Association of cohesin and Nipped-B with transcriptionally active regions of the *Drosophila melanogaster* genome

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Abstract

The cohesin complex is a chromosomal component required for sister chromatid cohesion that is conserved from yeast to man. The similarly conserved Nipped-B protein is needed for cohesin to bind to chromosomes. In higher organisms, Nipped-B and cohesin regulate gene expression and development by unknown mechanisms. Using chromatin immunoprecipitation, we find that Nipped-B and cohesin bind to the same sites throughout the entire non-repetitive *Drosophila* genome. They preferentially bind transcribed regions and overlap with RNA polymerase II. This contrasts sharply with yeast, where cohesin only binds between genes. Differences in cohesin and Nipped-B binding between *Drosophila* cell lines often correlate with differences in gene expression. For example, cohesin and Nipped-B bind the *Abd-B* homeobox gene in cells in which it is transcribed, but not in cells in which it is silenced. They bind to the *Abd-B* transcription unit and downstream regulatory region, and thus could regulate both transcriptional elongation and activation. We posit that transcription facilitates cohesin binding, perhaps by unfolding chromatin, and that Nipped-B then regulates gene expression by controlling cohesin dynamics. These mechanisms are likely involved in the etiology of Cornelia de Lange syndrome, in which mutation of one copy of the *NIPBL* gene encoding the human Nipped-B ortholog causes diverse structural and mental birth defects.

Introduction

Development of higher organisms requires tissue-specific activation and silencing of genes. Tissue-specific regulation is often mediated by sequences located several kilobases away from a gene, and the combined actions of transcriptional activators, silencing proteins, and factors that modify chromatin structure. Studies in *Drosophila* reveal that chromosomal proteins required for sister chromatid cohesion also play critical roles in control of gene expression during development. The *Drosophila* Nipped-B protein was discovered in a screen for factors that facilitate expression of the *cut* homeobox gene in the developing wing margin that is driven by a distant transcriptional enhancer located more than 80 kb upstream of the transcription start site (Rollins et al. 1999). Nipped-B is essential, and homozygous *Nipped-B* mutants die as 2nd instar larvae, while heterozygous *Nipped-B* mutations decrease expression of the *cut* and *Ultrabithorax* (*Ubx*) genes. These two genes, and some unknown developmental processes, are exquisitely sensitive to Nipped-B dosage: heterozygous *Nipped-B* null mutations reduce *Nipped-B* mRNA levels by only 25%, and a 50% reduction induced by RNAi is lethal (Rollins et al. 2004).

Homozygous *Nipped-B* mutants show sister chromatid cohesion defects as the maternally-provided Nipped-B wanes just prior to death (Rollins et al. 2004). Studies on Nipped-B orthologs in other organisms indicate that these defects result from a failure of the cohesin protein complex that mediates cohesion to bind to chromosomes (Arumugam et al. 2003; Ciosk et al. 2000; Gillespie and Hirano 2004; Seitan et al. 2006; Takahashi et al. 2004; Tomonaga et al. 2000; Watrin et al. 2006).

Cohesin binds to chromosomes throughout interphase when gene expression occurs. It contains four subunits, Smc1, Smc3, Rad21 and Stromalin (SA), which form a ring-like structure

(reviewed in Hirano 2006; Huang et al. 2005; Losada 2007; Nasmyth and Haering 2005). In most organisms, cohesin is loaded along chromosomes during telophase, and is removed from the arms at the subsequent prophase. A leading idea is that cohesin mediates cohesion by encircling both sister chromatids, although other mechanisms may also occur.

Heterozygous *Nipped-B* mutants do not show cohesion defects, indicating that their effects on gene expression are unlikely to be caused by a significant reduction in binding of cohesin to chromosomes. Changes in cohesin dosage, however, also affect *cut* expression, suggesting that Nipped-B's role in gene expression involves its ability to regulate cohesin binding. Although Nipped-B and cohesin are both needed for sister chromatid cohesion, they have opposite effects on *cut* expression. Reducing cohesin dosage increases *cut* expression in the developing wing margin, while reducing Nipped-B decreases expression (Rollins et al. 1999; Rollins et al. 2004; Dorsett et al. 2005). This gave rise to the idea that cohesin binds to *cut* and inhibits expression, possibly by interfering with enhancer-promoter communication, and that Nipped-B maintains a dynamic cohesin binding equilibrium to alleviate these effects (Dorsett 2004). Consistent with this idea, cohesin binds directly to *cut* regulatory sequences in cultured cells and to the *cut* locus in salivary gland chromosomes (Dorsett et al. 2005).

The *Drosophila* data suggest that birth defects associated with Cornelia de Lange syndrome (CdLS) stem from effects on gene expression. CdLS is caused by heterozygous loss-of-function mutations in the *Nipped-B-Like* (*NIPBL*) ortholog of Nipped-B, and in a few cases, by viable missense mutations in the *Smc1A* or *Smc3* cohesin subunit genes (Deardorff et al. 2007; Krantz et al. 2004; Musio et al. 2006; Tonkin et al. 2004). CdLS patients display slow growth, mental retardation, and defects in limbs and organs (Dorsett 2007; Jackson et al. 1993; Strachan et al. 2005). Most do not show cohesion defects (Kaur et al. 2005; Vrouwe et al. 2007), suggesting that

the diverse developmental deficits are caused by gene expression changes similar to those in *Drosophila*. The similar effects of reduced NIPBL activity and cohesin subunit missense mutations on human development in the absence of obvious effects on sister chromatid cohesion further suggest that Nipped-B/NIPBL are likely to dynamically regulate cohesin.

Another potential link between the effects of sister chromatid cohesion factors on development and effects on gene expression is provided by the finding that mice homozygous for a knockout of the *Pds5B* gene show developmental deficits reminiscent of some that occur in CdLS patients (Zhang et al. 2007). The Pds5 protein, which is also conserved from fungi to man, interacts with cohesin and plays roles in establishment and/or maintenance of sister chromatid cohesion (Dorsett et al. 2005; Hartman et al. 2000; Losada et al. 2005; Panizza et al. 2000; Stead et al. 2003; Sumara et al. 2000; Tanaka et al. 2001). In mammals, there are two Pds5 proteins, and the mice lacking Pds5B with developmental abnormalities do not have cohesion defects. In *Drosophila*, there is a single *pds5* gene, and heterozygous *pds5* mutations alter *cut* gene expression without the effects on cohesion seen in homozygous mutants (Dorsett et al. 2005), suggesting that changes in gene expression also likely underlie the effects of Pds5B on mouse development.

The binding of cohesin and the Scc2 ortholog of Nipped-B have been mapped genome-wide in *S. cerevisiae* (Glynn et al. 2004; Lengronne et al. 2004). Cohesin binds almost exclusively between genes in yeast, and most binding sites are between convergent transcription units. Coupled with the finding that Scc2 does not co-localize with cohesin, this led to the idea that cohesin loads onto chromosomes at Scc2 binding sites, and then is pushed to the ends of genes by RNA polymerase (Glynn et al. 2004; Lengronne et al. 2004).

The intergenic localization of cohesin in yeast, where it rarely overlaps regulatory sequences, and the lack of co-localization with Scc2, which is inconsistent with dynamic control by Scc2, are incompatible with the models for how Nipped-B/NIPBL and cohesin regulate *Drosophila* gene expression and human development. The yeast genome, however, is much more compact than that of higher eukaryotes, with smaller intergenic regions, few introns, and rare occurrence of long-range regulation. Thus the mechanisms that determine the location of cohesin binding sites are likely to differ in higher organisms. We mapped the Nipped-B and cohesin binding sites in the entire non-repetitive *Drosophila* genome to gain insights into how they interact with genes and how they might regulate gene expression. Strikingly, we find that in contrast their orthologs in yeast, Nipped-B and cohesin co-localize, and bind preferentially, but not exclusively to active transcription units.

