Dosage-Sensitive Regulation of Cohesin Chromosome Binding and Dynamics by Nipped-B, Pds5, and Wapl[†]

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The cohesin protein complex holds sister chromatids together to ensure proper chromosome segregation upon cell division and also regulates gene transcription. Partial loss of the Nipped-B protein that loads cohesin onto chromosomes, or the Pds5 protein required for sister chromatid cohesion, alters gene expression and organism development, without affecting chromosome segregation. Knowing if a reduced Nipped-B or Pds5 dosage changes how much cohesin binds chromosomes, or the stability with which it binds, is critical information for understanding how cohesin regulates transcription. We addressed this question by *in vivo* fluorescence recovery after photobleaching (FRAP) with *Drosophila* salivary glands. Cohesin, Nipped-B, and Pds5 all bind chromosomes in both weak and stable modes, with residence half-lives of some 20 seconds and 6 min, respectively. Reducing the Nipped-B dosage decreases the amount of stable cohesin without affecting its chromosomal residence time, and reducing the Pds5 dosage increases the amount of stable cohesin. This argues that Nipped-B and Pds5 regulate transcription by controlling how much cohesin binds DNA in the stable mode, and not binding affinity. We also found that Nipped-B, Pds5, and the Wapl protein that interacts with Pds5 all play unique roles in cohesin chromosome binding.

Cohesin, a structural maintenance of chromosome (SMC) protein complex, mediates sister chromatid cohesion and is conserved in structure and function from yeast to humans (reviewed in reference 33). Loss of cohesin results in precocious sister chromatid separation and defects in chromosome segregation upon cell division. Cohesin has a ring-like structure comprised of four subunits, including an Smc1-Smc3 heterodimer with two ATPase head domains, the Rad21 (Mcd1/Scc1) kleisin that bridges the Smc1 and Smc3 head domains, and stromalin (SA, Scc3, and Stag2), which interacts with Rad21. Cohesin can topologically encircle DNA (13, 16, 17). Thus, the leading ideas are that cohesion is mediated by individual cohesin rings encircling both sister chromatids or by interactions between cohesin rings encircling each sister (33).

Cohesin function is regulated by accessory factors. These include Nipped-B (Scc2, Mis4, and NIPBL), which is required for cohesin to bind to chromosomes (4, 12, 49, 50, 52). Nipped-B and its orthologs interact with the Mau-2 (Scc4) protein, which is required for Nipped-B to bind to chromosomes and load cohesin (2, 4, 44, 49, 55). Cohesin subunits can be detected in complexes with Nipped-B, and Nipped-B colocalizes extensively with cohesin on chromosomes, indicating that Nipped-B directly loads cohesin onto chromosomes (1, 10, 21, 30, 49). The ATPase activities of Smc1 and Smc3 are required for cohesin to bind chromosomes (1, 56). Thus, current data suggest that chromosome-bound Nipped-B/Mau-2

controls ATP hydrolysis and the opening of cohesin rings to allow chromosome encirclement.

The Pds5 protein also colocalizes with cohesin on chromosomes and helps establish and maintain sister chromatid cohesion (6, 14, 27, 34, 46, 51). Regulation of cohesion by Pds5 is not fully understood but involves interactions with cohesin and the Wapl (Rad61) protein. The Pds5-Wapl complex has paradoxical activities that include removing cohesin from chromosomes during mitosis, counteracting cohesion establishment during S phase, and promoting cohesin binding to chromosomes (9, 23, 40, 45, 48). In *Drosophila*, *wapl* and *pds5* mutants have opposite phenotypes: the absence of Pds5 causes complete loss of sister chromatid cohesion, and the absence of Wapl prevents separation of sisters in mitosis (6, 54).

Over the past decade it has become apparent that the sister chromatid cohesion factors regulate gene expression during development of metazoan organisms independently of their roles in sister chromatid cohesion and chromosome segregation. *Drosophila* Nipped-B was discovered in a genetic screen for factors that facilitate transcriptional activation of the *cut* and *Ultrabithorax* (*Ubx*) genes by long-range enhancers (39). Heterozygous null *Nipped-B* mutations reduce *Nipped-B* mRNA by 30% or less and alter *cut* and *Ubx* expression without measurable effects on chromatid cohesion, chromosome segregation, or viability (10, 38). Similarly, partial reductions of *Drosophila* Pds5 or cohesin levels also alter gene expression and development without causing cohesion or chromosome segregation defects (6, 38). Complete loss of cohesin alters gene expression and cell remodeling in nondividing *Drosophila* neurons, emphasizing that cohesin regulates genes independently of its chromosome segregation role during cell division (36, 43).

Cohesion factors also regulate vertebrate gene expression and development. Cornelia de Lange syndrome (CdLS), asso-

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ciated with diverse deficits in physical and mental development, is caused by heterozygous loss-of-function mutations in the human *NIPBL* ortholog of *Nipped-B* and by missense mutations in the *Smc1* or *Smc3* cohesin subunits (5, 22, 32, 53). The CdLS mutations do not overtly alter sister chromatid cohesion or chromosome segregation. Heterozygous *rad21* mutations in zebrafish and heterozygous *Nipbl* and *pds5* mutations in mice also alter gene expression and development without affecting chromatid cohesion (15, 19, 58, 59).

Current evidence argues strongly that cohesin directly regulates gene transcription. *Drosophila* *Nipped-B* and cohesin colocalize on chromosomes and preferentially bind a subset of active genes (10, 30). Genome-wide analysis shows that *Nipped-B* and cohesin regulate the same genes, and the set of genes that increase or decrease in expression in response to *Nipped-B* or cohesin reduction is highly enriched for genes that bind cohesin near their transcription start sites (42). Similarly, cohesin preferentially binds near the transcription start sites of active genes in human cells, and the set of genes dysregulated in CdLS cells is enriched for cohesin-binding genes (26).

