1.

At several loci there is transcription of ncRNA leading to heterochromatin formation and silencing

This suggests that mechanisms should exist linking these ncRNA to proteins that modify histones and DNA

In some cases, deletion of one component of the RNA interference pathway leads to defective heterochromatin establishment or spreading

2.
Heterochromatic domains and euchromaic domains are separated by "insulators" or "boundaries"

How do small RNAs generated by the RNAi machinery initiate heterochromatin assembly in fission yeast ?

RNAi-Mediated Targeting of Heterochromatin by the RITS Complex

André Verdel,[1] Songtao Jia,[2] Scott Gerber,[1,3] Tomoyasu Sugiyama,[2] Steven Gygi,[1,3] Shiv I. S. Grewal,[2]* Danesh Moazed[1]*

RNA interference (RNAi) is a widespread silencing mechanism that acts at both the posttranscriptional and transcriptional levels. Here, we describe the purification of an RNAi effector complex termed RITS (RNA-induced initiation of transcriptional gene silencing) that is required for heterochromatin assembly in fission yeast. The RITS complex contains Ago1 (the fission yeast *Argonaute* homolog), Chp1 (a heterochromatin-associated chromodomain protein), and Tas3 (a novel protein). In addition, the complex contains small RNAs that require the Dicer ribonuclease for their production. These small RNAs are homologous to centromeric repeats and are required for the localization of RITS to heterochromatic domains. The results suggest a mechanism for the role of the RNAi machinery and small RNAs in targeting of heterochromatin complexes and epigenetic gene silencing at specific chromosomal loci.

The Chp1 protein binds to centromeric repeats and is required for H3K9 methylation and for Swi6 (hu-HP1 homolog) binding.

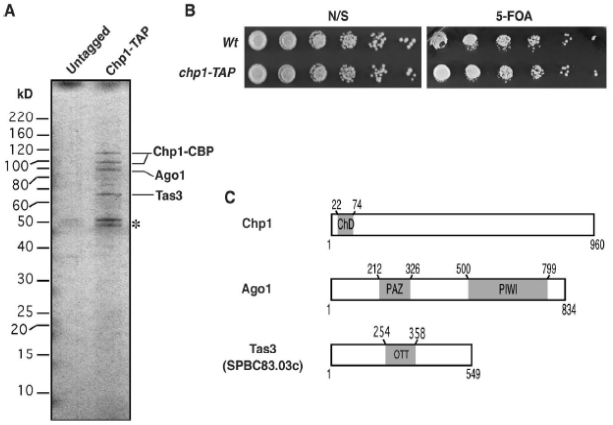
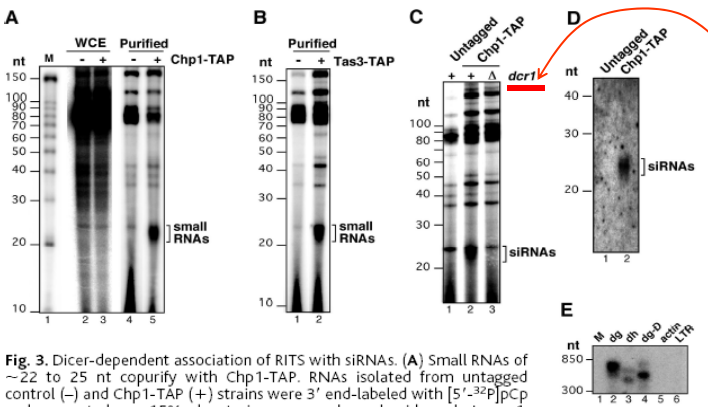


Fig. 1. Purification of Chp1-TAP and identification of associated proteins. Extracts from a Chp1-TAP strain and an untagged control strain were purified by the TAP procedure and applied to a 4 to 12% polyacrylamide gel, which was stained with colloidal Coomassie blue (**A**). The bands in the Chp1-TAP purification were excised from the gel and sequenced by tandem mass spectrometry (*22*). The identity of each band is based on multiple sequenced peptides and is indicated on the right. *Residual GST-TEV, the protease used for elution from the first affinity column. (**B**) The Chp1-TAP protein was fully functional for silencing of a centromeric *imr::ura4*$^{+}$ reporter gene as indicated by wild-type levels of growth on 5-FOA medium, which only allows growth when *ura4*$^{+}$ is silenced. N/S, nonselective medium. (**C**) Schematic diagram showing the subunits of the RITS complex and their conserved motifs. The chromodomain (ChD) in Chp1, the PAZ and PIWI domains in Ago1, and a region of sequence similarity between Tas3 and the mouse OTT (ovary testis transcribed) protein are indicated.

