Review

Interchromosomal association and gene regulation *in trans*

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The nucleus is an ordered three-dimensional entity, and organization of the genome within the nuclear space might have implications for orchestrating gene expression. Recent technological developments have revealed that chromatin is folded into loops bringing distal regulatory elements into intimate contact with the genes that they regulate. Such intrachromosomal contacts appear to be a general mechanism of enhancer–promoter communication *in cis*. Tantalizing evidence is emerging that regulatory elements might have the capacity to act *in trans* to regulate genes on other chromosomes. However, unequivocal data required to prove that interchromosomal gene regulation truly represents another level of control within the nucleus is lacking, and this concept remains highly contentious. Such controversy emphasizes that our current understanding of the mechanisms that govern gene expression are far from complete.

Interchromosomal gene regulation in mammals: an emerging field

We are only just beginning to realize the extent of organization within the mammalian nucleus and how this might shape transcriptional regulation. Loci can interact with sequences elsewhere in the genome, leading to the hypothesis that genes can be subjected to regulation *in trans* by regulatory elements on other chromosomes. This concept is not without merit, as a similar but well established phenomenon termed transvection takes place in *Drosophila melanogaster* (Box 1). However, unlike classical transvection, interchromosomal gene regulation in mammals is still highly controversial. We explore recent examples of interchromosomal associations (see Glossary) and discuss whether these represent a chance meeting of genes within the shared nuclear space or whether they provide evidence for functional regulation *in trans*.

Nuclear organization

More than a century ago, studies by Rabl and then Boveri suggested that chromatin was not distributed randomly within the nucleus but occupied distinct regions. However, only recent advances in technology have allowed the confirmation that metaphase chromosomes are indeed organized into discrete, non-overlapping 'territories' [1]. Furthermore, chromosomes adopt non-random positions within the nucleus with gene-rich chromosomes being located preferentially towards the center of the nucleus, an arrangement that is retained in many different cell types and appears to be conserved through evolution [2–11]. It is also well documented that heterochromatin and euchromatin segregate within the nucleus, forming chromatin neighborhoods with similar properties [12]. Within the relatively fixed nuclear positions of chromosome territories, loci undergo constrained diffusion within a small (<1 μm) corral [13]. However, gene activation and gene silencing events can be accompanied by dynamic chromatin movements (of up to 5 μm) that potentially determine access to the transcriptional machinery [13–16]. For example, on activation the major histocompatibility complex (MHC) genes form a large (megabase) chromatin loop that extends out from its chromosome territory [17]. By contrast, during T cell development, silencing of the recombination activating gene 1 (*Rag1*) is accompanied by its relocation to pericentromeric heterochromatin [18].

Glossary

Active chromatin hub (ACH): The clustering of active genes and *cis*-regulatory elements into a complex. It is thought that formation of an ACH increases the local concentration of transcription factors, allowing high transcription rates.
Chromosome territory: The discrete space or volume occupied by a single chromosome in the interphase nucleus. Chromosomal territories are essentially non-overlapping but their borders are not well defined and intermingling between chromosomes occurs at these junctions.
Enhanceosome: A multi-protein complex that binds to the enhancer region of a gene and stimulates transcription.
Homologous association: A meeting between identical sequences on homologous chromosomes.
Interchromosomal association: A meeting between sequences and/or genes on different chromosomes. Homologous associations also fall into this class. These can also be described as *trans*-associations.
Intrachromosomal association: A meeting between sequences and/or genes on the same chromosome. These can also be described as *cis*-associations.
Locus control region: A class of powerful *cis*-regulatory elements with functional properties overlapping with classical enhancers, insulators and boundary elements. They are defined by their ability to confer copy number-dependent, position-independent expression in transgenesis.
Nucleolar organizer region (NOR): A chromosomal region containing several tandem copies of ribosomal RNA genes around which the nucleolus forms. In humans, the NOR contains genes for 5.8 S, 18 S, and 28 S rRNA clustered on the short arms of chromosomes 13, 14, 15, 21 and 22.
Transcription factories: Nucleoplasmic complexes containing multiple molecules of RNA polymerase II. Transcription factories have been shown to assemble at new sites of transcriptional activity. Alternatively, it has been suggested that on activation, genes translocate to a limited number of preformed factories, which they are obliged to share with other active genes.
Transvection: A phenomenon where regulatory elements on one chromosome are able to regulate gene expression on another chromosome *in trans* (see Box 1).

