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Shuya Wang August 7, 2018

# The Role of Transcription Elongation, Cohesin, and Condensin II in Mediating Gene Looping in *Drosophila melanogaster*

by

Shuya Wang

Dr. Victor Corces
Adviser

Department of Biology

Dr. Victor Corces

Adviser

Dr. Levi Morran

Committee Member

Dr. Matthew Weinschenk

Committee Member

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Shuya Wang

Dr. Victor Corces

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## Abstract

### The Role of Transcription Elongation, Cohesin, and Condensin II in Mediating Gene Looping in *Drosophila melanogaster*

By Shuya Wang

Chromatin is a complex of DNA, proteins, and RNA that locate within the nucleus of eukaryotic cells. Investigating the mechanisms of the 3D organization of chromatin can refine our understanding of genomic processes, such as gene expression and replication. With the development of a technique, Hi-C, we are able to visualize the 3D folding of chromatin and chromatin organization features, including compartments and gene loops, were explored. My project focuses on how transcription elongation, cohesin, and condensin II interact with each in mediating gene looping in *Drosophila melanogaster*. The results suggest that transcription elongation and cohesin can independently promote loop formation in *Drosophila melanogaster*. By contrast, condensin II can independently counter such interactions.

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# Introduction

Chromatin is a complex of DNA, proteins, and RNA that is organized within the nucleus of eukaryotic cells (Mondal, et al., 2010). The three-dimensional organization of chromatin is a product of specific physical interactions between loci and the compartmentalization of the genome (Lieberman-Aiden et al., 2009). Deciphering the mechanisms of 3D chromatin folding will aid in understanding genomic processes, such as transcription and replication (Berkum et al., 2010). The development of a technique called Hi-C has enabled the visualization of the spatial arrangement of chromatin genome-wide. To perform Hi-C, chromatin is cross-linked with formaldehyde and then digested with a restriction enzyme, after which DNA fragments undergo ligation and are sequenced using high-throughput sequencing (Lieberman-Aiden et al., 2009). Hi-C data is typically shown as a heatmap, which displays the long-range contacts of each locus and is an efficient visualization method for genome interactions (Berkum et al., 2010).

Hi-C maps in mammals show a clear checkerboard pattern indicative of the segregation of active and inactive chromatin into A and B compartments respectively (Lieberman-Aiden et al., 2009). In *Drosophila melanogaster*, recent high-resolution Hi-C allowed the identification of much smaller compartments than those previously identified (Rowley et al., 2017). This fine-scale organizational pattern arises due to interactions among loci in the same chromatin states. The compartment pattern in *Drosophila* was shown to be most correlated with the segregation of active and inactive transcription such that contiguous stretches of the genome that are active form A compartmental domains while contiguous inactive stretches form B compartmental domains (Rowley et al., 2017).

If we further increase Hi-C resolution and zoom in on the heatmap, we are able to visualize intense interaction domains that correspond to individual genes (Rowley et al., 2017). The frequent intra-genic interactions between the transcription start sites (TSSs) and transcription termination sites (TTSs) are commonly referred to as gene loops and have been detected in other organisms including yeast and plants (O'Sullivan et al., 2004; Liu et al., 2016). Although gene loops are prevalent, the mechanism of loop formation has been unclear. A recent study has suggested that the strength of gene looping is correlated with expression and that inhibition of expression by the degradation of RNAPII via triptolide treatment disrupts *Drosophila* chromatin organization (Hug et al., 2017; Rowley et al., 2017). However, because triptolide treatment disrupts both transcription elongation and degrades paused RNAPII, it is unclear whether gene loops depend on RNAPII at promoters or on transcription elongation.

As part of this thesis, others in the lab tested the dependence of gene loops on transcription elongation. By performing RNAPII HiChIP, a method providing a view of long-range contacts associated with proteins of interest (Mumbach et al., 2016), they found high contact frequencies between TSSs and TTSs, and within gene bodies of elongating genes relative to the paused genes. Analysis of Hi-C, which lists the comprehensive chromatin contacts, has shown a similar pattern. Overall, this suggests that transcription elongation correlates with the propensity to form intra-genic interactions in *Drosophila*.