RITS complex

RNA-induced Initiation of Transcriptional Silencing



Deletion of Dicer abrogate small RNA component of RITS

Fig. 3. Dicer-dependent association of RITS with siRNAs. (**A**) Small RNAs of ~22 to 25 nt copurify with Chp1-TAP. RNAs isolated from untagged control (–) and Chp1-TAP (+) strains were 3′ end-labeled with [5′-^{32}P]pCp and separated on 15% denaturing urea polyacrylamide gel. Lane 1, [γ-^{32}P]ATP–labeled RNA markers (Ambion); lanes 2 and 3, labeling of RNA from whole-cell extract (WCE) (~1/2500 of input); lanes 3 and 4, labeling of RNAs after purification. Bracket on the right side indicates the position of small RNAs specifically associated with Chp1-TAP. (**B**) Copurification of small RNAs with Tas3-TAP. (**C**) No small RNAs are associated with RITS purified from *dcr1*Δ cells. Parallel purifications were performed from an untagged (control, lane 1) strain as well as *chp1-TAP, dcr1*$^{+}$ (lane 2) and *chp1-TAP, dcr1*Δ (lane 3) cells, and the associated RNAs were [5′-^{32}P]pCp labeled (compare lanes 2 and 3, bracket). (**D**) Northern blot showing that siRNAs associated with RITS hybridize to ^{32}P-labeled probes corresponding to centromeric repeat sequences. RNA from untagged control (lane1) and Chp1-TAP cells (lane 2), purified as described in (B), was separated on a denaturing gel and electrotransferred to a nylon membrane (*22*). DNA oligonucleotides with sequence complementary to the 12 heterochromatic siRNAs identified by Reinhart and Bartel (*16*) were 5′ labeled with [γ-^{32}P]ATP and used as probes for the Northern blot. (**E**) Southern blot showing that RITS contains siRNAs complementary to the outer centromeric repeats (*otr*). *dg* (lanes 2 and 4) and *dh* (lane 3) repeats, *actin* (lane 5), and LTRs (lane 6) were amplified by polymerase chain reaction (PCR) from genomic DNA, separated on 1.1% agarose gel, and transferred to nylon membrane. ^{32}P-labeled RITS siRNAs, obtained by labeling RNAs as described in (A), were separated on a denaturing urea gel, eluted, and used as probes for the blot.