Materials and methods

Chromatin immunoprecipitation was performed as described before (Dorsett et al. 2005; Schwartz et al. 2006). Nipped-B, SA and Smc1 antisera are described elsewhere (Dorsett et al. 2005; M. Gause, H.A. Webber, Z. Misulovin, G. Haller, J.C. Eissenberg, S.E. Bickel, and D. Dorsett, submitted for publication). RNA polymerase II (PolII) antibody was purchased from Babco (MMS-126R). For controls, we precipitated with Smc1 preimmune serum or rabbit IgG, or used input chromatin. Hybridization of probes prepared from the immune precipitated or input chromatin to tiled microarrays (Affymetrix no. 511262) was performed according to the manufacturer's directions. At least two independent precipitations using different chromatin

preparations were used to probe separate microarrays for each protein. All experiments used at least two control hybridizations.

Trimmed mean $\log_2$ IP/control ratios for microarray features were calculated from the IP and control hybridization intensities using sliding 675 bp windows with the TiMAT programs (http://bdtnp.lbl.gov/TiMAT/TiMAT2/index.html). TiMAT was also used to predict binding peaks and regions at 1% and 25% false discovery rates. Data was viewed with the Affymetrix browser (www.affymetrix.com/support/developer/tools/download_igb.affx), and the April 2004 *Drosophila* annotated genome. The R statistical environment (R Foundation for Statistical Computing, Vienna, 2007; ISBN 3-900051-07-0; www.R-project.org) was used to calculate correlation coefficients, plot $\log_2$ IP/control values for microarray features, and identify genes that differentially bind PolII, cohesin and Nipped-B.

Results

Nipped-B and cohesin co-localize genome-wide

We mapped binding sites for Nipped-B, and the Smc1 and SA cohesin subunits in the entire non-repetitive genome of *Drosophila* using chromatin immunoprecipitation and hybridization of the precipitated DNA to tiled microarrays (ChIP-chip), as described previously for Polycomb group (PcG) proteins (Schwartz et al. 2006). We used cultured cells instead of whole organisms, because cell lines should have less binding site heterogeneity. Three lines were used to look for differences in cohesin binding patterns. Two lines, the Sg4 subline (Schwartz et al. 2006) of

Schneider line 2 and the Kc167 subline of Kc cells (Echalier and Ohanessian 1970) are embryonic in origin, and the ML-DmBG3 line (BG3; Ui et al. 1994) is derived from 3rd instar central nervous system. The Affymetrix tiled microarray contains some $3x10^6$ 25 nt oligonucleotide features every 35 bp or so. Sliding windows of 675 bp were used to generate trimmed mean $\log_2$ IP/control ratios for the features, and statistical algorithms were used to predict binding peaks and regions at 1% and 25% false discovery rates.

We examined binding of Nipped-B, Smc1, and SA in Sg4 cells, Nipped-B and Smc1 in BG3 cells, and Smc1 in Kc cells. Cohesin and Nipped-B bind throughout the genome, and by visual inspection, their patterns are nearly identical. Figure 1 shows a 2 Mb region of chromosome 3L that illustrates several typical features. The Nipped-B, SA and Smc1 binding patterns in Sg4 cells are very similar, as are the Nipped-B and Smc1 patterns in BG3 cells. Cohesin binds to large regions that range in size from a few kb to more than 60 kb in length. There are also very long regions that show little cohesin binding. Near the middle of region of chromosome 3L shown in Figure 1 there are two long cohesin-free regions, each some 200 kb in size, separated by a small cohesin peak.

The peaks of Nipped-B and cohesin subunit binding sites predicted with a 1% false discovery rate are marked by vertical lines for Sg4 cells in Figure 1. The predicted peaks are very similar for Nipped-B, SA and Smc1, providing evidence for co-localization of Nipped-B and cohesin. Co-localization is also indicated by other analysis methods. Comparing the trimmed mean $\log_2$ IP/control values, the genome-wide correlation coefficient for Nipped-B and SA binding in Sg4 cells is 0.88, the Nipped-B-Smc1 correlation is 0.75, and the SA-Smc1 correlation is 0.71 (Table 1). In BG3 cells, the Nipped-B-Smc1 correlation is 0.92. To control for the possibility that systematic low-level signals might inflate the correlation, we compared Nipped-B in Sg4 cells to

the randomly-chosen Knirps protein binding in embryos measured using the same method (X.Y.L, M.D.B, unpublished). This gives a correlation of 0.11, indicating that systematic low-level signals do not make a significant contribution (Table 1).

Plots of the Nipped-B *vs.* SA or Smc1 trimmed mean $\log_2$ values further illustrate the binding correlation, with individual array features showing similar enrichment for Nipped-B and cohesin. Figure 2 shows these plots for the first 4.25 Mb of chromosome 2L in Sg4 and BG3 cells. Plots of other regions for both Sg4 and BG3 cells are very similar. A few Smc1 sites do not correlate with SA or Nipped-B (Fig. 2). We do not know if these are authentic, or sites for a protein that cross-reacts with the Smc1 antibodies. However, the high correlation between Nipped-B and cohesin, and between cohesin subunits indicates that the vast majority of sites are authentic, and that Nipped-B and cohesin bind the same sites.

The genome-wide correlation coefficients and plots confirm that although the similarities are predominant, there are also significant differences in cohesin binding between Sg4, Kc and BG3 cells. The genome-wide correlation for Smc1 binding between Sg4 and Kc cells is 0.58, and although plots reveal similar enrichment for many microarray features between the two cells, and there are also features that differ in binding (Fig. 3; Table 1). Similar correlation coefficients and plots are obtained when Nipped-B binding is compared between Sg4 and BG3 cells, or when Smc1 binding is compared between Sg4 and BG3 cells and between BG3 and Kc cells, indicting that there are similar differences in cohesin binding between all three cells lines (Table 1; Fig. 3). The correlation for Smc1 binding between BG3 and Kc cells is 0.55, and is 0.56 for Nipped-B between Sg4 and BG3 cells. As described below, many of these differences occur within genes. An example of one such difference between Sg4 and BG3 cells is shown in Supplemental Figure 1, and several differences are catalogued in Supplemental Table 1.

Nipped-B and cohesin bind transcribed regions

Nipped-B and cohesin localization in *Drosophila* contrasts sharply with that in *S. cerevisiae*. Cohesin binds every 10 kb or so in yeast, and the peaks are generally less than a few kb in width. The yeast Scc2 ortholog of Nipped-B binds different sites than cohesin, and almost all cohesin binds between genes (Glynn et al. 2004; Lengronne et al. 2004). As shown above, however, *Drosophila* Nipped-B co-localizes with cohesin, and there are large cohesin-binding and cohesin-free regions that extend for several kilobases.

Another key difference is that in contrast to the completely intergenic localization of cohesin in yeast, cohesin binds to many transcription units in *Drosophila*. We looked closely at the *cut* gene because it is regulated by Nipped-B and cohesin *in vivo*. The Smc1 binding between the wing margin enhancer and the *cut* transcription start site in Kc cells by ChIP-chip is virtually identical to that previously mapped by conventional ChIP, with relatively narrow peaks a few kilobases wide located 0.5 and 4 kb upstream of the transcription start site, and additional distal peaks between the wing margin enhancer and promoter (Fig. 4; Dorsett et al. 2005). The binding is very similar, but not identical in Sg4 cells. In both Kc and Sg4 cells, there are also multiple peaks of cohesin binding in the *cut* transcription unit. Cohesin also binds *cut* in BG3 cells, but in both the upstream regulatory region and transcription unit, the binding is more extensive, such that a 180 kb region starting upstream of the distal wing margin enhancer extending to the 3' end of the transcription unit is bound by cohesin and Nipped-B (Fig. 4). In contrast, the 25 kb wide cohesin binding region located 10 kb downstream of *cut*, like most sites in the genome, is very similar in all three cell lines, indicating that the increased cohesin and Nipped-B binding to *cut* in BG3 cells is authentic.

cut is one of 369 genes in the entire non-repetitive genome that bind cohesin within the transcription unit in all three cell lines (Supplementary Table 1). This group includes the *Act5C* actin gene, indicating that high transcription does not prevent cohesin binding (Supplementary Table 1; Supplementary Fig. 1). Thus, it is unlikely that in *Drosophila*, as proposed to explain cohesin localization in yeast (Glynn et al. 2004; Lengronne et al. 2004), that RNA polymerase pushes cohesin to the ends of genes.