To test whether gene loops depend on elongating RNAPII, people in our lab performed Hi-C after treatment with flavopiridol (Flv) which inhibits RNAPII Serine 2 phosphorylation and pauses RNAPII at TSSs (Chen et al., 2005). They found that elongating genes show decreased intra-genic interactions after Flv treatment despite retaining binding of RNAPII at TSSs as

shown in ChIP-seq. This finding shows that the physical contact between the TSS and TTS of a gene, defined as a gene loop in some studies (Chowdhary et al., 2017; O'Sullivan et al., 2004), depends on elongating RNAPII. Overall, their results show that the Flv-mediated inhibition of transcription elongation can interrupt intra-genic interactions in *Drosophila* cells.

While gene loops and compartmental domains seem to be a major form of genome organization in *Drosophila*, architectural proteins likely also play a role. In mammals, a mechanism based on the chromatin extrusion complex cohesin has been proposed to explain chromatin loop formation (Nasmyth et al., 2001). In the extrusion model, chromatin is passed through cohesin until meeting a barrier to form loops (Sanborn et al., 2015). In mammals, Hi-C has detected frequent point-to-point CTCF loops (Guo et al., 2015; Rao et al., 2014; Tang et al., 2015). The depletion of cohesin can eliminate such structures, indicating the necessity of cohesin in mediating loop formation (Nora et al., 2017; Rao et al., 2017; Schwarzer et al., 2017; Wutz et al., 2017). While CTCF loops are not observed in *Drosophila* Hi-C maps (Rowley et al., 2017), our lab has tested whether cohesin is required for intra-genic interactions in *Drosophila*, laying a basis for this thesis. They used RNA interference (RNAi) to knock down Rad21 (Rad21 KD) and examined its effects on intra-genic interactions in elongating genes. By comparing the changes in the Hi-C signal before and after Rad21 KD, they found a decrease in intra-genic interactions, especially the interactions between TSSs and TTSs. This is similar to the effect seen by inhibiting transcription elongation. In summary, the data indicate that both transcription elongation and cohesin can mediate intra-genic interactions.

Cohesin and condensin II are both structural maintenance of chromosomes (SMC) protein complexes found in eukaryotic cells (Rao et al., 2017; Schwarzer et al., 2017; Wutz et al.,

2017). Evidence has shown that the depletion of Cap-H2, a subunit of condensin II, causes an increase in enhancer-promoter interactions in *Drosophila*, suggesting that condensin II antagonizes enhancer-promoter interactions (Li et al., 2016). However, whether condensin II can disrupt gene looping in *Drosophila* remained to be explored. Therefore, others in the lab examined the role of condensin II in mediating intra-genic interactions in *Drosophila*. They first knocked down the subunit of condensin II, Cap-H2, and then performed a Hi-C analysis. The effect of Cap-H2 KD on intra-genic interactions was opposite to what we saw in Rad21 KD. Because Cap-H2 KD leads to an increase in intra-genic interactions, condensin II is likely to inhibit intra-genic chromatin interactions.

It was shown that both the inhibition of transcription elongation and the depletion of Rad21, a subunit of cohesin, can cause a decrease in gene looping, especially among elongating genes. Moreover, evidence shows that the induction of transcription in vivo leads to the relocation of cohesin in yeast (Davidson et al., 2016). Thus, I tested whether RNAPII mediates gene loop formation by pushing cohesin along chromatin. To test this, I examined the interplay between transcription elongation and cohesin occupancy in *Drosophila* cells. In addition, the increase in gene looping after Cap-H2 depletion and the structural similarity between cohesin and condensin II drove us to ask whether RNAPII can alter condensin II occupancy to affect intra-genic interactions.

# Result

## *The Relationship Between Cohesin and Transcription Elongation*

Inhibition of transcription elongation and depletion of the cohesin subunit Rad21 both result in decreased intra-genic interactions. Because it was found that RNAPII can relocate cohesin along the yeast genome (Ocampo-Hafalla et al., 2016), I questioned if transcription elongation can push cohesin along chromatin and thereby influence chromatin organization in *Drosophila*. To test this, I performed Rad21 ChIP-seq after Flv treatment to see whether there is a change in cohesin binding (Figure 1). However, the signal in my ChIP-seq displayed high background signal with few specific binding sites (Figure 1, middle track). This effect was not reproducible in replicates performed by others in the lab (Figure 1, bottom track). Analysis of these other replicates found no apparent change in cohesin occupancy after Flv treatment compared to the control, suggesting that transcription elongation does not cause cohesin repositioning in *Drosophila*.