Localization of RITS to heterochromatin

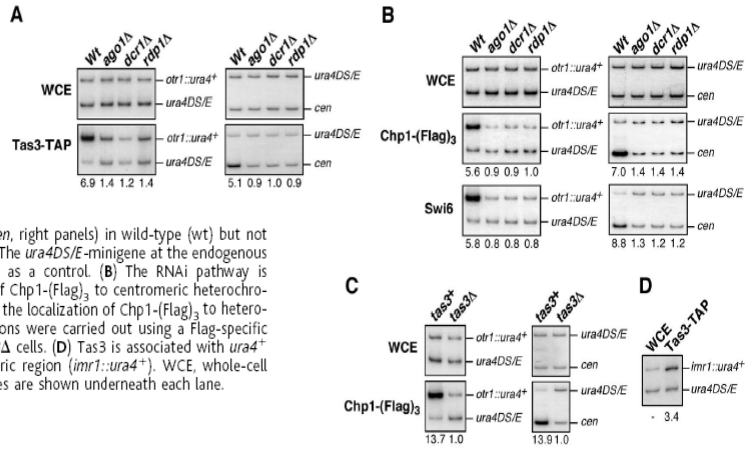


Fig. 4. The RNAi pathway is required for localization of RITS to heterochromatin. (**A**) ChIP experiments showing that Tas3-TAP is localized to centromeric heterochromatin in an RNAi-dependent manner. Tas3-TAP is associated with *ura4*$^+$ inserted at the *otr* centromeric repeats (*otr1::ura4*$^+$, left panels) and with native centromeric repeat sequences (*cen*, right panels) in wild-type (wt) but not *ago1*Δ, *dcr1*Δ, or *rdp1*Δ cells. The *ura4DS/E*-minigene at the endogenous euchromatic location is used as a control. (**B**) The RNAi pathway is required for the localization of Chp1-$(Flag)_3$ to centromeric heterochromatin. (**C**) Tas3 is required for the localization of Chp1-$(Flag)_3$ to heterochromatin. Immunoprecipitations were carried out using a Flag-specific antibody from *tas3*$^+$ and *tas3*Δ cells. (**D**) Tas3 is associated with *ura4*$^+$ inserted at the *imr* centromeric region (*imr1::ura4*$^+$). WCE, whole-cell extract. Fold enrichment values are shown underneath each lane.

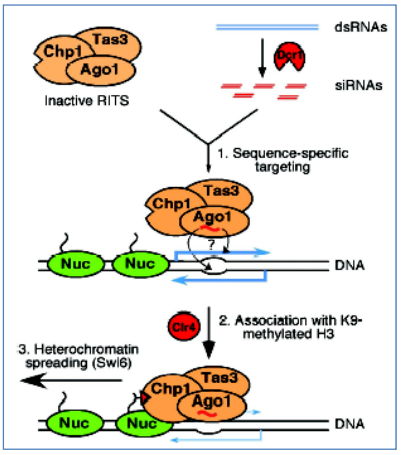


Fig. 5. A model for siRNA-dependent initiation of heterochromatin assembly by RITS. The RITS complex is programmed by Dcr1-produced siRNAs to target specific chromosome regions by sequence-specific interactions involving either siRNA-DNA or siRNA-nascent transcript (blue arrows) base pairing. Nuc, nucleosome; red triangle, K9-methylation on the amino terminus of histone H3. See text for further discussion and references.

Interaction of RITS with several chromatin-modifying enzymes (Histone Methyl Transferases HMT, DNA Methyl Transferases DMT) was demonstrated using both co-immunoprecipitation experiments and by showing binding of RITS together with HMTs and DMTs to the same regions of DNA, by chromatin immunoprecipitation (ChIP).

(Re-ChIP insert)

In S. pombe, the RdRP enzyme (not present in higher animals) is also localized to the centromere, thus suggesting a further amplification mechanism for centromeric siRNA.

