5*^{e3} null allele did not reduce staining for Smc1 or Stromalin, although the pattern appeared less discrete (Fig. 8). By contrast, *pds5*^{e6} mutant chromosomes showed strongly reduced staining for Smc1 and Stromalin (Fig. 8). Loss of cohesin staining was observed in multiple nuclei from multiple *pds5*^{e6} salivary glands. These results indicate that the *pds5*^{e6} mutant either blocks loading of cohesin onto chromosomes, or facilitates removal. The reduction of cohesin binding caused by *pds5*^{e6}, which dominantly increases *cut* expression, is consistent with the hypothesis that cohesin inhibits *cut* expression.

Discussion

Effects of cohesion proteins on *cut* gene activation

Drosophila Nipped-B was discovered in a screen for factors that facilitate activation of the *cut* and *Ultrabithorax* genes by enhancers located many kilobases from their promoters, and is a functional homolog of the yeast Scc2 cohesin-loading factor (Rollins et al., 1999; Rollins et al., 2004). Unexpectedly, small

sub-lethal reductions of Rad21 or Stromalin cohesin subunits by RNAi increased *cut* expression, an effect opposite to the decreases in *cut* expression caused by *Nipped-B* mutations or *Nipped-B* RNAi (Rollins et al., 1999; Rollins et al., 2004). The present data, showing that a mutation in the *smc1* cohesin subunit gene dominantly increases *cut* expression, confirm that the cohesin complex has a negative effect on *cut* expression in the developing wing margin.

The demonstration that cohesin binds to the *cut* locus in polytene chromosomes, and to multiple sites between the remote wing margin enhancer and the promoter in cultured cells, supports the hypothesis that the effects of cohesin on *cut* expression in the wing margin are direct. The wing margin enhancer does not activate *cut* in salivary glands or Kc cells, but it is not technically feasible to examine the association of cohesin with *cut* in the developing margin cells in which the wing margin enhancer functions. Based on the association of cohesin with *cut* in two diverse cell types, we posit that cohesin also binds *cut* in the developing wing margin cells, and inhibits activation by the wing margin enhancer. Such a direct effect of cohesin could explain why small reductions (<20%) in cohesin subunits induced by RNAi have detectable effects on *cut* expression (Rollins et al., 2004).

Both *pds5* mutations tested cause similar sister chromatid cohesion defects, but only *pds5*^{e6} reduces the binding of cohesin to chromosomes and increases *cut* expression. This provides additional evidence that binding of cohesin to chromosomes is required for it to inhibit *cut* activation, and also shows that Pds5 itself is not required to inhibit gene expression.

The negative effects of cohesin on *cut* expression raise the possibility that cohesin contributes to the silencing of euchromatic genes placed in heterochromatin. Cohesin binds more densely in centromeric heterochromatin in yeasts and metazoans, and, in *S. pombe*, heterochromatin proteins recruit cohesin (Bernard et al., 2001; Nonaka et al., 2002; Partridge et al., 2002). Moreover, RNAi-mediated silencing of a non-centromeric gene in *S. pombe* causes the recruitment of heterochromatin proteins and cohesin to the silenced gene (Schramke and Allshire, 2003).

Mechanisms for the effects of cohesin on gene expression

We favor the idea that cohesin inhibits enhancer-promoter communication in *cut*. This idea originates from the allele-specific effects of *Nipped-B* mutations on *cut* expression. The known *cut* regulators required for activation by the wing margin enhancer, including *scalloped*, *vestigial*, *mastermind*, *Chip*, *Nipped-A* and *l(2)41Af*, all display different *cut* allele specificities than *Nipped-B* (Morcillo et al., 1996; Morcillo et al., 1997; Rollins et al., 1999). In contrast to these factors, Nipped-B is most limiting when enhancer-promoter communication is partially compromised by a weak *gypsy* insulator, suggesting that Nipped-B facilitates long-range communication (Rollins et al., 1999).

Binding of cohesin to multiple sites between the wing margin enhancer and the *cut* promoter is consistent with the hypothesis that Nipped-B facilitates enhancer-promoter communication by regulating the binding of cohesin to chromosomes. To explain how Nipped-B aids activation, we theorize that Nipped-B can remove cohesin from chromosomes. In a simple model, Nipped-B facilitates the cohesin-binding equilibrium. When Nipped-B is partially reduced but not abolished, it takes longer to achieve equilibrium, but the extent of cohesin binding is not altered and there is little effect on sister chromatid cohesion. The reduced cohesin on-off rates, however, would diminish the opportunities for gene activation that require cohesin removal or repositioning.

We do not know how cohesin inhibits long-range activation, but we can envision mechanisms for various long-range activation models. For example, cohesin could inhibit the folding or looping of the chromosome that is required to bring the enhancer into contact with the promoter. Alternatively, cohesin could block a Chip-mediated spread of protein binding between the enhancer and promoter in linking models for long-range activation (Dorsett, 1999; Bulger and Groudine, 1999), or block the transfer or tracking of RNA polymerase from the enhancer to the promoter, as appears to occur in the chicken beta-globin gene locus (Zhou and Dean, 2004).