A critical question is how small changes in *Nipped-B*, cohesin, or *Pds5* activity alter transcription and development, without measurable effects on chromatid cohesion. It is unknown if reducing *Nipped-B* or *Pds5* levels affects the amount of cohesin that binds chromosomes or the affinity with which it binds. Here we used fluorescence recovery after photobleaching (FRAP) to determine how changes in *Nipped-B*, *Pds5*, and *Wapl* dosages affect cohesin chromosome binding and dynamics *in vivo*. We propose a model in which *Nipped-B* converts unbound or weak-binding cohesin to a stable DNA-encircling form. We provide evidence that *Wapl* is required to remove stable cohesin from chromosomes and that *Pds5* that is not interacting with *Wapl* inhibits formation of stable cohesin. Our findings support the idea that a reduced *Nipped-B* or *Pds5* dosage alters gene transcription by decreasing or increasing the amount of stable-binding cohesin, and not the binding affinity.

MATERIALS AND METHODS

Plasmid constructs. Plasmid constructs were made by standard molecular cloning procedures and sequenced. The plasmid DNA sequences are in the supplemental material. The constructs made were (i) pMet-EGFP-*Smc1*-PUROanti for transformation of cultured cells in which the metallothionein promoter (3) drives expression of enhanced green fluorescent protein (EGFP)-*Smc1* and a gene encoding puromycin *N*-acetyltransferase (PURO) is used for selection using puromycin (18); (ii) pMet-SA-EGFP-PUROanti; (iii) pSu(*Hw*)-XhoI-SA-EGFP-Casper, a P element germ line transformation vector using the *su(Hw)* gene promoter (20) to drive SA-EGFP expression; (iv) pChip-XhoI-EGFP-*Smc1*-Casper, a P element vector using the *Chip* gene promoter (44) to express the EGFP-*Smc1* fusion protein; (v) pChip-XhoI-*Nipped-B*-EGFP-Casper; (vi) pChip-XhoI-*Pds5*-EGFP-Casper; and (vii) pUAS-*Nipped-B*, a P element germ line transformation vector placing the *Nipped-B* coding sequence under the control of the upstream activation sequence (UAS), made using the pTFW Gateway vector (Drosophila Genomics Resource Center; donated by Terence Murphy).

Cell transformation, nuclear extracts, and immunoprecipitation. ML-DmD8 cells were cultured in Schneider's media with 10% fetal bovine serum and 10 μ g per ml insulin. Kc cells were cultured in Schneider's media containing 5% fetal bovine serum. Cells were transfected using Fugene (Roche) according to the manufacturer's directions. Transformed cells were selected using puromycin (18). To obtain cells with even expression levels, Kc cells transformed with the EGFP-*Smc1* expression vector were cloned in soft agar with pyridoxal supplementation (57). A line showing similar levels of expression in most cells was

chosen for FRAP experiments. Nuclear extracts of the transformed cells, immunoprecipitation with cohesin antibodies, and Western blotting were performed as previously described (10).

Germ line transformation and genetic crosses. P element germ line transformation of *y w* flies was performed by BestGene, Inc. (Chino Hills, CA). Genetic crosses were conducted at 25°C. Chromosomal linkage of insertions was determined by segregation using various dominant markers on chromosome II and III balancer chromosomes. Multiple insertions of each construct were examined for expression by Western blot analyses of brains extracted with radioimmunoprecipitation assay (RIPA) buffer and fluorescence microscopy of salivary glands. Specific transgene inserts of Chip-EGFP-*Smc1* and Chip-*Nipped-B*-EGFP on chromosome II and Chip-*Pds5*-EGFP and Su(*Hw*)-SA-EGFP on chromosome III were chosen for FRAP analysis. The abilities of Chip-EGFP-*Smc1*, Chip-*Nipped-B*-EGFP, and Chip-*Pds5*-EGFP transgene inserts to rescue mutant phenotypes were determined by crossing the transgenes into *smc1^{exc46}*, *Nipped-B⁴⁰⁷*, and *pds5^{ec3}* homozygous mutant backgrounds. The numbers of rescued 3rd-instar larvae and adults were compared to those of controls. The 3rd-instar brain lobe diameters were measured using dissected brains and a microscope eyepiece reticule. For FRAP analysis, the same Su(*Hw*)-SA-EGFP transgene on chromosome III was crossed into *hs-cyclin E*, *Nipped-B⁴⁰⁷/+*, UAS-*Nipped-B*, *Sg3-Gal4*, *pds5^{ec3}/+*, *wapl²/+*, *wapl²/Y*, *mcm6^{K1214}/mcm⁴*, and *dup^{PA77}/dup⁴³* genetic backgrounds, using appropriate balancer chromosomes and dominant markers. The *Sg3-Gal4* driver (stock no. 6870) was obtained from the Indiana University stock center. Western blot analyses were performed by extracting dissected salivary glands or brains with RIPA buffer. Seven glands or brains were loaded per lane.

FRAP. FRAP was performed at room temperature (22 to 24°C) using a Leica SP5 laser scanning confocal microscope. EGFP excitation and photobleaching was at 488 nm using an argon laser, and detection was at 500 to 575 nm. For Kc cells, EGFP-*Smc1* expression was induced using copper sulfate (3) for 48 h. Cells were then plated in Labtek no. 1 coverglass chambers coated with polylysine to improve adherence. FRAP was conducted using a 60 $\times$ oil immersion objective and a fully open pinhole. Three prebleach scans were performed at 400 Hz with the laser at 6% maximal power, and 6 bleaching scans of half the nucleus at 20% maximal power were performed. Postbleach scans were performed at 6% maximal laser power every 10 s for 5 min. Over 100 Kc cells were subjected to FRAP.

Salivary glands were dissected from late 3rd-instar larvae and placed in a Labtek no. 1 coverglass chamber with Schneider's media. Slivers of no. 1 coverglass were placed around glands to immobilize them. Glands were used for less than 2 h after dissection. FRAP was conducted using a 20 $\times$ objective and a fully open pinhole. Three prebleach scans were performed at 200 Hz at 9% maximal laser power before photobleaching half the nucleus seven times at 30% maximal power. Postbleach scanning was performed every 10 s for 10 min at 9% laser power. From 30 to 100 salivary nuclei were subjected to FRAP for each experimental group.