Swi6 in yeast = HP1

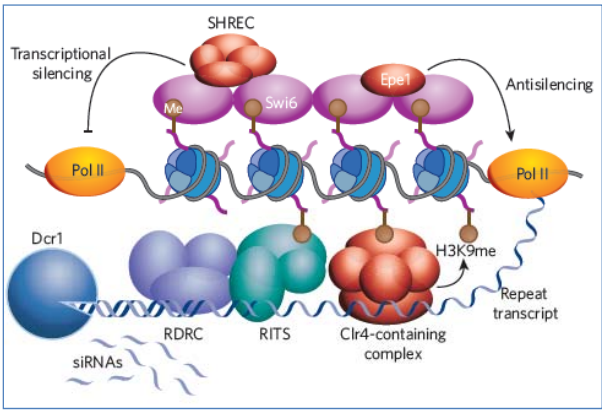


Figure 4 | Model showing RNAi-mediated heterochromatin assembly and silencing in **S. pombe**. Centromeric repeat (dg and dh) transcripts produced by Pol II are processed by the RNAi machinery, including the complexes RITS and RDRC (which interact with each other and localize across heterochromatic regions). The slicer activity of Ago1 (a component of RITS) and the RNA-directed RNA polymerase activity of Rdp1 (a component of RDRC) are required for processing the repeat transcripts into siRNAs. The siRNA-guided cleavage of nascent transcripts by Ago1 might make these transcripts preferential substrates for Rdp1 to generate double-stranded RNA, which in turn is processed into siRNAs by Dcr1. The targeting of histone-modifying effectors, including the Clr4-containing complex, is thought to be mediated by siRNAs. This process most probably involves the base-pairing of siRNAs with nascent transcripts, but the precise mechanism remains undefined. siRNAs produced by heterochromatin-bound RNAi 'factories' might also prime the assembly of RISC-like complexes capable of mounting a classic RNAi response. Methylation of H3K9 by Clr4 is necessary for the stable association of RITS with heterochromatic loci, apparently through binding to the chromo-domain of Chp1. This methylation event also recruits Swi6, which, together with other factors, mediates the spreading of various effectors, such as SHREC. SHREC might facilitate the proper positioning of nucleosomes to organize the higher-order chromatin structure that is essential for the diverse functions of heterochromatin, including transcriptional gene silencing. Swi6 also recruits an antisilencing protein, Epe1, that modulates hetero-chromatin to facilitate the transcription of repeat elements, in addition to other functions. A dynamic balance between silencing and antisilencing activities determines the expression state of a locus within a heterochromatic domain.

Tethering RITS to a Nascent Transcript Initiates RNAi- and Heterochromatin-Dependent Gene Silencing

Marc Bühler,[1] André Verdel,[1] and Danesh Moazed[1,*]
[1]Department of Cell Biology, Harvard Medical School, Boston, MA 02115, USA

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SUMMARY

In the fission yeast *Schizosaccharomyces pombe*, the RNA-Induced Transcriptional Silencing (RITS) complex has been proposed to target the chromosome via siRNA-dependent base-pairing interactions to initiate heterochromatin formation. Here we show that tethering of the RITS subunit, Tas3, to the RNA transcript of the normally active *ura4*$^+$ gene silences *ura4*$^+$ expression. This silencing depends on a functional RNAi pathway, requires the heterochromatin proteins, Swi6/HP1, Clr4/Suv39h, and Sir2, and is accompanied by the generation of *ura4*$^+$ siRNAs, histone H3-lysine 9 methylation, and Swi6 binding. Furthermore, the ability of the newly generated *ura4*$^+$ siRNAs to silence a second *ura4*$^+$ allele in *trans* is strongly inhibited by the conserved siRNA nuclease, Eri1. Surprisingly, silencing of tethered *ura4*$^+$, or *ura4*$^+$ inserted within centromeric heterochromatin, or some of the endogenous centromeric repeat promoters, is not associated with changes in RNA polymerase II occupancy. These findings support a model in which targeting of nascent transcripts by RITS mediates chromatin modifications and suggest that co-transcriptional processing events play a primary role in the silencing mechanism.

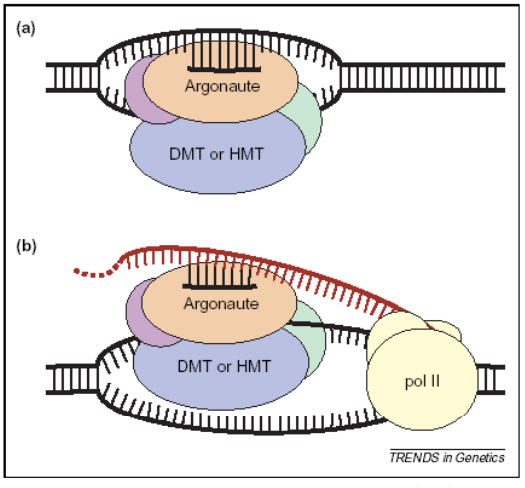


Figure 1. Models for siRNA-directed chromatin modification. DNA (DMT) or histone (HMT) methyltransferase activity is thought to be recruited to target loci by a RITS-like complex that includes an argonaute protein and an siRNA. Two potential mechanisms for target recognition are: **(a)** siRNA binding to target DNA; or **(b)** siRNA binding to nascent transcripts produced from target DNA by RNA polymerase II (pol II).