Binding of Nipped-B and cohesin to transcription units prompted us to compare their localization relative to RNA polymerase II (PolII). Genome-wide, the Nipped-B *vs*. PolII correlation is 0.62 in Sg4 cells and 0.51 in BG3 cells (Table 1). The antibody we used detects PolII with a hypophosphorylated C terminal domain, which generally localizes at promoters, while Nipped-B usually binds extended regions. Plots reveal extensive overlap in PolII and Nipped-B in both Sg4 and BG3 cells, but less direct correlation at individual features than between cohesin and Nipped-B (Fig. 5). Many features have low Nipped-B values and high PolII values and *vice versa*. The overlap with PolII in both cell lines, however, indicates that Nipped-B and cohesin bind many transcriptionally-active regions.

Indeed, detailed analysis shows that cohesin preferentially binds to active genes. We defined active genes as those that bind PolII at the transcription start site, and also have the histone H3 lysine 4 trimethylation (H3K4Me3) transcriptional elongation mark (reviewed by Shilatifard 2006; Y.B.S., T.G.K., V.P., unpublished) close to the promoter. Using 2-fold enrichment or greater for both as the criteria, there are 5,954 active genes in Sg4 cells. 423 of these overlap SA-binding regions with a 2-fold enrichment or greater. In contrast, 9,711 genes lack both PolII and H3K4Me3, and only 32 of these overlap SA-binding regions. Thus, by these criteria, active genes are more than 20-fold more likely to bind cohesin than are silent genes.

Genes are also more likely to have PolII downstream of the promoter when they bind cohesin. Thirty-five genes in the non-repetitive genome bind Nipped-B and cohesin in their transcribed regions in Sg4 but not in BG3 cells, and 80 bind cohesin in BG3 and not in Sg4 cells (Supplementary Table 1; see Supplementary Fig. 1 for an example). 33 of the 35 genes that bind cohesin in Sg4 and not in BG3 cells bind PolII in Sg4 cells, and PolII is also present more than a kilobase downstream of the promoter in 26 (74%) of these. In contrast, while 16 of these genes bind PolII in BG3 cells, only two (6%) have PolII downstream of the transcription start site. Similar results are seen for the 80 genes that bind cohesin in BG3 and not in Sg4 cells: 45 out of 80 (56%) have downstream PolII in BG3 cells, and only 2 (3%) have downstream PolII in Sg4 cells (Supplementary Table 1). Thus cohesin is more likely to bind a gene when it is actively transcribed.

As expected from the above analysis, Nipped-B and cohesin bind less to intergenic sequences than to genes, and within genes, they usually bind 5' UTRs and introns. These trends were quantified by determining the fraction of the Nipped-B and SA peaks predicted with a 25% false discovery rate for Sg4 cells that occur in intergenic sequences, introns, exons, and 5' and 3' UTRs (Supplementary Fig. 2). The results were normalized to the percent of the genome that consists of these features to calculate the binding preferences (Table 2). For example, 40 to 50% of Nipped-B and SA cohesin peaks occur in introns, which comprise about a third of the non-repetitive genome (Supplementary Fig. 2). Thus SA and Nipped-B bind slightly more to introns than expected if they bound at random, and the calculated preferences are 1.45 and 1.33, respectively (Table 2). About a third of Nipped-B and SA peaks occur in intergenic sequences and thus bind slightly less than expected on a random basis with preferences of 0.81 and 0.74 (Table 2; Supplementary Fig. 2). They occur 6 to 8-fold more than expected on a random basis in

5’ UTRs, and much less than expected in coding sequences (preference ratios of 0.27 and 0.18) and 3’ UTRs (ratios of 0.3 and 0.2). The preference for 5’ UTRs correlates with frequent occurrence of cohesin and PolII peaks at transcription start sites (see Fig. 4 and Supplementary Fig. 1 for examples), but the reason for the low preference of cohesin for coding sequences is unknown. It is also unknown what portions of the intergenic regions bound by cohesin might be transcribed, or are regulatory sequences, such as those upstream of *cut* (Fig. 4).

Nipped-B and cohesin binding correlates with *Abd-B* expression

Nipped-B facilitates expression of the *Ubx* gene of the bithorax complex (BX-C) *in vivo* (Rollins et al. 1999). Thus we closely examined binding of Nipped-B and cohesin to the BX-C, which also contains *abd-A* and *Abd-B*. In Sg4 cells, *Abd-B* is expressed, but PcG proteins silence *Ubx* and *abd-A* (Schwartz et al. 2006). Nipped-B, cohesin and PolII bind the transcribed *Abd-B* gene, but not the silent *Ubx* or *abd-A* genes, with the exception of Smc1-only sites near *abd-A* (Fig. 6). PolII, cohesin and Nipped-B bind the same 75 kb region starting near the upstream *Abd-B* promoter, extending past the 3’ end of *Abd-B* through the *iab-7* enhancer region, ending at the Fab-7 boundary (Fig. 6). This region is flanked on both sides by histone H3 lysine 27 trimethylation (H3K27Me3) domains associated with PcG silencing (Fig. 6; Kahn et al. 2006; Schwartz et al. 2006). The lack of cohesin binding to the silent *Ubx* and *abd-A* genes suggested that cohesin might bind *Abd-B* only when it is expressed. Indeed, cohesin does not bind *Abd-B* in Kc or BG3 cells, in which *Abd-B* is silent (Fig. 6).

Cohesin is also not found in other silenced regions, such as the entire Antennapedia complex in Sg4, BG3 and Kc cells (not shown). Like the silenced region of the BX-C, H3K27Me3 also

coats the Antennapedia complex in Sg4 cells (Kahn et al. 2006; Schwartz et al. 2006). The *cut* locus is also a Polycomb group (PcG) target in Sg4 cells (Schwartz et al. 2006; Schwartz and Pirrotta 2007), although this does not prevent binding of PolII to the *cut* promoter (Fig. 4). It is possible that the expansion of Nipped-B and cohesin binding to *cut* in BG3 cells relative to Sg4 cells may reflect loss of of PcG silencing of *cut* in BG3 cells, even though this does not result in increased PolII binding (Fig. 4). Significantly, Nipped-B binding anti-correlates with H3K27Me3 in Sg4 cells, with a genome-wide correlation coefficient of -0.30 (Table 1). This negative correlation is also illustrated by a plot of the trimmed mean log2 IP/control values for the microarray features, which reveals very little or no overlap in Nipped-B binding and H3K27Me3 (Fig. 5).

Discussion

Potential effects of cohesin and Nipped-B on gene expression

Based on effects of Nipped-B and cohesin on *cut* expression *in vivo*, it was originally proposed that cohesin binding to the *cut* regulatory region hinders enhancer-promoter interactions, and that Nipped-B alleviates this effect by dynamic control of cohesin binding (Dorsett 2004). The results reported here expand this model, by showing that transcription facilitates cohesin binding, and that in many genes, cohesin binds to the transcription unit where it can interfere with transcriptional elongation. The results also show that Nipped-B co-localizes with cohesin, consistent with the proposition that it maintains dynamic control of cohesin binding. As a general model, we envision that transcription facilitates cohesin binding, and that the cohesin that binds

then affects subsequent transcription. Nipped-B regulates these effects on transcription by dynamic control of cohesin binding or subunit interactions.