However, in turn, cohesin could possibly affect RNAPII occupancy and thereby subsequently influence intra-genic interactions. To test this, I performed RNAPII ChIP-seq after knocking down Rad21. First, I verified the knock-down efficiency of Rad21 in Kc167 cells by Western blot analysis after RNAi treatment. This indicated knockdown of Rad21 (Figure 2A). However, there were some other unexplained differences especially at larger sizes between RNAi treated cells and the control (Figure 2A). Therefore, knockdown of Rad21 cells was repeated and verified by others in the lab. I then performed RNAPII ChIP-seq in Rad21 KD cells and

observed high signal to background levels comparable to the control (Figure 2B). After others in the lab produced replicates of these data, it was found that there are no obvious changes of RNAPII binding in Rad21 KD (Figure 2B). Therefore, we conclude that elongating RNAPII and cohesin affect chromatin organization independently from each other.

*The effect of Transcription elongation on condensin II*

Because our lab has shown that Cap-H2 is enriched in gene bodies of elongating genes and condensin II shares structural similarities with cohesin, I then asked whether transcription elongation can relocate Condensin II. I performed ChIP-seq for Cap-H2 after Flv treatment but found little to no specific signal compared to previously obtained ChIP-seq data in control cells (Figure 3). Replicates done by others improved the signal and were subsequently used in the downstream analysis (Figure 3, bottom track). However, they found little change in Cap-H2 occupancy within the bodies of elongating genes.

They then tested whether Condensin II binding can influence elongating RNAPII but did not detect any change in the RNAPII positioning. Overall, the result suggests that transcription elongation and condensin II likely function independently in their effects on chromatin organization.

## Discussion

Our results indicate that transcription elongation and cohesin independently promote intra-genic interactions in *Drosophila* chromatin. Additionally, condensin II independently counters the formation of gene loops by inhibiting such interactions. A possible explanation would be that RNAPII and cohesin both play a partial role in mediating intra-genic interactions, and inhibiting either one would reduce gene looping but not eliminate it totally. Specifically, how these proteins maintain normal chromosome organization needs further clarification.

Last but not least, our findings shed light on the mechanisms by which short-range chromatin interactions in *Drosophila* are established. Besides intra-genic interactions, including gene loops, chromosomes are segregated into active A and inactive B compartments by long-range interactions (Lieberman-Aiden et al., 2009). Our lab has found that the knockdown of cohesin causes a decrease in interactions between A compartments in *Drosophila*, with similar effects observed when transcription elongation is inhibited. By contrast, condensin II depletion increases A-A compartmental interactions and gene loop formation. Identification of the exact mechanisms by which chromatin proteins, including cohesin and condensin II, form these compartmental interactions is of future interest.

# Methods

## *Drosophila cell culture and Flavopiridol (Flv) treatment*

Kc167 cells were acquired from the *Drosophila* Genomics Resource Center (DGRC), maintained at 27°C and subcultured regularly in Hyclone SFX insect culture media. To inhibit transcription elongation, Kc167 cells were treated with 1 μM flavopiridol for 3 h.

## *RNAi*

Rad21 dsRNA was generated using the Ambion MEGAscript Kit and incubated at 37°C overnight followed by deoxyribonuclease (DNase) treatment to remove the template DNA. dsRNA production was assessed by running 2 μL of each transcription reaction diluted 5-fold on a gel. Gels were stained with ethidium bromide and visualized on a UV transilluminator. Kc167 cells were split by 1:3 ratio the day before the first Rad21 knockdown treatment. On the first day of the experiment, Rad21 dsRNA was initially mixed with SFX media and then added to 20 million cells, with a final concentration of 3*10^-6 M. On the third day, the same process is repeated. On the fourth day, both control and treatment cells were transferred to T-75 flasks with the addition of another 20 μL of dsRNA. On the fifth day, cells were harvested and fixed for prospective ChIP-seq experiments. Approximately 2 million Kc167 cells were used for western blotting to verify the effectiveness of Rad21 knockdown on Kc167 cells. In this five-day treatment, I used 8 μl/ml of Cellfectin II reagent, a cationic-lipid formulation designed for optimal transfection of insect cells, to further improve the knockdown efficiency.