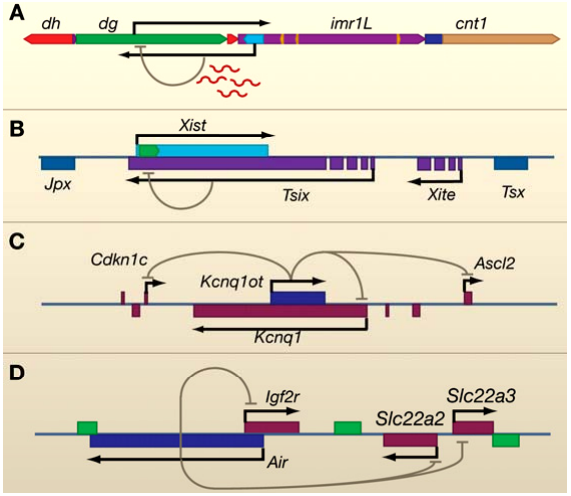
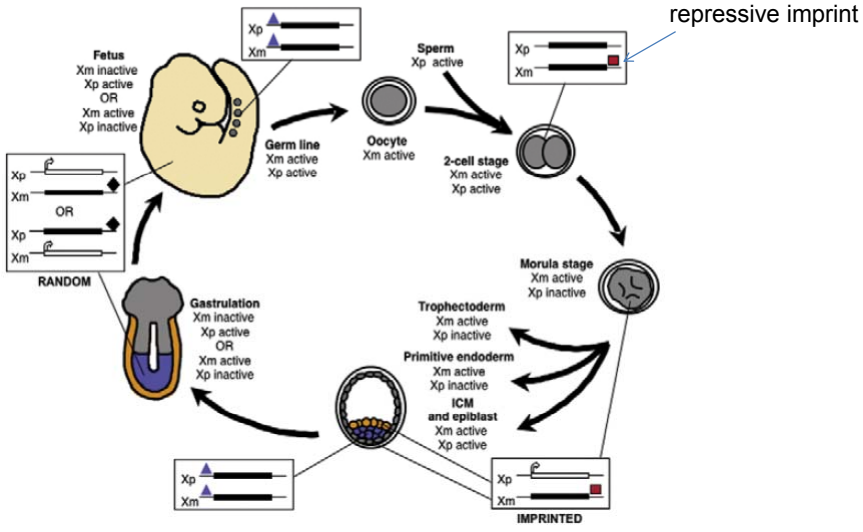


Figure 3. Silencing Transcripts at the S. pombe Centromere and Different Transcript-Mediated Silencing Systems

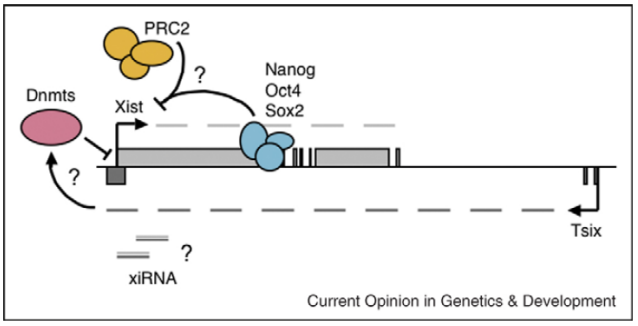
(A) S. pombe dg-imr transcript in centromere IL. The forward promoter is silenced by constitutive transcription and processing into siRNA of the reverse strand.

(B) The X inactivation center has several noncoding transcripts, and transcription of Tsix silences the Xist promoter.

(C and D) Paternal locus transcription of noncoding transcripts Kcnq1ot and Air influences the expression of overlapping and nonoverlapping genes in the imprinted gene cluster at the telomeric end of mouse chromosome 7 and the Igf2r locus, respectively. Paternally expressed genes are colored blue, maternally expressed genes are colored red, and ubiquitously expressed genes are colored green.



From: Senner, Current Opinion in Genetics & Development 2009, 19:122–126



Factors involved in regulation of the Xist promoter. *Xist* (light grey) is negatively regulated by its antisense transcript *Tsix* (dark grey). The balance of sense and antisense transcription across the promoter influences methylation of the *Xist* promoter by Dnmts (pink), through an as yet uncharacterised mechanism. It has been reported that *Xist* and *Tsix* RNAs form duplexes that are processed by RNAi enzyme Dicer to small xiRNAs. Whether or not xiRNAs regulate *Xist* expression is subject to debate. A *Tsix*-independent pathway also negatively regulates *Xist*. The pluripotency factors Nanog Oct4 and Sox2 (blue) have been shown to repress *Xist* and this is suggested to result from a direct interaction between binding sites for these factors in intron 1 of the gene and the *Xist* promoter. Polycomb repressor complex 2 (PRC2) (orange) has been shown to repress transcription from the *Xist* promoter in the absence of *Tsix* transcription, indicating that it may contribute to the *Tsix*-independent pathway.