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Box 1. Transvection in *Drosophila melanogaster*

The term transvection was introduced in 1954 by Ed Lewis to describe the phenomenon that upon homologous association of two alleles, an element on one chromosome can affect gene expression on the homologous chromosome [98]. In transvection, both enhancers and silencers can act to influence gene expression *in trans*[98]. For example, at specific *Drosophila* loci the pairing of alleles is required to achieve wild type levels of transcription, and deletion of regulatory elements on one chromosome can be rescued *in trans* by sequences on the homologous chromosome [98]. Similarly, the silencing effect of the polycomb response element (PRE) is enhanced by the pairing of two allelic copies of the PRE [98]. Interestingly, the degree of pairing in transvection is highly locus-, tissue-, and fly line-dependent, suggesting that, even though well established, the current model of transvection is incomplete and too simplistic [99].

Interestingly, when a gene is physically moved to a different genomic location (such as in transgenesis) it frequently becomes sensitive to 'position effects' resulting in aberrant expression. This might result from the influence of chromatin proximal to the site of integration and/or potentially from an altered position within the nucleus. Conversely, large BAC transgenes, transgenes containing locus control regions (LCR), or rearrangements caused by balanced translocations are frequently resistant to such position effects, even though they are introduced into both a novel genomic location and, presumably, an altered position within the nucleus [19,20]. This suggests that not all sequences are influenced by either their genomic or nuclear location. From a different perspective, artificially targeting loci to ectopic sites in the nucleus (such as the inner nuclear membrane) can also result in aberrant gene activation and/or silencing, providing evidence that gene positioning might actively regulate gene expression, rather than being a passive consequence of gene expression and/or silencing [21,22]. Thus, the nucleus is a highly structured organelle, and the ordering of the genome within the nuclear space possibly represents an additional level of gene regulation.

Intrachromosomal associations: looping *in cis*

Although the nucleus is a well organized 3D structure, transcriptional regulation in mammals was, until relatively recently, considered a linear process in which regulatory elements such as promoters and enhancers regulate proximal genes *in cis*. However, a strictly linear model is difficult to reconcile with the ability of enhancers to function when located more than a megabase away from their target gene [23]. Development of technologies such as chromosome conformation capture (3C) has enabled detailed analysis of chromosome folding, revealing for the first time how promoters communicate with distal regulatory elements. A brief overview of 3C-based methodologies is provided in Figure 1 [24–27]. In mammalian cells, 3C was first used to investigate promoter–enhancer communication at the β-globin locus [28]. These studies revealed that, through DNA looping, hypersensitive sites within the LCR come into close physical proximity with the active globin genes situated 40–60 kb away, forming a structure called the active chromatin hub (ACH). Additional distal hypersensitive sites were found in close association with the LCR and active globin genes, whereas