Features of the cohesin binding to the active *Abd-B* gene in Sg4 cells suggest that in some cases, cohesin could interfere with both transcriptional elongation and activation. Some cohesin and PolII peaks coincide in both the *Abd-B* transcription unit and 3' regulatory region, which contains intergenic transcription units likely involved in *Abd-B* regulation (Bae et al. 2002; Drewell et al. 2002). The cohesin in the regulatory region could hinder *Abd-B* activation by affecting this intergenic transcription. For instance, in the human *β-globin* gene, blocking intergenic transcription between the enhancer and promoter by insertion of a transcription terminator or an insulator reduces activation (Ling et al. 2004; Zhao and Dean 2004). Genes with distant regulatory elements, such as *cut* and *Ubx*, may be more sensitive to Nipped-B dosage because of combined effects on activation and elongation.

Cohesin might also have positive effects on gene expression in some cases. Although it is unknown if the effect is direct, reduction of Rad21 dosage decreases *runx* gene expression during early zebrafish development (Horsfield et al. 2007). The findings presented here do not provide an obvious explanation for how cohesin could directly facilitate gene expression, except the possibility that it might help maintain the chromatin in an unfolded state that is more conducive to transcription. Another possibility is that in specific cases, cohesin might contribute to chromatin boundary function to block the spread of silencing factors as it does at the *HMR* silent locus in yeast (Donze et al. 1999). There is a cohesin/Nipped-B peak at the known Fab-7 boundary element flanking the active *Abd-B* domain in Sg4 cells, and thus we cannot rule out the possibility that cohesin plays a role in defining chromatin domains conducive to gene expression.

Effects of transcription on cohesin binding

The data indicate that cohesin and Nipped-B bind preferentially, but not exclusively, to active genes. We speculate that transcription facilitates cohesin binding by unfolding chromatin to a 10 nm fiber that can fit into the 35 nm internal diameter of the cohesin ring (Anderson et al. 2002). Based on the anti-correlation with histone H3 lysine 27 trimethylation, it also appears likely that silencing, either by preventing transcription, or through an independent effect on chromatin structure, inhibits cohesin binding.

Transcription is neither necessary nor sufficient for cohesin binding, because some poorly-expressed genes such as *cut* bind cohesin, and some active genes, such as *SA*, do not. In the case of *cut*, PolII binds primarily at the promoter in both Sg4 and BG3 cells. There is little downstream polymerase in the *cut* transcription unit either cell type, yet there is substantially more cohesin binding to the transcription in BG3 cells. Thus there must be additional factors besides transcription that regulate cohesin binding.

Implications for Cornelia de Lange syndrome

Association of cohesin and Nipped-B with many genes suggests that the diversity of CdLS phenotypes stems from effects on multiple genes. Many of the genes bound by cohesin in *Drosophila* cells encode evolutionarily-conserved transcription factors and receptors that control limb, organ, peripheral and central nervous system development (see Supplementary Table 1). These include the genes encoding the Notch receptor, its Serrate and Delta ligands and mastermind coactivator, the thickvein TGFβ receptor and the Mad DNA-binding protein that

mediates TGFβ signaling, the patched hedgehog receptor, the ecdysone steroid hormone receptor, and the EGF receptor. Homeobox genes bound by cohesin include *cut*, *Lim1*, *Distal-less* (*Dll*), *homeobrain* (*hbn*), *Abd-B*, *invected* (*inv*), *homothorax* (*hth*), and *C15*, among others. There are also multiple zinc finger protein genes that bind cohesin, include the *pannier* (*pnr*) GATA1 ortholog and its interaction partner *u-shaped* (*ush*). In BG3 cells, the entire *achaete-scute* gene complex encoding multiple HLH transcription factors involved in nervous system development is bound by cohesin and Nipped-B.

The finding that cohesin binding to *Abd-B* correlates with *Abd-B* expression, and the variation in cohesin binding between the three cell lines indicate that many other genes are also likely to bind cohesin in other cell types. Thus identification of target genes that cause specific CdLS phenotypes will require mapping cohesin binding and gene expression patterns in affected tissues at critical stages of development. Because many genes are bound by cohesin in each cell type, it also appears likely that many individual patient phenotypes stem from simultaneous effects on expression of multiple genes.

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Table 1. Genome-wide correlation coefficients for protein binding.

Protein[1]	Nipped-B-Sg4	SA-Sg4	Smc1-BG3	Smc1-Kc	PolII-BG3
Smc1-Sg4	0.75[2]	0.71	0.47	0.59	-
SA-Sg4	0.88	-	-	-	-
Nipped-B-BG3	0.56	-	0.92	-	0.51
Smc1-BG3	-	-	-	0.55	-
PolII-Sg4	0.62	-	-	-	-
H3K27Me3-Sg4[3]	-0.30	-	-	-	-
Knirps-embryo[4]	0.11	-	-	-	-

[1]Indicates the protein and cell type: Sg4, Kc, BG3 (ML-DmBG3) or embryo.

[2]Correlation coefficients were calculated using the trimmed mean $\log_2$ IP/control ratio values for the microarray features.

[3]histone H3 lysine 27 trimethylation (Schwartz et al. 2006).

[4]Li et al., submitted for publication.

Table 2. SA cohesin subunit and Nipped-B binding preferences for genome features.

		binding preference ratio[1]	
Genome feature	fraction of genome	SA	Nipped-B
Intergenic	0.41	0.74	0.81
Intron	0.33	1.45	1.32
Coding	0.17	0.27	0.18
3' UTR	0.03	0.34	0.2
5' UTR	0.02	6.0	8.3

[1] Ratios greater than 1 indicate that binding is more than expected on a random basis, and values less than 1 indicate less binding. These values were calculated from data in Supplementary Figure 1 by dividing the fraction of the top-ranked half of the peaks predicted at a 25% false discovery rate occurring in a genome feature by the fraction of the non-repetitive genome (column 2) containing that feature.

Figure legends

Fig. 1. Binding of Nipped-B, cohesin subunits, and PolII to a 2 Mb region of chromosome 3L determined by genome-wide chromatin immunoprecipitation (ChIP). This region was chosen to illustrate typical features of cohesin and Nipped-B binding patterns seen throughout the genome. The eight tracks at the top graph the trimmed mean $\log_2$ IP/control ratios for the microarray features on a scale of -0.5 to 3.0. The top four tracks (black) show RNA polymerase II (PolII), Nipped-B, and SA and Smc1 cohesin subunit binding in Sg4 cells of embryonic origin. The red track shows Smc1 binding in Kc cells of embryonic origin, and the three blue tracks show the PolII, Nipped-B and Smc1 binding in ML-DmBG3 cells derived from 3rd instar central nervous system. The vertical lines underneath the Sg4 Nipped-B, SA and Smc1 tracks indicate microarray features predicted by TiMAT analysis to be binding peaks with a 1% false discovery rate. The map of the chromosome 3L region (April 2004 release of the *Drosophila melanogaster* genome; Celniker et al. 2002; Berkeley Drosophila Genome Project, personal communication) is shown below the ChIP tracks. The key features to note are that Nipped-B binding is virtually identical to that of the SA and Smc1 cohesin subunits, that the cohesin/Nipped-B binding patterns are very similar but not identical between the three cells lines, and that cohesin binds large regions ranging in size from a kilobase up to more than 60 kb in length. There are also large regions, such as the 400 kb gene-poor domain near the middle of the graph, that are nearly devoid of cohesin and Nipped-B.