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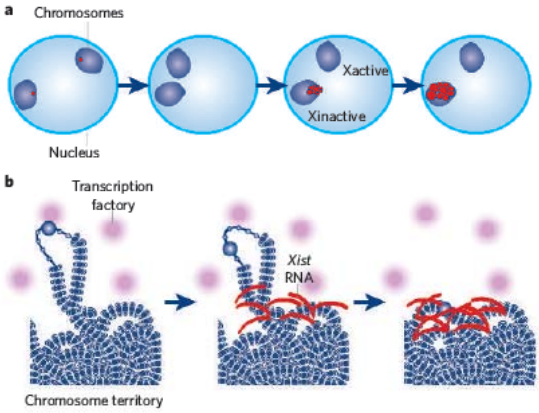
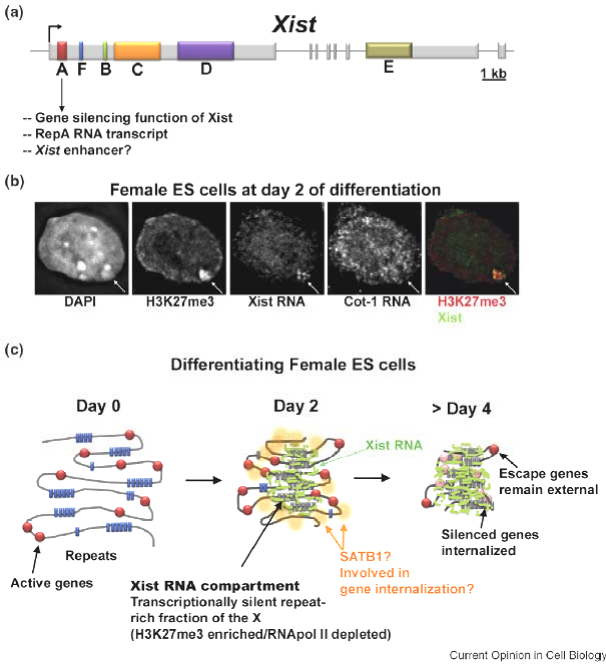


from Frasor, 2007

Figure 1 | Events of nuclear reorganization during X-chromosome inactivation. **a**, Soon after female embryonic stem cells start to differentiate, the two X chromosomes (purple) come together in the nucleus, and the X-inactivation centres, which initiate X-chromosome inactivation, interact[9,10]. These events occur concomitantly with the process of X-chromosome counting and choice[38] and lead to upregulation of *Xist* transcription (red) from the future inactive X chromosome (Xinactive). **b**, The coating of the inactive X chromosome by *Xist* RNA molecules excludes Pol II and the transcriptional machinery (pink) from the inactive X-chromosome territory[22]. Genes initially located outside the domain (purple circles) coated by *Xist* RNA are retracted back inside the *Xist* compartment as they become silenced through a mechanism dependent on the A repeats of *Xist* RNA[22].



(a) Structure of the Xist gene with the conserved repeat regions labeled A–F. The A region (red) denotes the conserved A-repeat region essential for gene silencing.
(b) Combined RNA-immuno-fluorescence on day 2 differentiated female ES cells, showing the Xist-coated transcriptionally silent compartment which is enriched for H3K27me3.
(c) Model. Xist coating induces the formation of a transcriptionally silent repetitive compartment. As genes are silenced they are recruited into this compartment. A possible mediator for this internalization may be the matrix-associated protein SATB1/2.