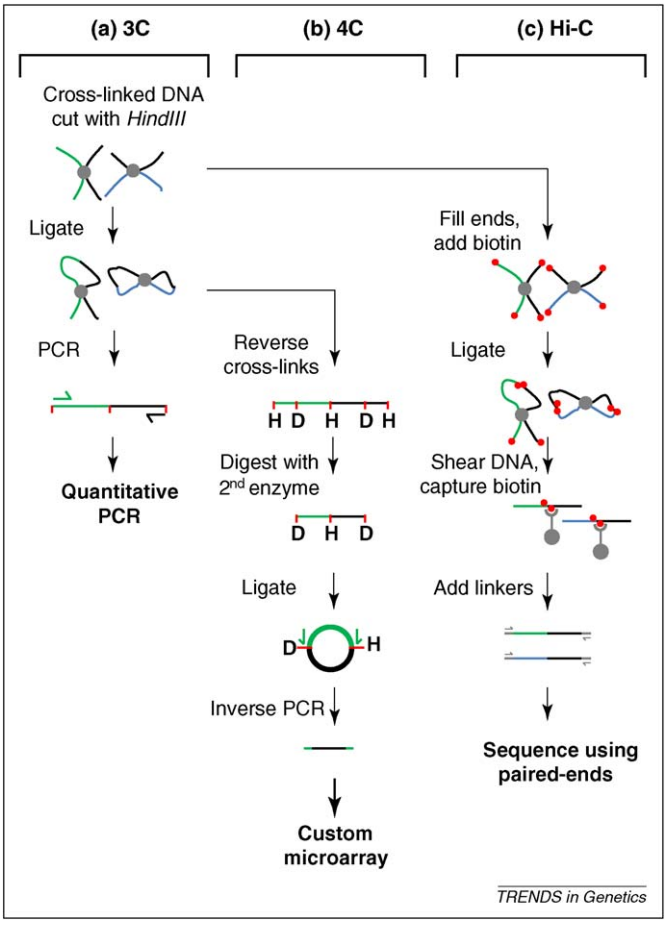

Figure 1. The development of 3C technology has enabled detailed analysis of chromosome conformations for the first time. (**a**) 3C begins with the treatment of living cells with formaldehyde, which cross-links DNA sequences in close proximity at the time of fixation. Cross-linked DNA is purified and subjected to restriction digest, in this case *HindIII* (H). Fragments are diluted and incubated with DNA ligase resulting in intramolecular ligation of cross-linked fragments. Ligation of pre-selected genomic regions is quantified using locus-specific PCR primers [24,28]. 3C is thus limited to the analysis of pre-determined regions of interest. (**b**) 4C is a variation of 3C. Cross-linked and ligated templates are generated as in 3C. The cross links are then reversed and the template is digested with a second frequent cutting restriction enzyme such as *DpnII* (D) to reduce fragment sizes before a second round of ligation. This generates small DNA circles that form the template for inverse PCR using primers designed within the bait region (green). The resulting PCR products are hybridized to custom arrays (or sequenced), allowing interrogation of genome-wide associations for a single pre-selected bait sequence [26]. (**c**) Hi-C is the most recent adaptation of 3C and potentially allows analysis of all associations genome wide. In Hi-C, cross-linked, digested 3C material is tagged with biotin and the 'sticky ends' generated by restriction digestion are filled in. The material is ligated and then sheared to generated small fragments, which are captured using streptavidin beads. Linkers are added and the material is subjected to paired-end sequencing [25].

intervening sequences and olfactory receptor genes were looped out of this complex (Figure 2) [28]. Using the complementary technique called RNA TRAP, a second group independently reported looping between the β-globin gene and its LCR [29]. It has been suggested that clustering of regulatory elements within the ACH increases the local concentration of transcription factors and maintains active chromatin domains, facilitating high levels of transcription [28,30].

Similar findings have subsequently been noted for many other genes, in some instances with enhancers being reported to 'loop' into contact with regulated genes from more than one megabase away [31]. Thus,