For experiments to measure SA-EGFP dynamics in G and S phase, control and *hs-cyclin E* larvae were placed on moist 3MM paper in 1.5-ml microcentrifuge tubes and heat shocked at 37°C for 30 min in a water bath. After recovery for 2.5 h at 25°C on regular *Drosophila* media, the salivary glands were dissected in Schneider's media containing 0.1 mM EdU (5-ethynyl-2'-deoxyuridine; Invitrogen) and immobilized in a microscopy flow chamber (Warner Instruments catalog no. RC-30) with a custom 0.1-mm gasket. EdU is incorporated into DNA during DNA synthesis and provides a marker for cells in S phase (41). FRAP was conducted in the chamber using the conditions described above on 6 nuclei per gland, and the glands were then fixed in the chamber by flowing in phosphate-buffered saline (PBS) containing 2% formaldehyde for 20 min. After being washed extensively with PBS, they were permeabilized for 20 min with PBS containing 0.5% Triton X-100 and washed extensively again with PBS. The incorporated EdU was detected using Click-iT reaction buffer with Alexa Fluor 594 azide (Invitrogen A10270) according to the manufacturer's directions. After extensive washing with PBS, the glands were counterstained for DNA using PBS containing 0.3 μ g per ml DAPI (4',6-diamidino-2-phenylindole). Alexa Fluor 594 and DAPI were visualized using helium and UV diode laser excitation.

FRAP data analysis. Background fluorescence was subtracted, fluorescence intensities were corrected for bleaching that occurred during postbleach scans, and the fluorescence intensities in the bleached and unbleached halves were normalized to the last prebleach scan as described previously (37). Loss of fluorescence in the unbleached half of the nucleus was used to determine the amount of unbound protein (11).

The difference in fluorescence between the unbleached and bleached halves of the nuclei over time (11) was analyzed mathematically as a logarithmic decay, which could contain one or more components with different half-lives. To initially determine the minimum number of components needed to provide a

satisfactory fit, the data for all cells in an experimental group were averaged. The average curves were then fit to one-, two-, and three-component models, using Scientist (MicroMath) curve-fitting software, using residual analysis and other goodness-of-fit measures. In all cases, a two-component model, $\Delta F = Ae^{-ka \cdot t} + Be^{-kb \cdot t}$, where ΔF is the difference in fluorescence between the bleached and unbleached halves of the nucleus, A and B are the relative starting amounts of each component, e is the natural log base, ka and kb are the decay rate constants for each component, and t is time postbleaching, provided a significantly better fit than a single-component model. A three-component model did not provide a statistically significantly better fit. A three-component model usually split the fast component into two components with very similar rate constants. Starting the analysis 60 s after photobleaching did not significantly change the rate constants, indicating that unbound protein did not make a significant contribution to the fast component. Three-component models combining the high-rate constant with two low-rate constants measured for different groups (group I Kc cells, group II Kc cells, wild-type salivary glands, and *wapl*²/Y salivary glands) either could not be fit or gave fits in which one of the slow components was present in negligible amounts.

After initial analysis to determine the optimal model, each cell in an experimental group was fit individually to the model to calculate the values for A , B , ka , and kb . A scatter plot of ka versus kb was conducted for each experimental group to determine if cells fell into one or more distinct categories with different rate constants. This revealed two clusters of Kc cells, with significantly different rate constants for the slow component. A single cluster was detected for all salivary gland experimental groups. Individual cells with a ka or kb value more than two standard deviations from the mean were discarded from the analysis, and the values for A , B , ka , and kb for the group were averaged, and the standard errors of the means calculated. Chromosomal half-lives were calculated from the ka and kb values, and the distributions of the total protein into unbound, weakly bound, and stably bound fractions were calculated using the values for A and B .

RESULTS

EGFP-tagged sister chromatid cohesion proteins are functional. To conduct FRAP, we made fusions of EGFP to cohesion proteins. These included EGFP-Smc1, SA-EGFP, Nipped-B-EGFP, and Pds5-EGFP. SA-EGFP and EGFP-Smc1 expressed in transfected cultured cells from the metallothionein promoter were incorporated into cohesin as determined by their coimmunoprecipitation from nuclear extracts with antibodies against other subunits (Fig. 1A).

To conduct FRAP *in vivo*, we made transgenic lines expressing the EGFP-tagged proteins from the *Su(Hw)* or *Chip* gene promoters that are active in most cells. The fusion proteins localize primarily to the nucleus, and by Western blot analyses, their levels were substantially lower than the endogenous proteins in 3rd-instar brains. Examples of SA-EGFP and Nipped-B-EGFP are shown in Fig. 1B.

Where possible, we tested if the fusion proteins rescue mutant phenotypes. EGFP-Smc1 transgenes allowed flies homozygous for the *smc1*^{exc46} null allele, which die as 2nd-instar larvae, to survive to adults. Nipped-B-EGFP transgenes rescued *Nipped-B*⁴⁰⁷ mutant flies, which normally die at 2nd instar, to pupariation at half the expected ratio, with developmental delays. Inability of Nipped-B-EGFP to rescue completely likely reflects low expression relative to endogenous levels and perhaps a lack of expression in some cells. *pds5*⁵³/*pds5*⁵⁶ lethality at the 3rd-instar stage was rescued to adulthood by Pds5-EGFP transgenes (C.-Y. Pai, personal communication). The small brain phenotype of *pds5*⁵³ homozygous null mutants (6) was rescued by a Pds5-EYFP transgene. Based on the immunoprecipitation and genetic data, we conclude that the EGFP fusion proteins are functional.