Fig. 2. Co-localization of Nipped-B and cohesin subunit binding sites. The plots compare enrichment values for the SA and Smc1 cohesin subunits and Nipped-B at individual microarray

features. The trimmed mean $\log_2$ IP/control values of the features for chromosome 2L extending from nt 5,522- 4,254,929 for the indicated proteins and cells are plotted against each other. Plots of similar-sized regions across the genome are very similar. The correlation coefficients (r) for the plotted region are given, which are similar to those calculated for the entire non-repetitive genome (Table 1). Nipped-B, SA and Smc1 values for individual microarray features correlate well with each other except for a few Smc1-positive features that are low for SA and Nipped-B (arrows). The white masses centered close to $\log_2$ values of 0 for both proteins represent the majority of features that have low binding for both proteins.

Fig. 3. Similarity of cohesin binding in different cell lines. The Smc1 (Sg4, Kc, BG3) or Nipped-B (Sg4, BG3) trimmed mean log2 IP/control values of individual microarray features for different cell lines are plotted against each other. The plots cover the same region used in Figure 2 (chromosome 2L nt 5,522- 4,254,929), and other regions show very similar results. The correlation coefficients (r) for the plotted regions are similar to those for the entire genome (Table 1). In all cases, the similarities in cohesin binding between cell lines predominate, with many features showing similar values in the cells being compared, but there are also features that have significant values in one cell type and not the other.

Fig. 4. Binding of Nipped-B and cohesin to the *cut* gene regulatory region and transcription unit. Tracks above the chromosome map show cohesin subunit, Nipped-B and PolII binding as trimmed mean $\log_2$ IP/control values (scale -0.5 to 3) for Sg4 (black), Kc (red) and BG3 (blue) cells. The peaks predicted with a 1% false discovery rate for Nipped-B, SA, and Smc1 in Sg4 cells are indicated with vertical lines underneath the tracks. The extent and direction of *cut*

transcription is indicated with an arrow, and the distal wing margin enhancer is indicated by a box (wm). Nipped-B and cohesin bind to the upstream regulatory region and *cut* transcription unit in all three cell lines, but the binding is more extensive in BG3 cells. PolII is found predominantly at the promoter in both Sg4 and BG3 cells, indicating that the difference in cohesin binding between the two cell types is unlikely to reflect a substantial difference in transcription.

Fig. 5. Overlap of Nipped-B and RNA polymerase II (PolII) binding and lack of Nipped-B binding to regions enriched in histone H3 lysine 27 trimethylation (H3K27Me3). The Nipped-B trimmed mean $\log_2$ IP/control values for the individual microarray features are plotted against those for PolII or H3K27Me3. The same 4.25 Mb region of chromosome 2L used for Figures 2 and 3 is plotted for each comparison, but other regions of the genome show a nearly identical pattern. The top two panels compare Nipped-B to PolII and H3K27Me3 in Sg4 cells, and the bottom panel compares Nipped-B and PolII in BG3 cells. The correlation coefficients (r) for the plotted region are similar to those calculated for the entire non-repetitive genome (Table 1). The plots show that many sequences are enriched by both Nipped-B and PolII immunoprecipitation, but there is less direct correlation in enrichment values at individual features than between Nipped-B and cohesin subunits (Fig. 2). The middle plot shows that there is essentially no Nipped-B binding to regions with high levels of H3K27Me3.

Fig. 6. Binding of Nipped-B and cohesin to the active *Abd-B* gene in Sg4 cells. The tracks show histone H3 lysine 27 trimethylation (H3K27Me3), PolII, Nipped-B, SA, and Smc1 localization in the bithorax complex (BX-C) for Sg4 cells (black and gray), Smc1 binding in Kc cells (red), and

PolII, Nipped-B and Smc1 binding in BG3 cells (blue). Trimmed mean $\log_2$ IP/control values are plotted on a scale from -0.5 to 3. The H3K27Me3 data is from Schwartz et al. 2006. The direction of transcription for the BX-C is indicated with an arrow. An expanded map of *Abd-B* showing the regulatory region (Akbari et al. 2006; Maeda and Karch 2006) with enhancers (*iab*), boundary elements (Fab) and promoter-targeting sequences (PTS) with Nipped-B and PolII binding overlaid on each other illustrates the coincidence of some Nipped-B and PolII peaks. At the lower right is a northern blot showing *Abd-B* transcripts in Sg4 cells and their absence in Kc cells. The blot was reprobed for *RpL32* as a loading control. Nipped-B and cohesin bind to the *Abd-B* transcription unit and downstream regulatory region in Sg4 cells where *Abd-B* is active, but not in Kc or BG3 cells where it is silent. The *Ubx* and *abd-A* genes, which are actively silenced by Polycomb group proteins in Sg4 cells, as indicated by H3K27Me3 (Schwartz et al. 2006), do not bind Nipped-B and cohesin in any of the cell lines, although *Ubx* is regulated by Nipped-B *in vivo* (Rollins et al. 1999).

Fig. 1

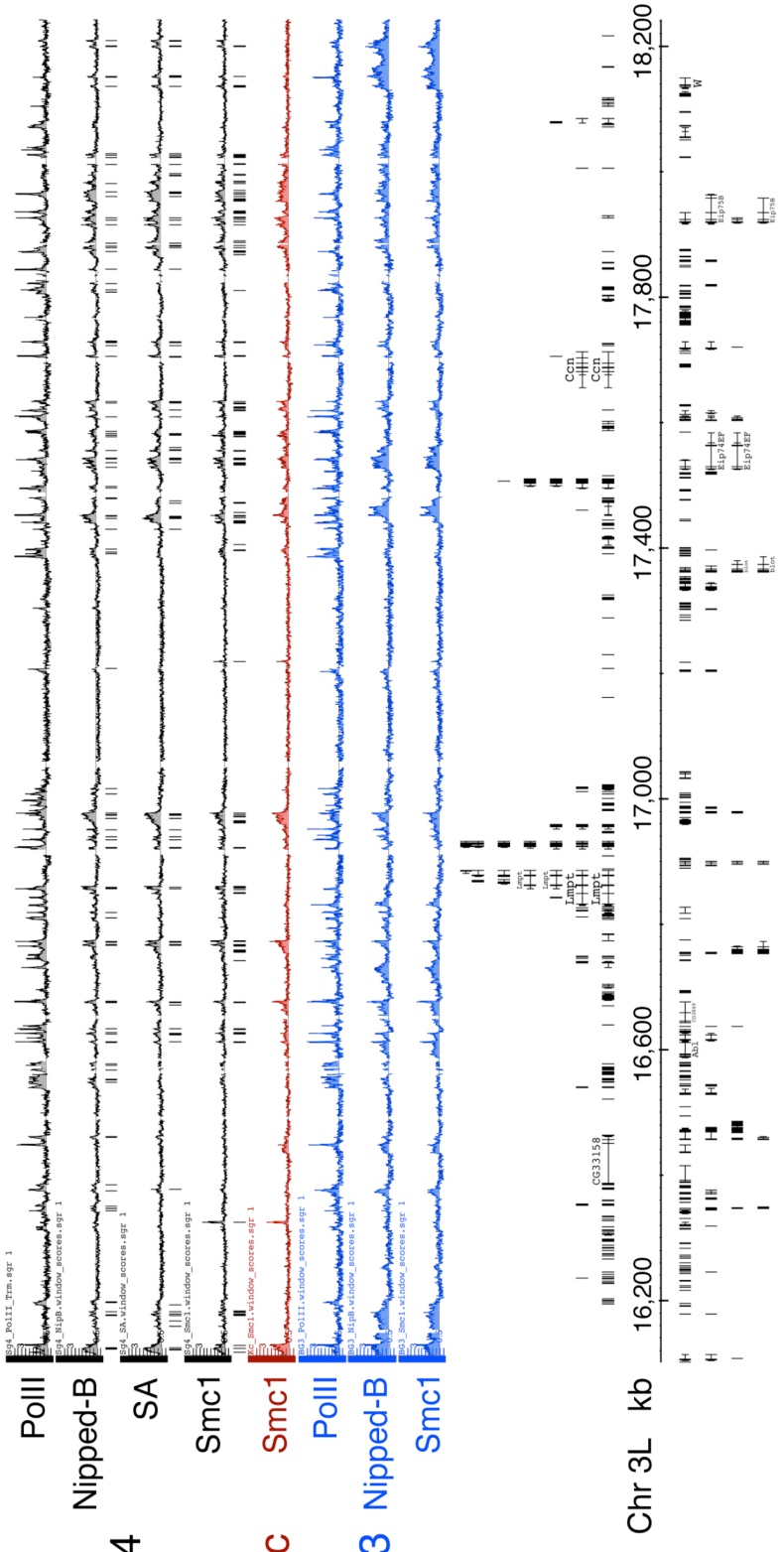

Sg4
PolII
Nipped-B
SA
Smc1
Kc
Smc1
BG3
PolII
Nipped-B
Smc1
Chr 3L kb
16,200
16,600
17,000
17,400
17,800
18,200