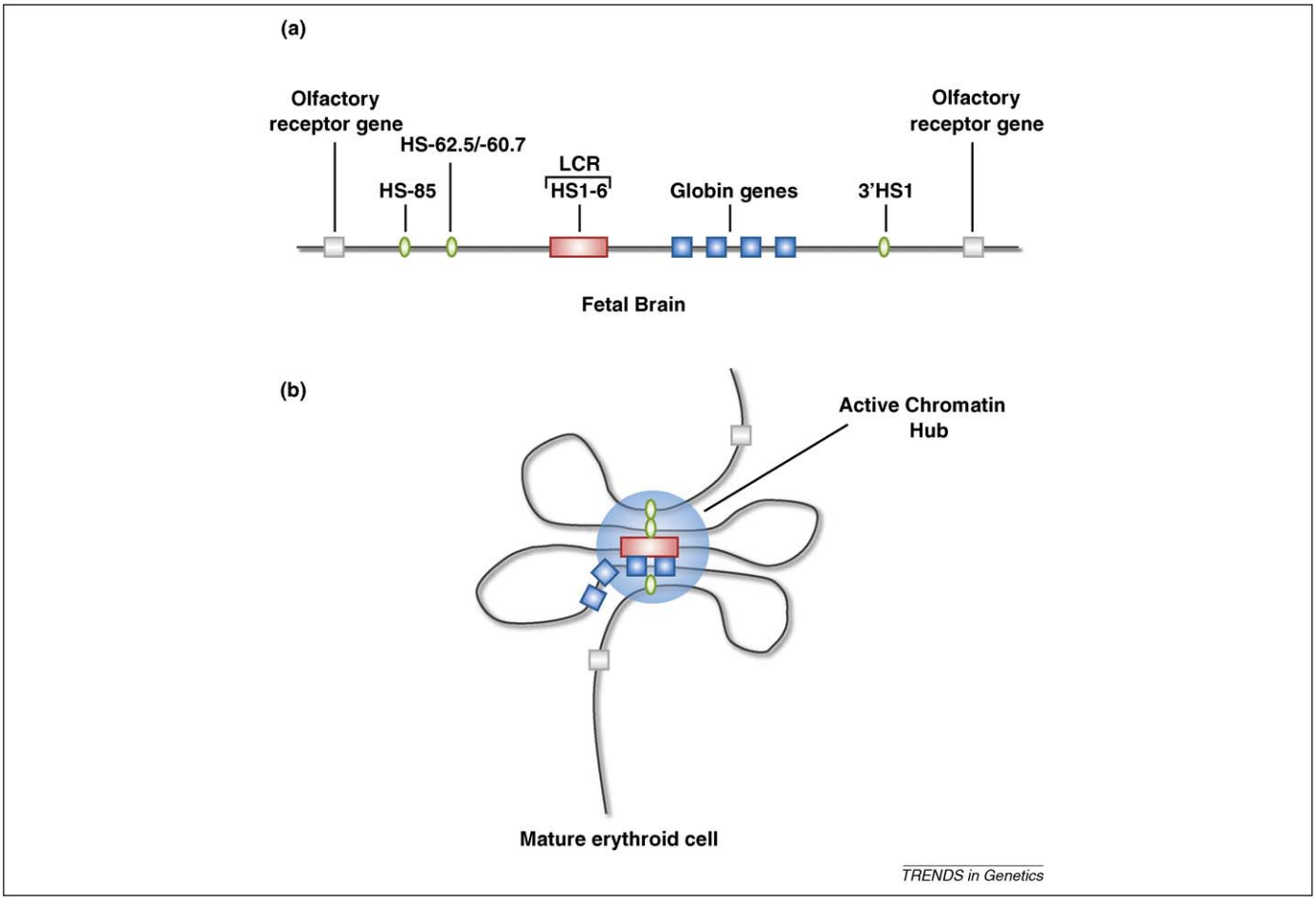

Figure 2. *Cis* looping: the active chromatin hub (ACH). (**a**) In fetal brain, where the β-globin genes are not expressed, the locus adopts a linear conformation. (**b**) In mature erythroid cells, where the β-globin gene is expressed, the locus forms chromatin loops that bring the LCR and distal hypersensitive sites (upstream HS-85/HS-62.5/HS-60.7 and downstream HS1) into proximity with the expressing globin gene, to form an active chromatin hub. Intervening sequences and olfactory receptor genes are looped out of this complex [28].

intrachromosomal looping interactions appear to be a general phenomenon of long-range enhancer–promoter communication.

Interchromosomal associations

The ability of regulatory sequences to control transcription *in cis* through long-range looping intrachromosomal interactions combined with the observation that certain genes and/or sequences can adopt preferred locations within the nucleus raises the exciting possibility that regulatory elements, such as enhancers or LCRs, located on one chromosome could coordinately regulate genes on a different chromosome through interchromosomal associations. In potential support of this hypothesis, studies of promoter–enhancer communication have revealed that in living cells or nuclear extracts, transcription from plasmids containing promoter sequences can be activated *in trans* by enhancer sequences on separate plasmids [32–34]. Furthermore, *in vitro* experiments have shown that RNA polymerase II (Pol II) can be transferred from a plasmid containing the β-globin LCR to a second plasmid containing the β-globin gene, in a process that is facilitated by the erythroid transcription factor NFE2 [35]. Although highly artificial, these experiments reveal that there is no absolute prerequisite for enhancer and promoter sequences to be present on the same DNA strand. Subsequently, specific interchromosomal associations have been identified in the mammalian nucleus; the function of these associations is discussed below.