Cohesin binds salivary gland polytene chromosomes with dynamics similar to those in G₂ cells. We conducted FRAP *in*

FIG. 1. Expression of EGFP fusion cohesion proteins. (A) SA-EGFP and EGFP-Smc1 interact with cohesin. Nuclear extracts of control cells (+) or ML-DmD8 cells expressing SA-EGFP or Kc cells expressing EGFP-Smc1 were immunoprecipitated (IP) with anti-Smc1 or anti-Rad21 and subjected to Western blot analysis with anti-SA or anti-Smc1, showing that the fusion proteins interact with cohesin. (B) Whole-cell extracts of 3rd-instar control (+) brains or brains expressing SA-EGFP or Nipped-B-EGFP were subjected to Western blot analysis with the indicated antibodies.

in vivo to determine the effects of various mutations that alter *in vivo* gene expression on cohesin binding and dynamics. We used salivary gland polytene cells because the fluorescent signals are strong, and more importantly, the chromosomes are not highly mobile. In most tissues, high chromosome mobility confounds FRAP analysis (8).

We considered the possibility that cohesin dynamics in the polytene salivary cells, which have a G-S endocycle, could differ from those in cells with a full mitotic cycle. Thus, as a control, we first measured EGFP-Smc1 dynamics in Kc cultured cells. To conduct FRAP, we photobleached half the nucleus and then measured the decreasing difference in fluorescence intensity between the bleached and unbleached halves over time. The amount of unbound EGFP-Smc1 was determined by measuring the loss of fluorescence intensity in the unbleached half of the nucleus in the first postbleach measurement (11). The decreasing difference in the fluorescent intensities between the bleached and unbleached halves of the nuclei (Fig. 2B) was treated mathematically as a logarithmic decay. We found that these “recovery” or “equilibration” curves fit a model in which EGFP-Smc1 binds chromosomes in two modes with short (weak-binding) and long (stable-binding) chromosomal half-lives. A model with a single binding mode gave a poor fit, and a three-mode model did not significantly improve the fit.

The Kc cell nuclei fell into two categories (Fig. 2B), differing in the chromosomal half-life of the stable-binding form of EGFP-Smc1. In more than two-thirds of the cells (group II), the half-life of the stable mode was 550 s and the weak-binding

FIG. 2. EGFP-Smc1 dynamics in Kc cells. (A) FACS analysis of Kc cells before or after copper (Cu^{2+}) induction of EGFP-Smc1 expression. (B) FRAP recovery of EGFP-Smc1 after photobleaching in group I (G1) and group II (G2) cells, which represent some 30% and 70% of cells, respectively. The difference in fluorescence (ΔF) between the unbleached half and the bleached half of the nucleus is plotted versus the time postbleaching. We assume that group I cells are in G₁ and group II are in G₂. (C) Half-lives of weak- and stable-binding modes of EGFP-Smc1 chromosome, determined with a two-binding mode model. (D) Distribution of EGFP-Smc1 into unbound, weak binding, and stable binding. Unbound EGFP-Smc1 was determined by measuring the loss of fluorescence in the unbleached half of the nucleus. (E) Ratios of weak-binding EGFP-Smc1 to stable-binding and unbound EGFP-Smc1. Error bars are standard errors of the mean.

half-life was 5 s (Fig. 2C). In group I, the half-life of the stable-binding mode was shorter, approximately 120 s. Based on fluorescence-activated cell sorter (FACS) analysis (Fig. 2A), we conclude that group II represents G₂ and possibly late-S-phase cells and that group I comprises G₁ and possibly early-S-phase cells. These findings agree with studies showing that mammalian cohesin has a longer binding half-life in G₂ (11). There were minor differences in the distribution of the total EGFP-Smc1 into unbound and weak- and strong-binding fractions between groups I and II.

EGFP-Smc1 in 3rd-instar salivary gland cells also binds in both weak and strong modes, with chromosomal half-lives of approximately 25 and 425 s (Fig. 3). Thus, the EGFP-Smc1 dynamics in salivary glands closely resemble those in G₂ Kc cells. As described below, some 95% of 3rd-instar salivary gland cells are in G phase.

The difference in the half-lives of weak-binding EGFP-Smc1 between Kc cells and salivary cells (5 to 10 s versus 25 s) likely reflects recapture of cohesin released from chro-

mosomes before it diffuses to the other half of the nucleus. This can cause overestimation of chromosomal residence times close to the diffusion rate (31). Salivary nuclei are substantially larger than Kc nuclei (20 to 25 versus 6 to 8 μm in diameter), and thus the half-life of the weak-binding form is likely overestimated for salivary cells. The very long half-life of stable cohesin, the large difference in the half-lives of the stable forms between G₁ and G₂ Kc cells, and the slightly shorter half-life of stable cohesin in salivary cells compared to G₂ Kc cells indicate that, as expected, recapture is not a significant factor in the measurement of stable cohesin residence times.

Nipped-B-EGFP and Pds5-EGFP have dynamics similar to those of cohesin. We compared the dynamics of the SA-EGFP, Nipped-B-EGFP, or Pds5-EGFP protein to those of EGFP-Smc1 in salivary gland cells. In all cases, a dual weak- and strong-binding model provided a good fit, and all proteins had similar chromosomal half-lives (Fig. 3B). The dynamics for SA-EGFP and Nipped-B-EGFP were nearly indistinguishable

FIG. 3. SA-EGFP, EGFP-Smc1, Nipped-B-EGFP, and Pds5-EGFP dynamics in 3rd-instar salivary gland cells. (A) FRAP recovery curves for SA-EGFP, EGFP-Smc1, Nipped-B-EGFP, and Pds5-EGFP. The SA-EGFP, EGFP-Smc1, and Nipped-B-EGFP curves are overlapping, while Pds5-EGFP shows faster recovery. (B) Chromosomal half-lives for weak- and strong-binding modes of SA-EGFP, EGFP-Smc1, Nipped-B-EGFP, and Pds5-EGFP. (C) Distribution of SA-EGFP, EGFP-Smc1, Nipped-B-EGFP, and Pds5-EGFP into unbound and weakly and strongly bound fractions. (D) Ratio of weak-binding SA-EGFP, EGFP-Smc1, Nipped-B-EGFP, and Pds5-EGFP to stable binding and unbound forms. Error bars are standard errors of the mean.

from those of EGFP-Smc1 (Fig. 3A). Pds5-EGFP showed a slightly faster recovery (Fig. 3A), primarily because the ratio of weak to stable binding was higher (Fig. 3C and D).