*Western Blotting*

I used 2 million Kc167 cells for SDS PAGE analysis. Cells were first resuspended in 2X sample buffer (125 mM Tris pH = 6.8, 4% SDS, 20% Glycerol, 10% BME, and bromophenol blue) and boiled for 10 min. Then gel electrophoresis was run at 120 V to separate protein macromolecules. Subsequently, the separated proteins were transferred onto a nitrocellulose or polyvinylidene difluoride (PVDF) membrane. Next, the PVDF membrane was blocked for 30 min with blocking solution (5% milk in PBS) to prevent any non-specific binding of antibodies to the surface of the membrane. The membrane was then incubated with the primary antibody specific to the protein of interest and a secondary antibody specific to the host species of the primary antibody. The chromogenic substrate, which was obtained from ThermoFisher, was added to the membrane and interacts with the secondary antibody to form chemiluminescent signal. The signal output was finally captured on film.

*Chromatin Immunoprecipitation Sequencing (ChIP-seq)*

*Cell Lysis and Chromatin Sanitation*

First, formaldehyde was added to about 1x10^6 Kc167 at a final concentration of 1%. Then cells were rotated at room temperature for 10 min. Glycine (0.125 M final concentration) was added to quench crosslinking. Kc167 cells were incubated at room temperature for 5 minutes with rotation. Cells were centrifuged at 2000 rpm 2 min at 4°C and washed twice with cold PBS. Then cells were lysed in cell lysis buffer with protease inhibitors and incubated on ice for 15 min. I collected nuclei by centrifugation at 4000 rpm for 8 min at 4°C. Each ChIP was added with 100 uL cold IP dilution buffer with 1 uL protease inhibitors prior to sonication.

Samples were sonicated in a Bioruptor (Output setting High, 30/60 28 cycles). Lysates were centrifuged at max speed in a microcentrifuge for 10 minutes at 4 °C to pellet cellular debris and supernatants were transferred to a clean tube and flash frozen in liquid nitrogen or went directly into the ChIP protocol.

*Chromatin Immunoprecipitation*

Protein A or G beads were washed five times with IP dilution buffer. The supernatant (sheared chromatin) was added to IP-dilution buffer and Protein A or Protein G Dynabeads (Life Technologies). The mixture was incubated for 2 h at 4°C with rocking to preclear the chromatin. Beads were collected on a magnet. Antibody (3 µl) was added to chromatin and incubated at 4°C overnight with rotation. The following antibodies were utilized: Rad21, and RNAPII. Protein A or G Dynabeads were added for 2 h at 4°C and beads were collected on the magnet. I performed 3X washing with the low salt wash buffer, each time with 5 min of rotation at RT. Then followed 2X washing with a high salt wash buffer, 2X washing with the LiCl wash buffer, and 1X with TE as before. All buffer was supplemented with proteinase inhibitors. Then samples were eluted twice in 400 µL freshly prepared IP Elution Buffer. After elution, samples were placed at 68°C for 15 min and vortexed for every 2 min. After centrifugation, samples were collected on magnetic stands and supernatant was transferred to clean tubes. 20 µL of 5 M NaCl, 8 µL of 0.5 M EDTA, 16 µL of 1 M Tris, and 8 µL of 10 mg/mL RNaseA were added and incubated for 6 h at 65 °C to reverse cross-linking. 8 µl of 10 mg/ml Proteinase K was added and incubated at 50°C for 2 h. I extracted DNA with Phenol:Chloroform:Isoamyl alcohol (25:24:1). Then added glycogen and NaAc prior to the addition of 2.5 volumes ice-cold EtOH. Samples were incubated

at -80°C for 30 min. DNA was isolated by centrifugation, washed with 70% EtOH, and air dried. DNA was resuspended in 26.5 µl Elution Buffer for library generation.