Alternative expression of cytokine genes

One of the first systems for which interchromosomal associations have been reported is the differentiation of naïve $CD4^+$ T helper cells into TH1 and TH2 subsets. In mice, the TH2 cytokines interleukin 4 (*Il4*), interleukin 5 (*Il5*) and interleukin 13 (*Il13*) and their LCR are located in a single gene cluster on chromosome 11, whereas the TH1 cytokine interferon gamma (*Ifng*) is located on chromosome 10. In murine naïve $CD4^+$ T cells, which do not express any of these genes, there is an interchromosomal association between the regulatory regions of the TH2 cytokine locus and the *Ifng* promoter region [36]. Polarization towards either the TH1 or TH2 fate results in initiation of appropriate cytokine gene expression, paralleled by a loss of interchromosomal associations. It was postulated that this intimate association is responsible for poising the two classes of cytokine genes for immediate expression (within 3–6 h) upon T cell receptor (TCR) stimulation of naïve

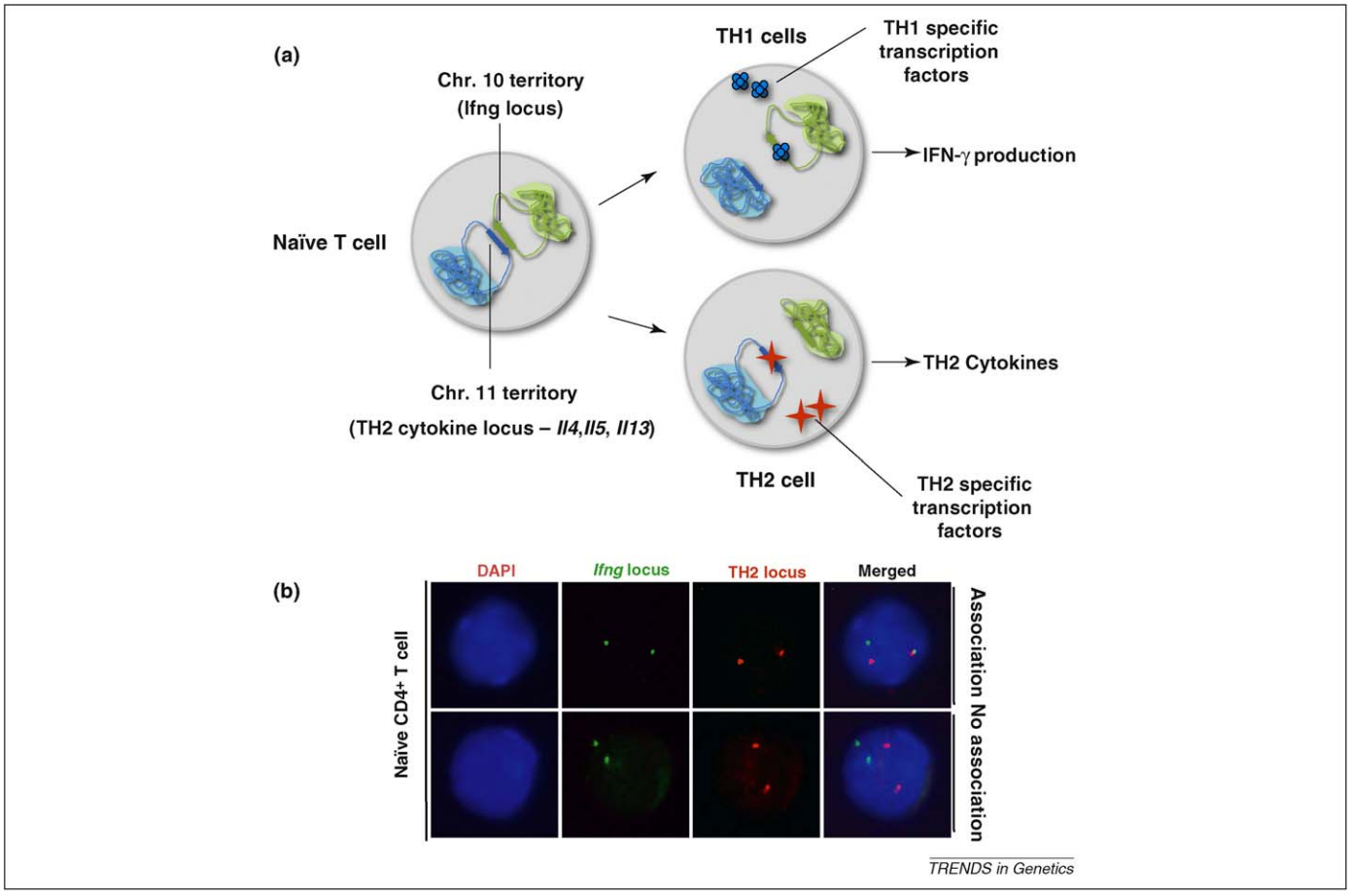

Figure 3. Interchromosomal associations of cytokine genes. (**a**) In murine naïve CD4$^+$ T cells, the TH2 locus (containing *Il4, Il5, Il13* and *Rad50*) on chromosome 11 associates with the *Ifng* gene on chromosome 10. Differentiation into TH1 or TH2 effector cell results in loss of interchromosomal associations concomitant with induction of *Ifng* or TH2 cytokine expression, respectively [36]. (**b**) Representative 3D fluorescence *in situ* hybridization (3D-FISH) and confocal analysis of associations in naïve CD4$^+$ T cells. Red represents a TH2 BAC probe, and green represents an *Ifng* BAC probe. DNA staining with DAPI is shown in blue. Each spot represents one allele. The upper panel shows a representative nucleus displaying an interchromosomal between a single allele of *Ifng* and a single allele of the TH2 locus. The lower panel shows a representative example nucleus displaying no interchromosomal association between the *Ifng* and TH2 loci.

CD4$^+$ cells [36]. In support of this hypothesis, mutations within the TH2 LCR affect TH2 cytokine expression and influence expression of *Ifng* in stimulated naïve T cells and polarized effector TH1 cells (Figure 3) [36]. Since these studies, similar *trans* associations have been detected between the TH2 locus and the tumor necrosis factor alpha/lymphotoxin (*Tnfα/Lt*) locus on chromosome 17, and also between the TH2 locus and the interleukin 17 (*Il17*) locus on chromosome 1 (C.G.S. and Lark Kyun Kim, unpublished observations).

Olfactory receptor choice