The similarity in *in vivo* dynamics of SA-EGFP and EGFP-Smc1 was expected because both are core cohesin subunits. It was unexpected, however, that Nipped-B-EGFP and Pds5-EGFP would have similar dynamics. Compared to most DNA binding proteins, the chromosomal half-life of stable cohesin is unusually long. We hypothesize that stable binding involves topological entrapment of DNA within the cohesin ring. Nipped-B and Pds5 are not predicted to encircle DNA, and thus, we suggest that they interact strongly enough with chromosome-bound cohesin for cohesin to determine their half-lives.

Cohesin has similar dynamics in the endocycle G and S phases. Five percent or less of late 3rd-instar salivary gland cells are in the endocycle S phase (Fig. 4). Thus, we assumed that the S-phase cells, even if they had different cohesin dynamics, would not have a significant impact on the chromosomal residence times determined in the above experiments. To determine directly if cohesin dynamics are different during S phase, we induced cyclin E under the control of a heat shock promoter in a transgene, forcing S-phase entry (47). We measured SA-EGFP dynamics after heat shocking larvae to induce cyclin E and recovery for 2.5 h. After FRAP, we performed EdU staining to confirm which nuclei were in S phase. As a control, we performed heat shock and recovery with larvae that

did not contain the cyclin E transgene. EdU staining showed that at least 80% of the salivary gland cells with the cyclin E transgene were in S phase, compared to less than 5% in control glands (Fig. 4A). With data only from nuclei positive for EdU staining, cohesin showed a slightly slower recovery (Fig. 2B) but still had both weak and stable binding, with a slightly shorter half-life for stable binding (Fig. 4C). The proportion of stable SA-EGFP binding increased to 25%, with a decrease in unbound cohesin. Thus, entry into S phase induces small but significant changes in cohesin dynamics. SA-EGFP dynamics in control heat-shocked salivary glands (Fig. 4), using only nuclei that did not stain with EdU, did not significantly differ from those in glands that were not heat shocked (Fig. 3), except that the weak-binding half-life was slightly shorter.

In *Xenopus* oocyte extracts, licensing of replication origins by assembly of the prereplication complex (pre-RC) is required for binding of the Nipped-B/Mau-2 complex to chromatin, which then loads cohesin (12, 49, 50). Cohesin colocalizes with origin recognition complex (ORC) components of the pre-RC and replication origins in *Drosophila* cells, but cohesin binding does not require pre-RC components (28). Although some ORC components are reported not to be essential for endoreduplication, at least some Mcm complex components and the Cdt1 (Double-parked [Dup]) subunits of the pre-RC are required (35). We tested if SA-EGFP cohesin dynamics in salivary glands were altered with mutations affecting Mcm6 or Dup activity. Heteroallelic combinations of lethal alleles with

FIG. 4. SA-EGFP dynamics in G- and S-phase salivary gland cells. (A) (Top) A salivary gland in which S phase has been induced by the hs-cyclin E transgene. The left column (green) shows EGFP-SA fluorescence, the middle (red) shows EdU staining, which indicates DNA synthesis, and the right shows DNA DAPI staining. (Bottom) A control salivary gland subjected to the same heat shock without the hs-cyclin E transgene. (B) FRAP recovery curves for G-phase nuclei in control salivary glands (blue diamonds) and S-phase nuclei induced by cyclin E expression (red circles). (C) Half-lives of weak and stable SA-EGFP chromosome binding in G- and S-phase nuclei. (D) Distribution of SA-EGFP into unbound, weakly bound, and strongly bound forms in G- and S-phase nuclei. (E) Ratios of weakly bound SA-EGFP to the stably bound and unbound.

viable female-sterile alleles (*mcm6*^{K1214}/*mcm6*^Δ and *dup*^{PA77}/*dup*^{Δ3}) had no significant effect on the distribution of SA-EGFP into the different fractions or SA-EGFP dynamics, except that the *mcm6* mutations slightly reduced the chromosomal half-life of the stable-binding fraction from 327 ± 30 s to 272 ± 25 s (not shown; standard errors of the mean shown). We conclude that reduced activity of these pre-RC components does not substantially alter cohesin binding or dynamics, with the caveat that these mutations do not strongly affect DNA replication.

Nipped-B dosage regulates stable cohesin binding. In contrast to the lack of significant effects of *Mcm6* and *Dup* mutations, a heterozygous *Nipped-B* null allele (*Nipped-B*^{Δ407/+}) reduces the fraction of SA-EGFP that binds in the stable mode by a third, from 18% to 12%, with a corresponding increase in unbound SA-EGFP (Fig. 5). The half-life of strong SA-EGFP binding increased slightly from 337 to 410 s. Thus, the largest effect is a decrease in the amount of stable cohesin.

We measured Nipped-B protein levels in salivary glands by

Western blotting and discovered that relative to that of 3rd-instar brains, the ratio of Nipped-B to the Rad21 and SA cohesin subunits is some 100-fold lower (Fig. 5E). Immunostaining with antisera against the N terminus of Nipped-B showed that Nipped-B colocalizes with cohesin on salivary gland chromosomes, and immunostaining glands with an antibody against a central Nipped-B domain detected nucleoplasmic Nipped-B (6, 38). Reverse-transcription (RT)-PCR of RNA isolated from salivary glands and the central nervous system confirmed that *Nipped-B* RNA is present at similar levels in both tissues, relative to *RpL32* ribosomal protein transcripts (not shown). This suggests that Nipped-B protein levels are regulated translationally or posttranslationally in salivary glands, but we cannot rule out the possibility that Nipped-B is modified in a manner that reduces recognition of the denatured protein on Western blots.