from Chow, Current Opinion in Cell Biology 2009, 21:359–366

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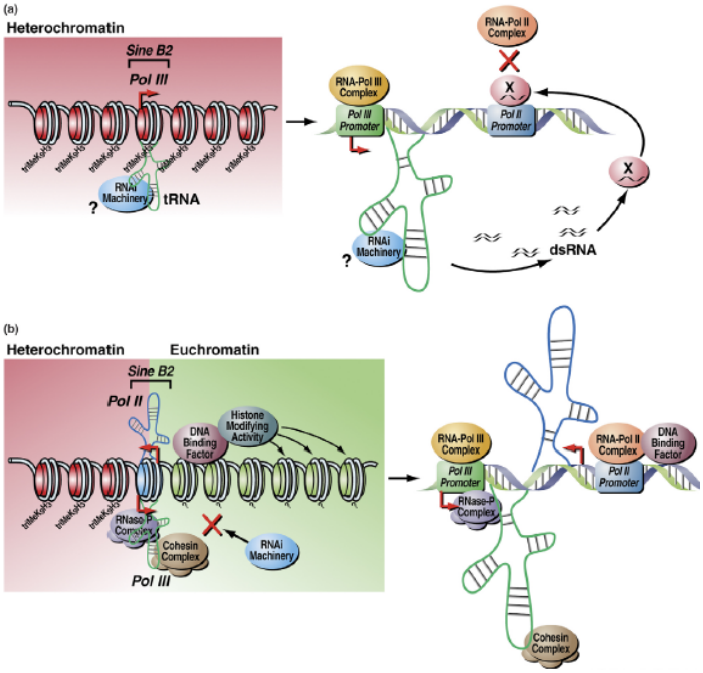
Boundaries. Boundaries... Boundaries???

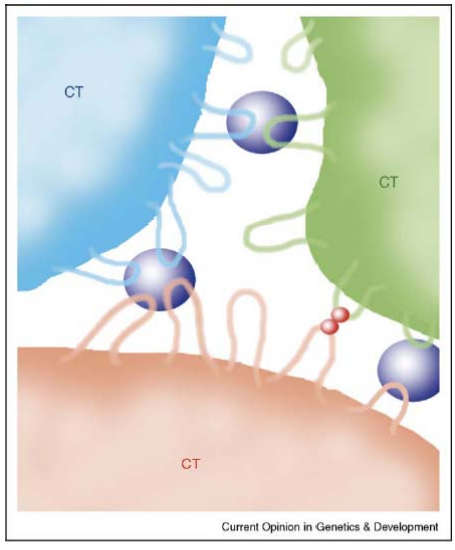
Victoria V Lunyak

One way to modulate transcription is by partitioning the chromatin fiber within the nucleus into the active or inactive domains through the establishment of higher-order chromatin structure. Such subdivision of chromatin implies the existence of insulators and boundaries that delimit differentially regulated chromosomal loci. Recently published data on transcriptional interference from the repeated component of the genome fits the classic definition of insulator/boundary activity. This review discusses the phenomena of transcriptional interference and raises the question about functionality of genomic "junk" along with the need to stimulate a dialogue on how we would define the insulators and boundaries in the light of contemporary data.Rule 19 (a) (Boundaries)"Before the toss, the umpires shall agree the boundary of the field of play with both captains. The boundary shall, if possible, be marked along its whole length"Rules of Cricket

Current Opinion in Cell Biology 2008, **20**:281–287

review





Nuclear organization. Three different chromosome territories (CTs) are shown. Potentially active genes in *cis* and *trans* dynamically engage transcription factories (blue spheres). Most 'active' genes spend the majority of their time outside transcription factories and are transcriptionally inactive. Other types of regulatory interactions (red spheres) might possibly sequester genes or inhibit mobility and factory contact.

review

Nuclear organization of the genome and the potential for gene regulation

Peter Fraser[1] & Wendy Bickmore[2]

Much work has been published on the *cis*-regulatory elements that affect gene function locally, as well as on the biochemistry of the transcription factors and chromatin- and histone-modifying complexes that influence gene expression. However, surprisingly little information is available about how these components are organized within the three-dimensional space of the nucleus. Technological advances are now helping to identify the spatial relationships and interactions of genes and regulatory elements in the nucleus and are revealing an unexpectedly extensive network of communication within and between chromosomes. A crucial unresolved issue is the extent to which this organization affects gene function, rather than just reflecting it.