There are >1300 olfactory receptor genes dispersed over several mouse chromosomes, but only a single olfactory receptor is ever expressed in any one neuron. It has been proposed that a single enhancer element on chromosome 14 (called the H enhancer) stochastically establishes an interchromosomal association with any one of the 1300 olfactory receptor genes, resulting in the exclusive expression of that receptor [37]. Subsequently, however, it was reported that deletion of the H enhancer affected the expression of only a small number of proximal *cis*-linked olfactory receptor genes and did not affect those on other chromosomes [38,39]. In addition, in heterozygous mice, expression of olfactory receptor genes proximal to the H^- mutant allele was not rescued by the wild type H^+ allele *in trans* [39]. Whereas the latter data show convincingly that the H enhancer is not required for olfactory receptor gene expression and choice *in trans*, it is possible that the additional compensatory enhancer elements exist in the genome. Further experimentation using genome-wide, 3C-based methods could be informative in determining whether such regulatory elements actually exist.

Imprinting

Genomic imprinting is a regulatory mechanism that establishes parent of origin-specific gene expression patterns [40]. Imprinted genes are expressed from only one of the two alleles depending on the parental origin, leading to monoallelic expression [40]. One of the first indications that interchromosomal associations might be important in imprinting came from observations made by LaSalle *et al.* in 1996. These authors noted that the imprinted 15q11-q13 region in human T lymphocytes undergoes transient homologous association during late S phase [41]. Mutations within this region result in the genetic disorders Prader-Willi syndrome (PWS) and Angelman syndrome (AS) [42]. Interestingly, cells from either PWS or AS patients did not

show homologous associations, revealing that homolog pairing might play a role in establishing and/or maintaining imprinting [41]. Homologous associations were shown to occur at the Beckwith-Wiedemann syndrome locus (BWS) of human chromosome 11p15, which contains imprinted insulin-like growth factor 2 (*IGF2*) and *H19* (a non-coding RNA) genes, suggesting that these associations might represent a general mechanism for regulating imprinting [41]. However, when Teller *et al.* revisited these findings they found no evidence for an increase of the fraction of nuclei with paired, oppositely imprinted AS/PWS or BWS loci at late S phase [43]. However, in agreement with LaSalle *et al.,* they did observe a significant homologous association between the centromeres of chromosome 11 (approximately 4 megabases away from the AS/PWS locus) during late S phase. Teller *et al.* predicted that this is mediated by a nucleolar organizer region (NOR: which are known to undergo pairing events) linked to the centromere of chromosome 15 (and by default the AS/PWS region) [43]. Indeed, in an analysis of lymphoblastoid cells from *Gorilla gorilla*, in which the AS/PWS region is not linked to a NOR they actually observed increased distances between AS/PWS loci during late S phase [43]. This led the authors to suggest tentatively that the associations reported by LaSalle *et al.* result from a side effect of the conversion of NORs, and have nothing to do with an imprinting mechanism [43].