Fig. 2

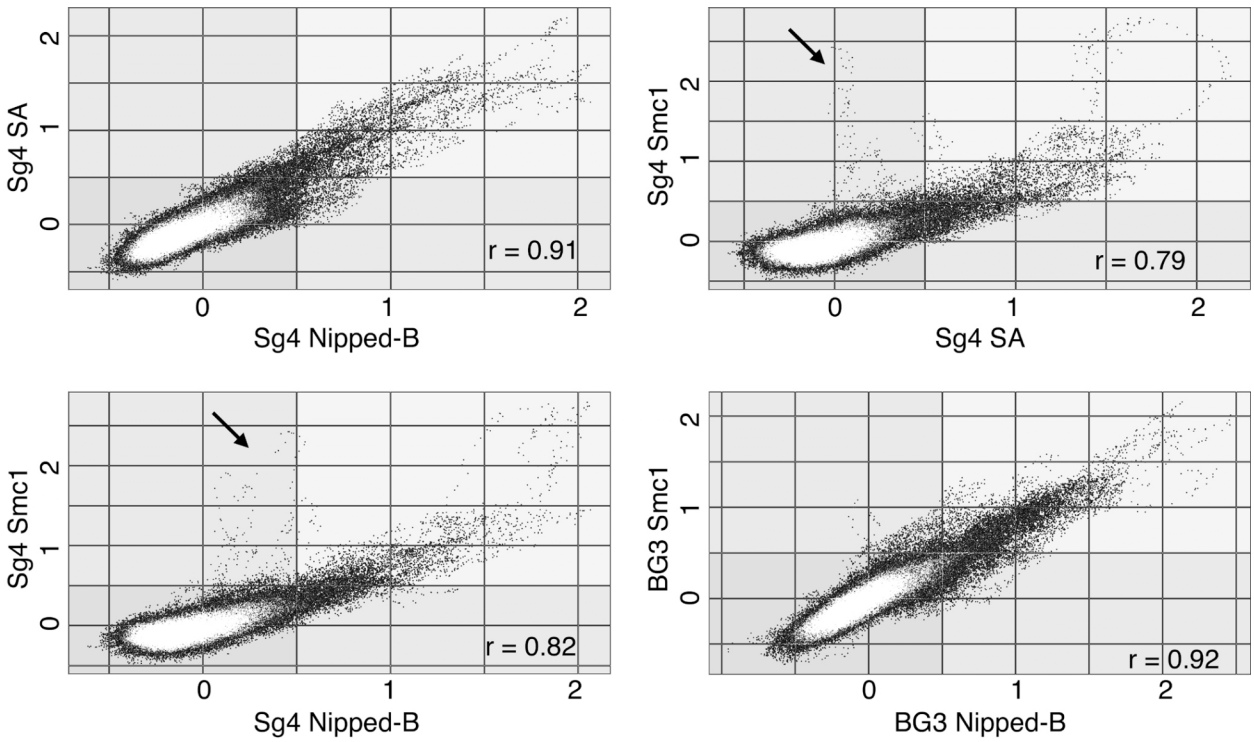

Sg4 SA
Sg4 Nipped-B
r = 0.91
Sg4 Smc1
Sg4 SA
r = 0.79
Sg4 Smc1
Sg4 Nipped-B
r = 0.82
BG3 Smc1
BG3 Nipped-B
r = 0.92
0
1
2

Fig. 3

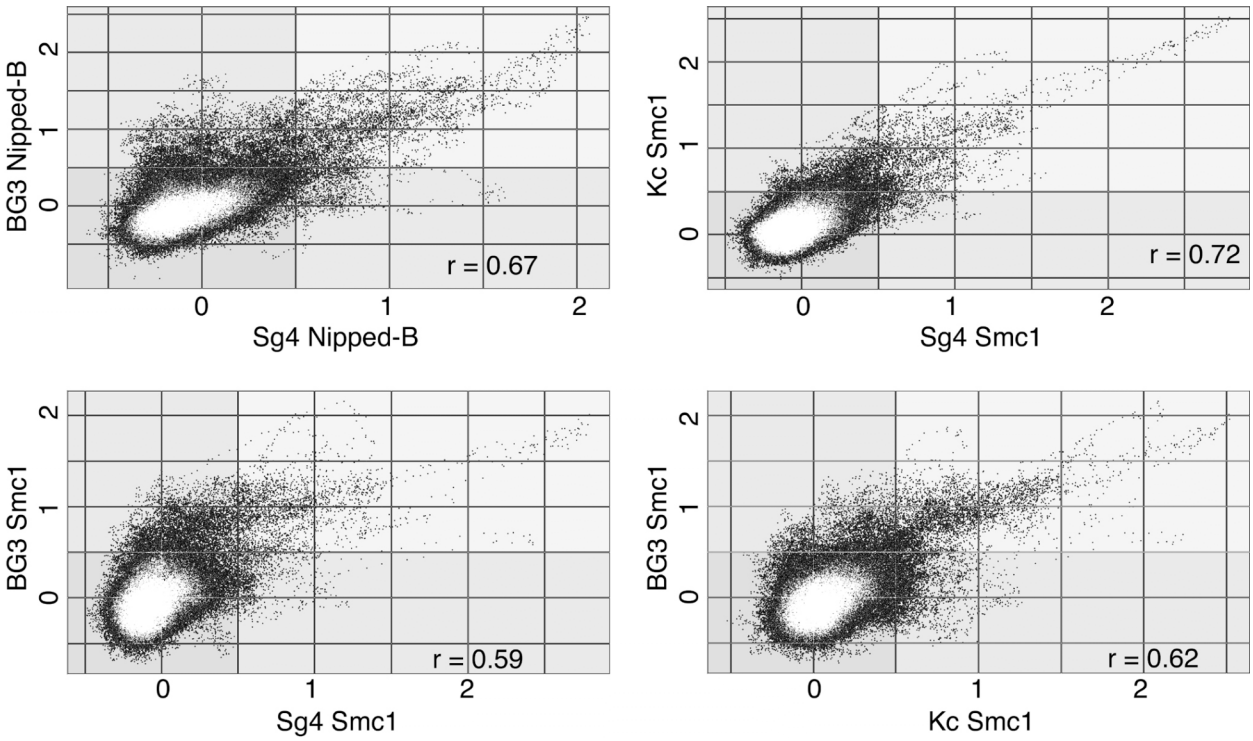

BG3 Nipped-B
Sg4 Nipped-B
r = 0.67
Kc Smc1
Sg4 Smc1
r = 0.72
BG3 Smc1
Sg4 Smc1
r = 0.59
BG3 Smc1
Kc Smc1
r = 0.62
0
1
2