We made flies with a UAS-Nipped-B transgene to determine if Nipped-B overexpression alters cohesin binding or dynamics in salivary glands. In the presence of a salivary gland-

FIG. 5. Effects of Nipped-B dosage on SA-EGFP dynamics in salivary gland cells. (A) FRAP recovery curves for SA-EGFP in wild-type salivary cells (SA-EGFP) and *Nipped-B*^{407/+} mutants (Nipped-B -/+) and with overexpressed Nipped-B (UAS-Nipped-B; Sgs3-Gal4). (B) Chromosomal half-lives for weak- and strong-binding SA-EGFP. (C) Distribution of SA-GFP into unbound, weakly bound, and strongly bound forms. (D) Ratio of weak-binding SA-EGFP to stably bound and unbound. (E) Western blots of salivary glands (sg) and brains (br) comparing expression of Nipped-B, Rad21, and SA, relative to actin, in sg and br. All lanes on left are the same Western blot. The right lanes compare levels of expression in sg without and with the UAS-Nipped-B; Sgs3-GAL4 overexpression transgenes.

specific Gal4 driver, Nipped-B levels are greatly increased (Fig. 5). This had no measurable effect on the size of nuclei or chromosome morphology but nearly doubled the chromosomal half-life of stable SA-EGFP to 650 s, without increasing the amount of the stable fraction. We did not simultaneously overexpress the Mau-2 protein that interacts with Nipped-B, and thus, Mau-2 may be limiting for how much stable cohesin can be formed. We speculate that overexpressed Nipped-B increases the stable residence time by displacing other factors that destabilize cohesin binding.

Using broadly expressed Act5C-Gal4 or tubulin-Gal4 drivers, Nipped-B is overexpressed severalfold in other tissues, such as those of the central nervous system (Fig. 5E). Survival to adulthood was decreased by 37% to 80%, depending on the combination of the UAS-Nipped-B transgene and the Gal4 driver (not shown). Most of the surviving adults have mild abdominal segmentation defects. Thus, Nipped-B overexpression also affects development.

Pds5 and Wapl have multiple effects on cohesin binding and dynamics. In *Drosophila*, Pds5 and Wapl have opposite effects on sister chromatid cohesion. *Drosophila pds5* null mutants

show loss of cohesion, while *wapl* loss-of-function mutants fail to lose cohesion at mitosis (6, 54). In flies heterozygous for the *pds5*^{e3} null allele, stable SA-EGFP increased substantially from 17 to 28% of the total, with a decrease in the weak-binding fraction, but there were no significant changes in the half-life (Fig. 6). In contrast, the heterozygous *wapl*² loss-of-function mutation decreased the amount of stable cohesin, with an increase in unbound cohesin, also without an effect on half-life.

The effects of reduced Pds5 and Wapl dosages on SA-EGFP dynamics were surprising, because one might expect that stable cohesin mediates sister cohesion. Thus, stable cohesin would be expected to decrease with the Pds5 dosage and increase with a reduced Wapl dosage, but the opposite was seen in both cases. Effects of *pds5* and *wapl* mutations on sister cohesion, however, are seen only with homozygous mutants. We could not examine homozygous *pds5* mutants, because they produce small salivary glands and have altered polytene chromosome morphology (6). We found, however, that the hemizygous *wapl*² mutation had dramatic effects opposite to those of the heterozygous *wapl*² mutation, increasing both the unbound and strong-binding fractions of SA-EGFP, at the expense of

FIG. 6. Effects of Pds5 and Wapl dosages on SA-EGFP dynamics in salivary gland cells. (A) SA-EGFP FRAP recovery curves for wild-type (SA-EGFP), *pds5*^{+/+} (*pds5*^{-/+}), *wapl*^{+/+} (*wapl*^{-/+}), and *wapl*^{2/Y} (*wapl*^{-/Y}) salivary cells. (B) Chromosomal half-lives for weak and stable SA-EGFP. (C) Distribution of SA-EGFP into unbound, weakly bound, and strongly bound fractions. (D) Ratio of weakly bound SA-EGFP to stably bound and unbound. Error bars are standard errors of the mean.

the weak-binding fraction (Fig. 6). Strikingly, the half-life of strong-binding cohesin increased more than 3-fold to 1,060 s. This is consistent with the findings that *Drosophila wapl*² mutants do not properly separate sisters and that knockdown of Wapl in mammalian cells increases the cohesin chromosomal half-life and blocks sister separation (10, 23, 54).

DISCUSSION

Figure 7 summarizes our current model for regulation of cohesin chromosome binding by Nipped-B, Pds5, and Wapl based on the *in vivo* FRAP data. This model incorporates published data and has several features. (i) Cohesin exists in at least three dynamic forms in cultured cells and salivary glands, which we interpret as unbound, weakly bound to chromosomes, and stably bound. (ii) The DNA-bound Nipped-B/Mau-2 complex converts unbound or weakly bound cohesin to stable-binding cohesin, which we posit is cohesin encircling DNA, based on data from yeast (13, 16, 17). (iii) Cohesin mediating sister chromatid cohesion (cohesive stable cohesin), which may encircle two sister chromatids or interact with cohesin encircling another sister, has a longer chromosome residence time than cohesin encircling a single chromatid (non-cohesive stable cohesin). (iv) Wapl, as part of the Pds5-Wapl complex, removes cohesin to produce unbound or weakly bound cohesin. (v) Pds5 is in equilibrium with the Pds5-Wapl complex, and Pds5 that is not complexed with Wapl inhibits conversion of cohesin to a stably bound form. (vi) A fraction of the Nipped-B/Mau-2 and Pds5-Wapl complexes interact tightly

with cohesin on chromosomes. Below we discuss the evidence for this model and the implications for published data showing that changes in Nipped-B, Pds5, and cohesin dosages alter gene expression and development, without effects on sister chromatid cohesion.