Recently, using the 3C-based associated chromosome trap method, the laboratory of Andrew Hoffman identified an interchromosomal association between the murine *Igf2/H19* imprinting control region (ICR) on the maternal chromosome 7 and an intergenic sequence between the WD repeat and SOCS box-containing 1 (*Wsbl*) and neurofibromin 1 (*Nf1*) genes on the paternal chromosome 11 [44]. Knockdown of the zinc finger-containing CCCTC-binding factor (CTCF) or deletion of the maternal ICR from chromosome 7 (but not the paternal ICR) abolished these associations and reduced expression of *Wsb1* and *Nf1* from chromosome 11, suggesting that the ICR mediates interchromosomal gene regulation [44]. However, as CTCF is known to interact with, and recruit Pol II, these findings must be interpreted with care [45]. Indeed, the involvement of CTCF in mediating associations might stem from its role in regulating transcription; associations could be lost simply as the consequence of reduced transcription resulting from deletion of CTCF or loss of the maternal ICR CTCF sites.

Using 4C (Figure 1), the laboratory of Rolf Ohlsson has extended these studies and identified >100 chromosomal fragments associating with the maternal allele of the *H19* ICR [27]. Moreover, within this panel of association partners imprinted regions were over-represented [27]. Mutation of CTCF sites within the maternal *H19* ICR (but not the paternal allele) abolished these associations, resulting in dysregulated expression of normally associated genes and loss of asynchronous replication at numerous *trans*-associated imprinted loci [27,46]. These observations led the authors to speculate that the *H19* ICR is a hub for the 'transvection of parent of origin-specific effects to non-allelic loci on other chromosomes' [46]. Taken together, these data indicate the interchromosomal regulation is important in directing imprinting and that the *H19* ICR could be the 'master regulator' of these events. Furthermore, although these studies highlight CTCF as a central player in this process, its exact role is unclear as associated alleles do not show enrichment for the presence of CTCF sites [27,46].

X chromosome inactivation

In mammals, equivalent gene 'dosage' between XY males and XX female cells is achieved by chromosome-wide transcriptional silencing of one of the two female X chromosomes [47]. This silencing event is exquisitely controlled by a small region of the X chromosome termed the X inactivation center (XIC). The XIC contains the non-coding RNA gene named X (inactive)-specific transcript (*Xist*), which is expressed specifically from the inactive X chromosome and triggers widespread gene silencing [47]. Two other non-coding RNAs, XIST antisense RNA (*Tsix*) and X-inactivation intergenic transcription element (*Xite*), are expressed solely from the active X chromosome and are important for choice (which chromosome to silence) and counting (how many chromosomes to silence) events [48]. Recent evidence suggests that immediately before initiation of X inactivation the two X chromosomes undergo transient (<1 h) homologous association mediated by the XIC [49–51]. Deletion of either *Tsix* or *Xite* from the XIC inhibits homologous association, concomitant with a failure in counting and choice, resulting in random X inactivation of neither, one or both X chromosomes [48–51].

Interestingly, multi-copy transgene arrays comprised of short sequences containing *Tsix* or *Xite* are sufficient to initiate *de novo* ectopic paring between the autosomal site of integration and the endogenous X chromosome [51]. Furthermore, these new associations are formed at the expense of endogenous homologous associations resulting in a failure of X inactivation [48,51,52]. Knockdown of either CTCF or Oct4 (also known as POU class 5 homeobox 1) precludes homologous association; surprisingly, depletion of CTCF results in a loss of X inactivation, and depletion of Oct4 results in silencing of both X chromosomes [52,53]. However, interpretation of these findings is complicated by the role of CTCF in mediating transcription and recruitment of Pol II [45]. This is especially relevant, given that transcription itself is required for X chromosome pairing [52].

Together, these findings provide considerable data to support the notion that although homologous interchromosomal association is not essential for X inactivation *per se*, it is likely to play a role in chromosome counting and choice. Furthermore, deletion and transgenic analysis imply that discrete sequences mediate these associations. The mechanism behind how the alleles locate each other and how homologous association regulates counting, choice and inactivation has yet to be resolved.