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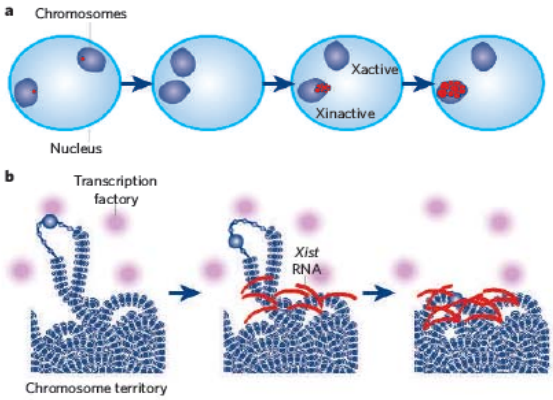

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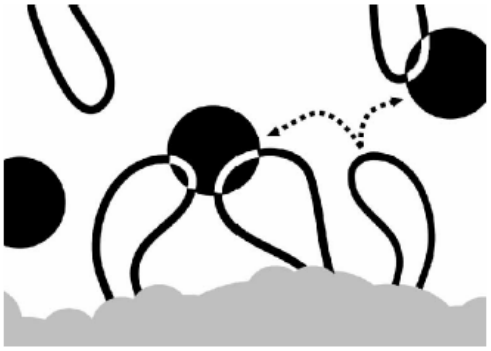

Figure 8 Model of dynamic associations of genes with transcription factories. Schematic representation of chromatin loops (black) extruding from a chromosome territory (gray). Transcribed genes (white) in RNAP II factories (black circles). Potentiated genes (free loops) that are not associated with RNAP II factories are temporarily not transcribed. Potentiated genes can migrate to a limited number of preassembled RNAP II factories to be transcribed (dotted arrows). We propose that both *cis* and *trans* associations are possible.

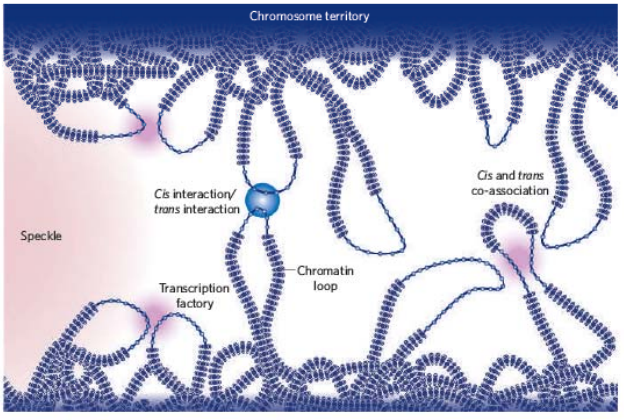

Figure 2 | Colocalization of genes in the nucleus for expression or coregulation. Active genes on decondensed chromatin loops that extend outside chromosome territories can colocalize both *in cis* and *in trans* at sites in the nucleus with local concentrations of Pol II (namely transcription factories; dark pink) and adjacent to splicing-factor-enriched speckles (pale pink). Interactions can also occur between regulatory elements and/or gene loci and lead to coregulation in *trans* (blue circle).

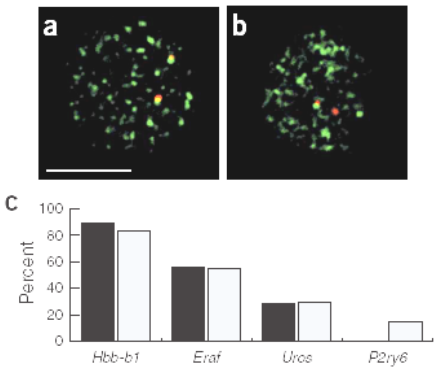

Figure 4 Actively transcribed genes associate with RNAP II foci. (**a**) RNA immuno-FISH of *Hbb-b1* transcription (red) with RNAP II staining (green) in anemic spleen erythroid cells. Scale bar, 5 μm. (**b**) DNA immuno-FISH of *Eraf* (red) with RNAP II staining (green). (**c**) Comparison of the percentage of alleles exhibiting a gene transcription signal by RNA FISH (black), with the percentage of loci that overlap with an RNAP II focus by DNA FISH (gray) for *Hbb-b1* ($n = 83$), *Eraf* ($n = 59$), *Uros* ($n = 47$) and *P2ry6* ($n = 79$).

The 3C assay:
Chromosome Conformation Capture

Find a protocol... and discuss it!