Fig. 4

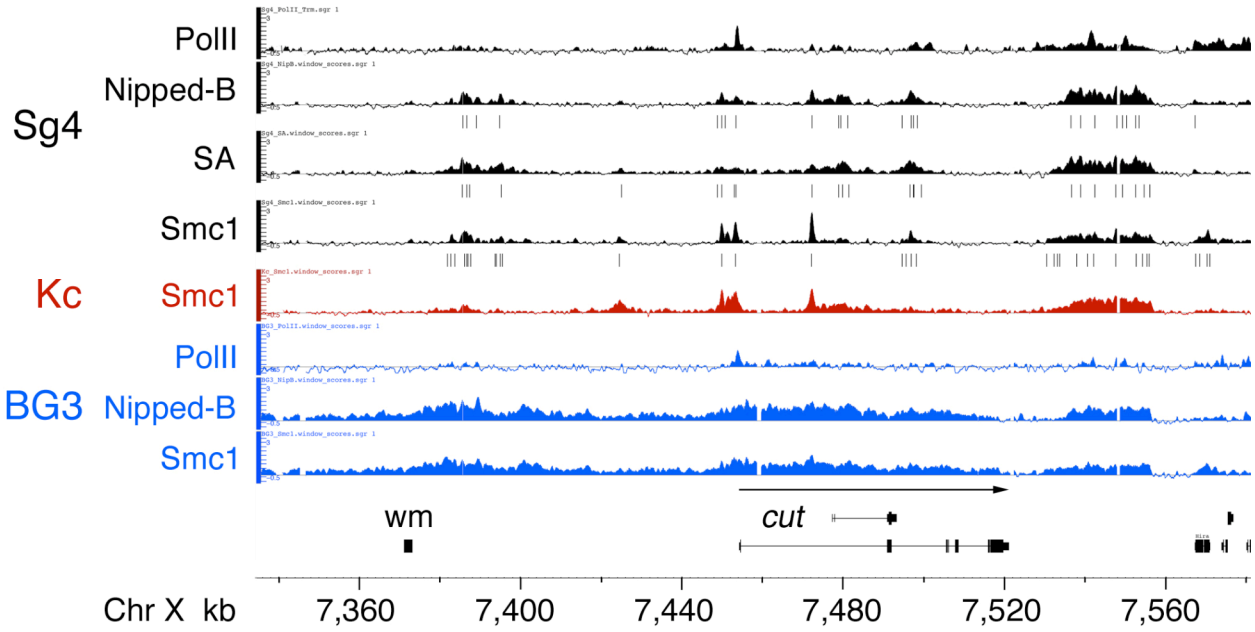

Sg4
PolII
Nipped-B
SA
Smc1
Kc
Smc1
BG3
PolII
Nipped-B
Smc1
wm
cut
Chr X kb
7,360
7,400
7,440
7,480
7,520
7,560

Fig. 5

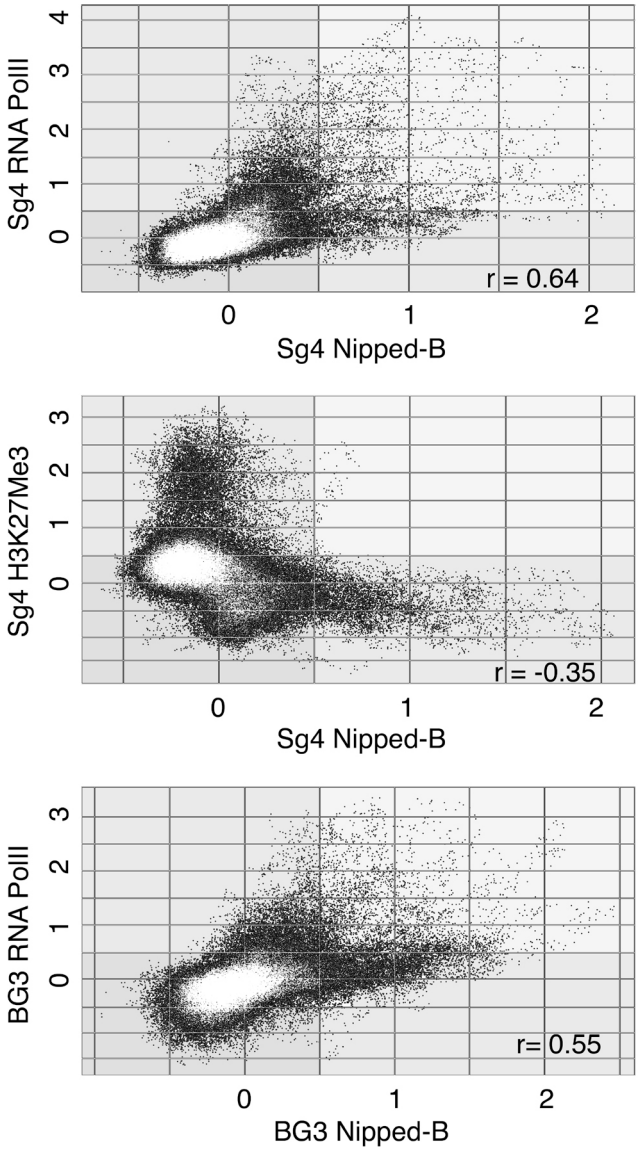
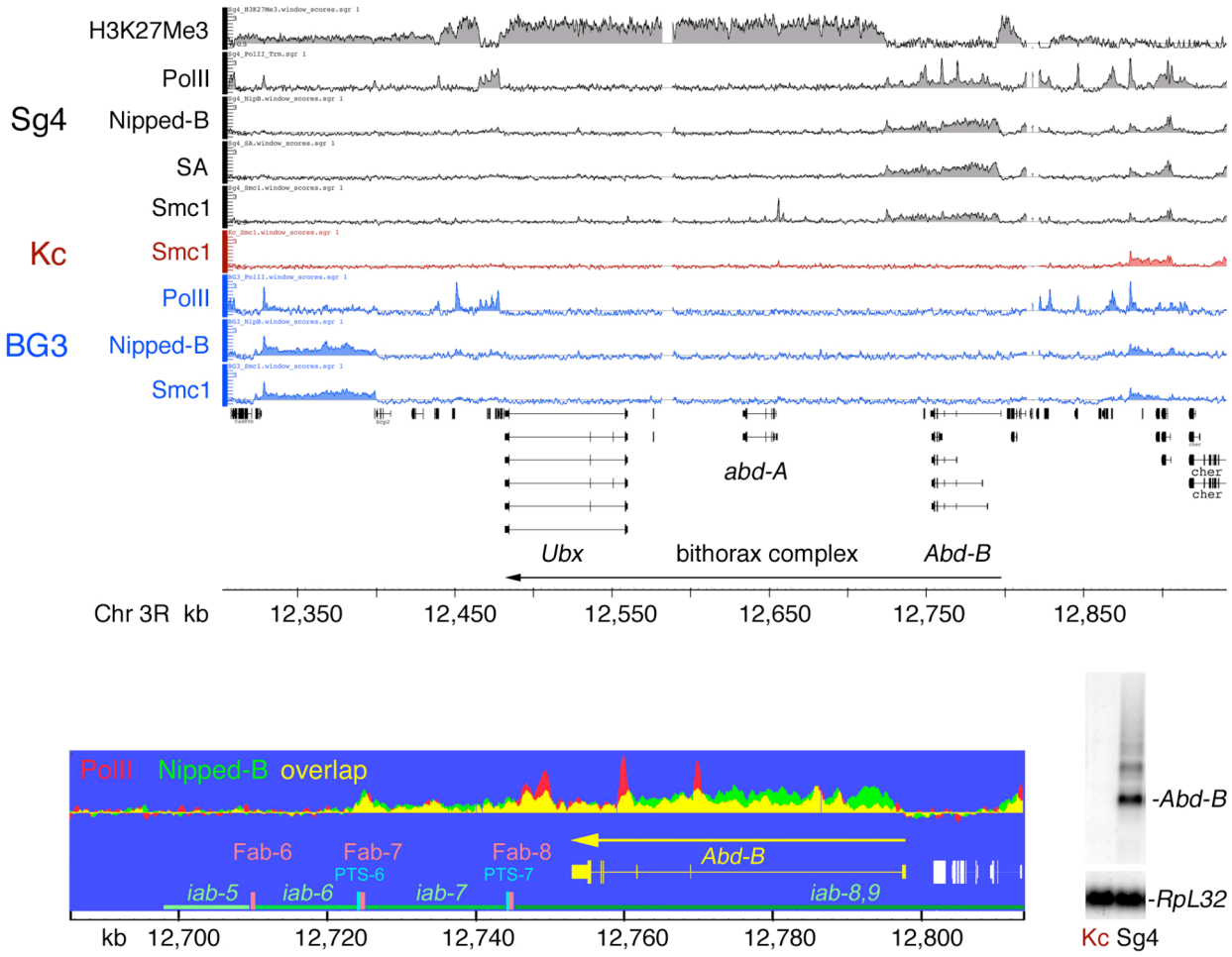