Effects of Pds5 on cohesin function

We found that *Drosophila* Pds5 is required for sister chromatid cohesion, consistent with studies on fungal Pds5 (Hartman et al., 2000; Panizza et al., 2000; Tanaka et al., 2001). To explain the effects of the *pds5*^{e6} mutation on cohesin chromosome binding and *cut* expression, we propose that it produces a mutant protein that blocks cohesin binding, or causes cohesin to be released from chromosomes. This agrees with observations on vertebrate and *S. pombe* Pds5 suggesting that Pds5 has both positive and negative effects on cohesion, possibly by regulating the association of cohesin with chromosomes (Losada et al., 2005; Tanaka et al., 2001).

Vertebrates contain two Pds5 isoforms that associate with chromosomal cohesin (Losada et al., 2005; Rankin et al., 2005; Sumara et al., 2000). Reduction of Pds5 partially decreases sister chromatid cohesion (Losada et al., 2005). Consistent with our finding that the *pds5*^{e3} null mutation does not reduce the binding of cohesin to polytene chromosomes, and with previous work on *S. cerevisiae* and *S. pombe* Pds5 (Hartman et al., 2000; Stead et al., 2003; Tanaka et al., 2001), *Xenopus* Pds5 is not required for the binding of cohesin to chromosomes (Losada et al., 2005). One report suggested that *S. cerevisiae* Pds5 is required for the association of cohesin with chromosomes (Panizza et al., 2000), but it is possible that this discrepancy might be caused by differences in the mutant alleles, similar to the differences we find between *pds5*^{e3} and *pds5*^{e6}.

Depletion of Pds5 from *Xenopus* extracts increases the amount of cohesin associated with chromatin (Losada et al., 2005). A similar increase in cohesin binding could explain the slight decrease in *cut* expression caused by the *pds5*^{e3} null allele. Consistent with the idea that wild-type Pds5 partially reduces cohesin binding, deletion of *S. pombe pds5* partially suppresses a temperature-sensitive mutation in *mis4*, which encodes the homolog of the Nipped-B and Scc2 cohesin-loading factors (Tanaka et al., 2001).

Because wild-type Pds5 appears to partially reduce the binding of cohesin to chromosomes, we speculate that the *pds5*^{e6} mutation increases this activity, which may be related to the cohesin-loading function of Nipped-B/Scc2. Scc2 interacts with cohesin, and is thought to open the cohesin ring

(Arumugam et al., 2003). In synchronized yeast cells, cohesin loads at Scc2-binding sites and translocates away (Lengronne et al., 2004). Like Nipped-B/Scc2, Pds5 contains several HEAT repeats (Neuwald and Hirano, 2000), and thus might also open the cohesin ring during DNA replication to allow it to encompass both sister chromatids. It could play a similar role in the snap model (Milutinovich and Koshland, 2003), in which cohesin complexes bound to the two sisters interlock to hold the sisters together. If the *pds5*^{e6} mutant protein interacts with cohesin non-productively, it could block access to Nipped-B and prevent loading. Alternatively, when the mutant Pds5 attempts to establish cohesion, it might fail, releasing cohesin from the chromosome. Wild-type Pds5 might partially reduce cohesin binding by competing with Nipped-B for cohesin, or by occasionally failing to establish cohesion.

Implications for Cornelia de Lange syndrome

The effects of cohesin on *cut* expression are likely pertinent to the etiology of Cornelia de Lange syndrome (CdLS) (reviewed by Strachan, 2005). CdLS is caused by mutations in the *Nipped-B-Like* (*NIPBL*) human homolog of *Nipped-B* (Krantz et al., 2004; Tonkin et al., 2004). Most missense mutations that cause CdLS affect residues conserved in Nipped-B (Borck et al., 2005; Gillis et al., 2004; Krantz et al., 2004; Miyake et al., 2005; Tonkin et al., 2004). CdLS is characterized by several physical and mental deficits, including slow growth, mental retardation, and upper limb, gastroesophageal and cardiac deformities (de Lange, 1933; Ireland et al., 1993; Jackson et al., 1993). Heterozygous loss-of-function *NIPBL* mutations cause CdLS, and thus the developmental changes likely reflect gene expression effects similar to those caused by heterozygous *Nipped-B* mutations (Rollins et al., 1999). At least some birth defects in CdLS, such as limb truncations and cardiac abnormalities, could be caused by changes in expression of the known homeotic genes. The observations presented here indicate that cohesin likely plays a role in CdLS by inhibiting the long-range gene control of homeotic genes.

The possibility that some developmental changes in CdLS reflect reduced sister chromatid cohesion cannot be ruled out. A recent study found evidence for cohesion deficits in 41% of CdLS patients compared with in 9% of controls (Kaur et al., 2005). Also, the autosomal recessive Roberts syndrome has some similarities to CdLS, and is caused by mutations in a human homolog of the Eco1/Eso1/Deco cohesion factor (Vega et al., 2005). Cells from Roberts patients display defects in sister chromatid cohesion. Homozygous *Drosophila deco*[1] mutants appear to affect cohesin binding only at centromeric regions (Williams et al., 2003), and, as described above, we did not see dominant effects of *deco* mutations on *cut* gene expression, leading us to favor the idea that most CdLS developmental deficits reflect changes in gene expression instead of in sister chromatid cohesion.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/21/4743/DC1

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