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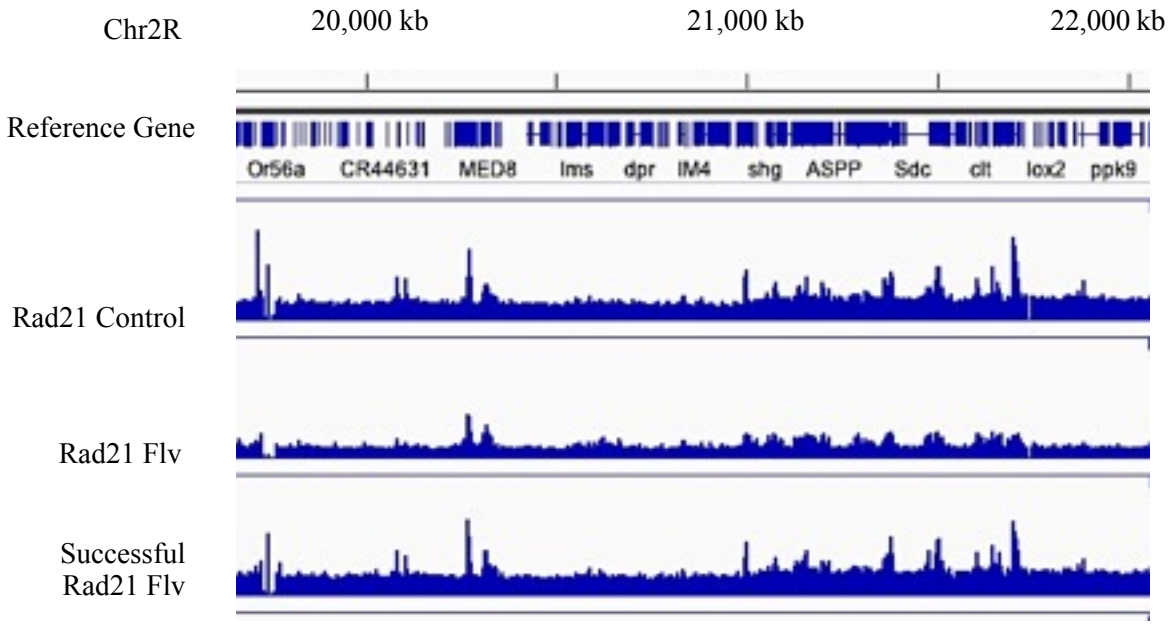


Figure 1. The effect of transcription elongation on cohesin occupancy.

IGV tracks including the Rad21 ChIP-seq in the control done by others (top track) and the Rad21 ChIP-seq after Flv treatment done by myself (middle track) or by others (bottom track).