Sg4 RNA PolII
0
1
2
3
4
r = 0.64
0
1
2
Sg4 Nipped-B
Sg4 H3K27Me3
0
1
2
3
r = -0.35
0
1
2
Sg4 Nipped-B
BG3 RNA PolII
0
1
2
3
r= 0.55
0
1
2
BG3 Nipped-B

Fig. 6

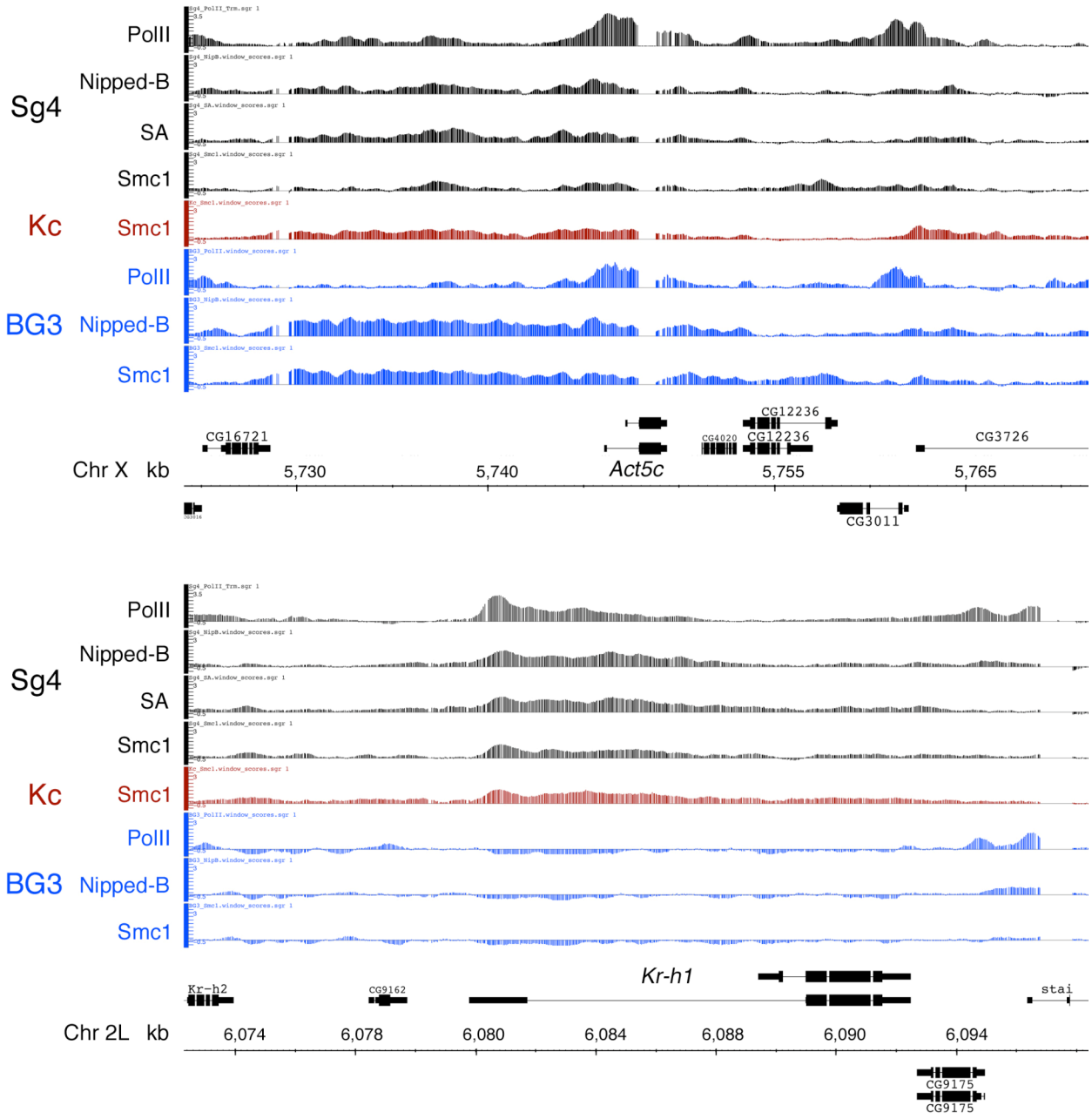

H3K27Me3
PolII
Sg4
Nipped-B
SA
Smc1
Kc
Smc1
PolII
BG3
Nipped-B
Smc1
abd-A
cher
cher
Ubx
bithorax complex
Abd-B
Chr 3R kb
12,350
12,450
12,550
12,650
12,750
12,850
PolII Nipped-B overlap
Fab-6
Fab-7
PTS-6
Fab-8
PTS-7
Abd-B
iab-5
iab-6
iab-7
iab-8,9
kb
12,700
12,720
12,740
12,760
12,780
12,800
-Abd-B
-RpL32
Kc Sg4

Supplementary Fig. 1. Binding of Nipped-B and cohesin to transcribed genes. The top panel shows the binding of RNA polymerase II (PolII), Nipped-B and cohesin subunits to the expressed *Act5c* actin gene in Sg4, Kc and ML-DmBG3 (BG3) cells. The bottom shows the binding of PolII, Nipped-B and cohesin to the *Kr-h1* gene in Sg4 cells, and lack of binding to the same gene in BG3 cells. The Sg4 tracks are in black, the Kc track is red, and the BG3 tracks are blue. The trimmed mean $\log_2$ IP/control values are plotted on a scale of -0.5 to 3.0, except for the Sg4 PolII track, which is on a scale of -0.5 to 3.5. These examples provide evidence that transcription does not push cohesin off the ends of genes, and another case of cohesin binding that correlates with gene transcription.

Supplementary Fig. 2. Nipped-B and cohesin bind preferentially to 5' UTRs and introns. The plots compare the positions of predicted Nipped-B and SA peaks to annotated *Drosophila* genome features (Berkeley Drosophila Genome Project, April 2004 release; Celniker et al. 2002). Nipped-B and SA peaks predicted with a 25% false discovery rate using the TiMAT program are taken in rank order starting with the most significant from left to right in sliding windows of 500 peaks. For each window, the fraction of peaks that are in intergenic sequences, introns, coding sequences, and 3' and 5' UTRs are calculated. The color-coded straight lines labeled on the right show the fraction of the non-repetitive genome that corresponds to each genome feature. These data show that the largest fraction of the highest-ranked SA and Nipped-B peaks are in introns, followed by intergenic, 5' UTR, coding, and 3' UTR sequences. Relative to the fraction of the genome, SA cohesin and Nipped-B binding sites are most over-represented in 5' UTRs, followed by introns, and are most under-represented in coding

sequences, followed by 3’ UTRs and intergenic sequences (see Table 1). The top half of the peaks was used to calculate the binding preference ratios in Table 1.

Supplementary Fig. 1



Supplementary Fig. 2