Cohesin binds chromosomes in multiple modes. With all cells examined, we resolved two chromosome binding forms of Nipped-B, Pds5, and the Smc1 and SA cohesin subunits with short and long equilibration half-lives that we interpret as weak and stable binding (Fig. 7). In no case did we detect more than two binding forms, although we would not detect forms that have very similar chromosomal residence times or represent a very small fraction of total cohesin. It is highly unlikely that these represent binding to nuclear structures other than chromosomes. By genome-wide chromatin immunoprecipitation with cultured cells, and immunostaining of both salivary and meiotic chromosomes, we found that Nipped-B, Pds5, and cohesin colocalize and bind extensively to extended domains on chromosomes (6, 10, 30). Immunostaining of whole salivary glands and cultured cells has not revealed evidence for specific binding to other nuclear structures.

The half-lives of the chromosome binding modes differed with the cell type and stage of the cell cycle. In G₁ Kc cells, we saw stable binding, with a half-life of 120 s. In G₂ Kc cells, the stable-binding half-life increased to 550 s, suggesting that cohesive stable cohesin binds more stably than noncohesive cohesin. In the endocycling salivary gland G-phase cells, the stable form had a half-life of 340 to 425 s, similar to that in G₂ Kc cells. Polytene chromosomes have many copies of both

FIG. 7. Models for the roles of Nipped-B, Pds5, and Wapl in regulating cohesin chromosome binding and dynamics. We hypothesize that weak-binding cohesin with a chromosomal half-life ($t_{1/2}$) of approximately 20 s associates with DNA without encircling it. The Nipped-B/Mau-2 complex binds DNA and facilitates opening of the cohesin ring to allow it to encircle DNA. With excess Pds5, and sister chromatids, this is rapidly converted to stable “cohesive” cohesin with a measured half-life of approximately 340 s. The cohesive mode could consist of a single cohesin ring encircling two chromatids as illustrated or two interacting cohesin rings that each encircle a chromatid. The Pds5-Wapl complex can remove stable cohesin to generate unbound cohesin or weakly bound cohesin. We postulate that free Pds5 interferes with cohesin loading by Nipped-B/Mau-2, perhaps by competing for binding to cohesin. The Pds5-Wapl complex does not inhibit loading, but a decrease in Wapl increases the amount of free Pds5, thereby reducing loading. In the absence of Wapl, free Pds5 still makes cohesive cohesin, which binds with a half-life of approximately 1,060 s.

sisters; thus, we presume that the stable form in these cells is cohesive. We did not detect stable cohesin in G₂ Kc cells or salivary glands with a half-life similar to that in G₁ Kc cells, indicating that virtually all stable cohesin is converted from noncohesive to cohesive when there are two or more sister chromatids (Fig. 7).

Comparison of cohesin chromosome binding patterns and dynamics in salivary cells and cultured *Drosophila* and human cells argues that cohesin binding and regulation are nearly identical in polytene and nonpolytene cells. Cohesin associates preferentially with interbands in salivary polytene chromosomes, which are regions with active genes, and also prefers active genes in cultured *Drosophila* and human cells (6, 26, 30, 36). These studies also showed that Nipped-B and cohesin colocalize completely on both polytene and nonpolytene chromosomes. Finally, the chromosomal half-life of stable cohesin in salivary gland G cells (5.5 to 6.8 min) is close to that seen in G₂ Kc cells (9.2 min) and human G₂ cells (8.4 min) (23).

There is a noticeable difference in cohesin dynamics between *Drosophila* and mammalian cells. Twenty percent of bound cohesins in G₂ mammalian cells have a half-life measured in hours (11, 23), but we did not detect such extremely stable cohesin in either Kc cells or salivary glands. The percentage may be too low to detect. One possibility is that the extremely stable cohesin binds heterochromatin around centromeres, which is a smaller fraction of the *Drosophila* genome and is also underreplicated in salivary cells. In *Saccharomyces cerevisiae*, bulk cohesin binding has a half-life of 55 s, and centromeric cohesin has a half-life of 93 s, supporting the idea that cohesin residence time is longer around centromeres (29).

Do Nipped-B and Pds5 interact tightly with cohesin on chromosomes? We were surprised to find that a significant fraction

of Nipped-B-EGFP and Pds5-EGFP had chromosomal half-lives similar to that of stable cohesin, which is much longer than those of most DNA-binding proteins. Nipped-B and Pds5 both colocalize with cohesin on chromosomes (6, 10, 30), and thus, a simple idea is that a fraction of the Nipped-B/Mau-2 and Pds5-Wapl complexes interact tightly with stable cohesin. If so, the half-lives of these interactions must be equal to or longer than those of stable cohesin with chromosomes. In *S. cerevisiae*, Pds5 and the Scc2 ortholog of Nipped-B also have dynamics similar to that of cohesin (29). Initial reports were that Scc2 does not colocalize with cohesin, which argues against the idea that Scc2-cohesin interactions determine the Scc2 chromosomal half-life, but another study finds substantial Scc2-cohesin overlap, consistent with the possibility of tight Scc2-cohesin interactions on DNA (21, 24).

Nipped-B/Mau-2 converts cohesin to the stable-binding form. A heterozygous *Nipped-B* null mutation, which reduces *Nipped-B* expression by 30% or so and alters gene expression but not sister chromatid cohesion (38), decreased the fraction of cohesin that binds stably by a third, from 18 to 12% of total cohesin. This agrees with observations indicating that the Nipped-B/Mau-2 complex is required for binding of cohesin to chromosomes (2, 4, 12, 44, 49, 50, 52, 55) and is the first evidence that the Nipped-B dosage determines the extent of cohesin binding. The FRAP data indicate more precisely that Nipped-B converts weak-binding or unbound cohesin to the stable form (Fig. 7).