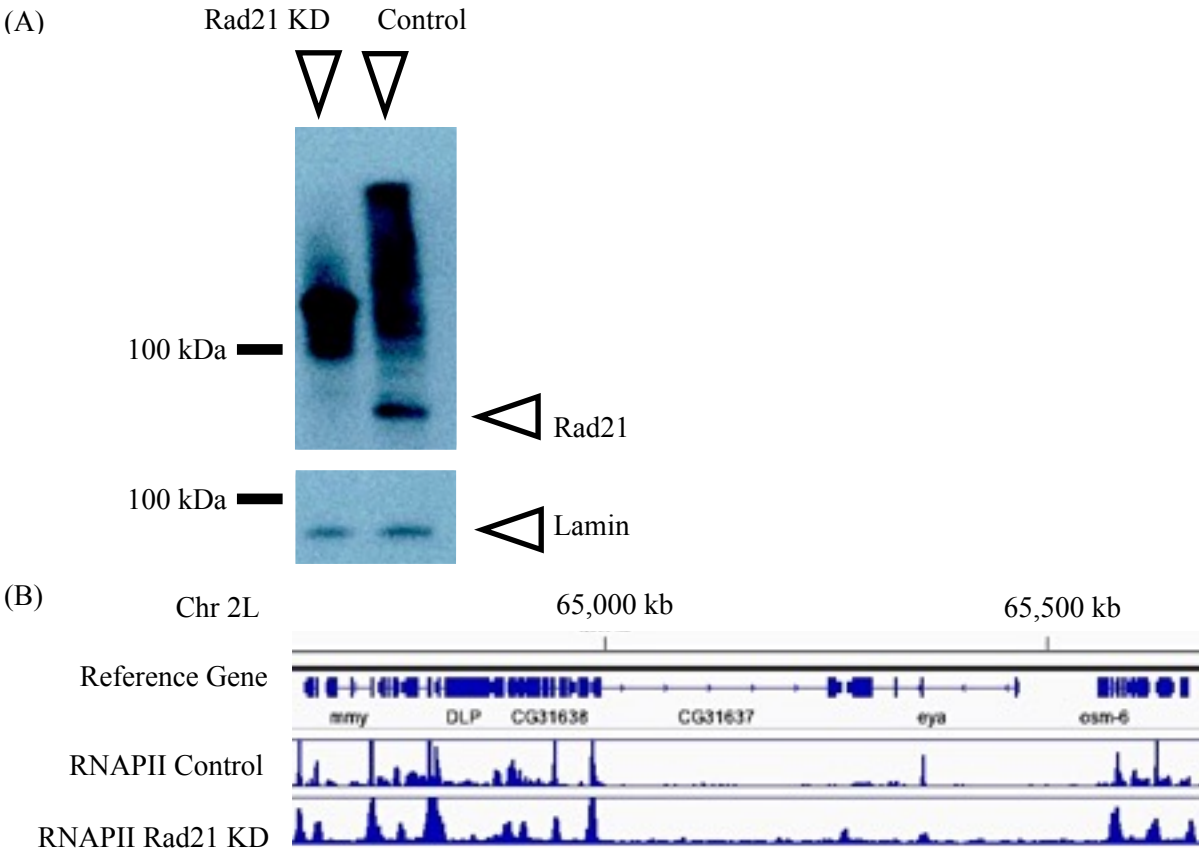


Figure 2 The effect of cohesin KD on RNAPII.

(A)Western blot of Rad21 in Rad21 KD and control Kc167 cells. Arrows on the right indicate the protein of interest. Lamin is used for a loading control.

(B) IGV tracks including the RNAPII ChIP-seq in the control done by others (top track) and in the Rad21 KD Kc167 cells done by me (bottom track)。

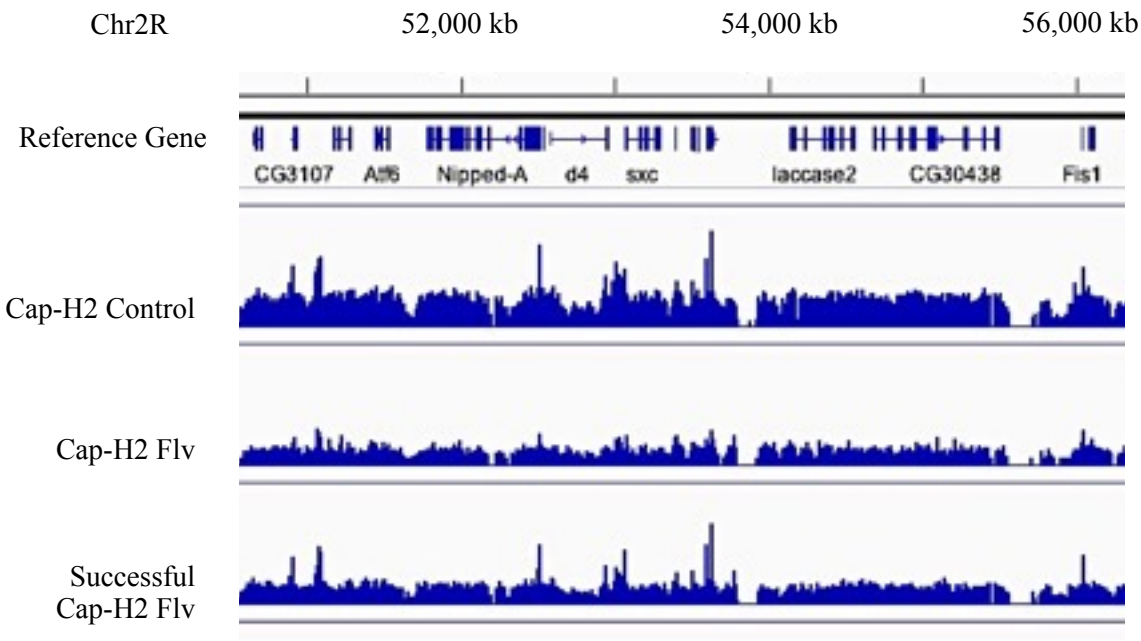


Figure 3 The effect of transcription elongation on condensin II.

IGV tracks include the Cap-H2 ChIP-seq on the control done by others (top track) and after Flv treatment done by me (middle track) or by others (bottom track).