We found that the Nipped-B-to-cohesin ratio is much lower in the salivary gland than in the brain or Kc cells, while the fraction of EGFP-Smc1 that is stable is only slightly lower. The ratio of weak-to-stable EGFP-Smc1 in salivary cells, however, is 2-fold higher than in G₂ Kc cells, suggesting that conversion

of weak-binding to stable-binding cohesin is less efficient. Higher Nipped-B levels may be needed to maintain the level of stable cohesin in brain cells, which divide very rapidly. The highest levels of *Nipped-B* mRNA and high levels of protein are also found in early embryos, in which nuclei divide very rapidly (39, 44). We do not know, however, what fraction of Nipped-B binds chromosomes or is functional in nonpolytene cells.

The FRAP data indicate that Nipped-B is not a significant pathway for cohesin removal. If this were so, reducing the Nipped-B dosage would substantially increase the cohesin chromosomal residence time. There was a slight increase in the SA-EGFP half-life, from 337 ± 23 to 410 ± 33 s, but this is still less than the EGFP-Smc1 half-life (425 ± 45 s). This argues against the idea that the Nipped-B dosage determines the on-off rates of cohesin binding, which was put forth to explain the opposing effects of reductions in Nipped-B and cohesin dosages on *in vivo* expression of the *cut* gene (38). It thus supports the alternative idea that a small reduction in cohesin binding can have the opposite effect of a large reduction on the transcription of some genes, as reported for the *Enhancer of split* gene complex (42).

Nipped-B overexpression increased the half-life of stable cohesin, suggesting that free Nipped-B can inhibit cohesin removal by Pds5-Wapl. We speculate that Nipped-B, which similar to Pds5 contains several HEAT repeats, competes with Pds5-Wapl for binding to stable cohesin. We did not see an increase in the amount of stable cohesin with Nipped-B overexpression. However, Mau-2, which is required for Nipped-B to bind to chromatin (49), was not overexpressed, and thus, we deduce that Nipped-B that is not tethered to chromatin does not convert cohesin to the stable-binding form.

Pds5 and Wapl play multiple roles in cohesin chromosome binding. The complete loss of Wapl caused a striking increase in both the amount and half-life of stable cohesin and a substantial decrease in the amount of weak-binding cohesin. This is consistent with the lack of sister chromatid separation in *wapl* mutants and the effects of Wapl knockdown on cohesin dynamics in mammalian cells (23, 54). We conclude that Wapl, in complex with Pds5, is required to remove cohesin from chromosomes during interphase (Fig. 7). The half-life of the stable cohesin in *wapl* mutant salivary glands is strikingly close to that determined for human cells lacking Wapl (17.7 versus 18.2 min), providing further evidence that regulation of cohesin chromosome binding in salivary glands is nearly indistinguishable from that in nonpolytene cells.

We were initially surprised to discover that the heterozygous *wapl* mutation in females had an effect opposite to that of the hemizygous *wapl* mutation in males and decreased the amount of stable cohesin. It is highly unlikely that this reflects a difference in cohesin regulation between males and females, because sister chromatid separation is required during cell division in both sexes. A clue to an explanation for this unexpected finding arose from another unexpected finding, which is that the heterozygous *pds5* mutation increased the amount of stable cohesin. This was unexpected because homozygous *pds5* mutations cause the loss of sister chromatid cohesion (38), which is presumably mediated by stable cohesin. To explain the opposing and unexpected effects of decreased Pds5 and Wapl dosages on cohesin dynamics, we propose that free Pds5 that is

not in a complex with Wapl inhibits conversion of weak-binding to strong-binding cohesin by Nipped-B, which may involve binding of free Pds5 to weakly bound cohesin (Fig. 7). With a reduced Pds5 dosage, there is less free Pds5 to inhibit formation of stable cohesin. Reducing Wapl increases the amount of free Pds5, decreasing formation of stable cohesin but leaving sufficient Pds5-Wapl complex to remove stable cohesin from chromosomes. Loss of Wapl increases cohesion (54), and thus, we also posit that free Pds5 can establish cohesion to make hyperstable cohesin (Fig. 7).

Cohesin dynamics and gene expression. We undertook the FRAP studies to increase understanding of how changes in Nipped-B, Pds5, and cohesin dosages alter gene expression without overtly affecting sister chromatid cohesion or chromosome segregation in *Drosophila*, zebrafish, mice, and humans. A strong genome-wide correlation between cohesin binding near transcription start sites measured by chromatin immunoprecipitation and microarray analysis (ChIP-chip) and significant effects on transcript levels in both *Drosophila* and human cells argues that cohesin directly regulates transcription of hundreds of genes (26, 42). The FRAP studies imply that the stable-binding form of cohesin is most important for regulating transcription. This is because stable cohesin showed larger changes than weak-binding cohesin with heterozygous *Nipped-B* and *pds5* mutations that alter *in vivo* gene expression. The *Nipped-B* mutation reduced the stable cohesin fraction by a third from 18 to 12% of total cohesin, and the *pds5* mutation increased the stable cohesin fraction 1.6-fold to 28% of total cohesin. The *Nipped-B* mutation, however, did not significantly affect the fraction of weak-binding cohesin, while the *pds5* mutation decreased this fraction by a quarter, from 49% of the total to 37%. How stable cohesin regulates transcription remains to be determined, and we also do not know if the global changes measured by FRAP occur at all cohesin-binding genes.

We also note that the effects of the heterozygous *wapl* mutation on cohesin binding are very similar to those of the *Nipped-B* mutation, decreasing the amount of stable cohesin, without affecting the weak-binding fraction. *NIPBL* mutations have been discovered in slightly more than half of clinically diagnosed cases of CdLS, and amino acid-changing mutations in *SMC1A* account for only 5% of cases (reviewed in references 7 and 25). Thus, the FRAP analysis suggests that heterozygous *WAPL* mutations might be responsible for some of the CdLS cases in which *NIPBL*, *SMC1A*, or *SMC3* mutations have not been found.

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