Estrogen-responsive genes

Recent data suggest that interchromosomal associations can form rapidly in response to extracellular cues. For example, estrogen-inducible genes are rapidly inducible by treatment with 17β-estradiol (E2). However, most estrogen receptor (ER-α) binding sites are intergenic and distal

from E2-inducible genes, suggesting they form long-range looping associations [54–56]. To explore this possibility, Hu *et al*, developed a novel variant of 3C technology termed 'deconvolution of DNA interactions by DSL' (3D) [57]. Using this method, they identified a series of intrachromosomal associations between the E2-regulated trefoil factor 1 (*TFF1*) gene on human chromosome 21 and other ER-α bound loci on the same chromosome [57]. In addition, they identified an interchromosomal association between *TFF1* and an E2-regulated gene, named gene regulated by estrogen in breast cancer protein (*GREB1*), on chromosome 2. In untreated cells these associations were absent but formed rapidly (within 15 min) on treatment with E2. Remarkably, these associations were paralleled by relocalization of the entire nuclear territories of chromosome 21 and chromosome 2, which became intimately associated [57]. Formation of E2-inducible interchromosomal associations was dependent upon nuclear actin and nuclear myosin-I, suggesting coalescence might be mediated through active and directed large-scale nuclear reorganization events [57]. Although this represents an exciting possibility it must be noted that actin and myosin have been shown to be functional components of Pol II and chromatin remodeling complexes [58,59]. Thus, if associations result from stochastic events driven by transcription and/or chromatin remodeling, then manipulating actin or myosin will affect transcription and will indirectly influence these associations.

Finally, providing some evidence that these associations might have functional significance is the observation that expression of associated alleles was increased dramatically relative to non-associated alleles [57]. However, at this stage the evidence is correlative and does not prove that interchromosomal association between these two loci regulates their transcription.

Viral induction of IFN-β

The human antiviral response is initiated by transcriptional activation of type I interferons, including interferon beta (IFN-β). Viral infection activates NF-kB (and other factors), resulting in enhanceosome assembly on the *IFNB* enhancer, inducing stochastic expression of *IFNB* from a single allele. Using 4C to study how this activation is rendered monoallelic, Apostolu *et al.* identified three unique sequences on different chromosomes that rapidly (within 2 h) associate with the expressing *IFNB* allele following viral infection [60]. These three sequences were each composed of Alu repeats that contained a functional NF-kB binding site. Transfected plasmids containing these sequences associated with the endogenous *IFNB* gene in an infection-dependent manner, driving elevated levels of *IFNB* expression. Mutation of the NF-kB site suppressed this effect. The authors suggest a model in which viral infection induces NF-kB binding to these three sequences, which in turn, through intrachromosomal and interchromosomal association deliver NF-kB to a single *IFNB* allele initiating monoallelic expression [60]. Thus, the coordinated engagement of multiple transcription factor binding sites with a single allele, through intrachromosomal and interchromosomal associations, might represent a general mechanism in which gene expression is rendered monoallelic. However, such a mechanism is difficult to reconcile with the extremely short residence times of most transcription factors [61].

Erythropoiesis

In mice and humans, multiple genes required for erythropoiesis have been reported to associate within RNA polymerase II transcription factories or at splicing component 35 (SC35)-enriched splicing speckles when expressed [62–64]. Of particular interest is the interchromosomal association between the human α-globin and β-globin genes. As the expression patterns of these two genes are similar during adult erythropoiesis and their gene products are required in equimolar amounts, it is tempting to speculate that their association is important in their coordinate regulation. However, a number of observations reveal that this is unlikely. For example, the murine globin genes do not show *trans*-association to the same degree as in humans [62,63]. Furthermore, when the mouse α-globin locus is replaced with the human α-globin cluster, this humanized allele associates much less frequently with murine β-globin locus, but is still regulated appropriately [62]. Finally, deletion of the human α-globin locus does not affect β-globin expression [65]. This implies that these associations are likely to represent sharing of common resources, such as transcription factories or splicing speckles, rather than providing evidence of interchromosomal gene regulation. Recruitment of related genes to such specialized factories could help in coordinating gene expression and possibly increasing efficiency of transcription. Indeed, there is some precedent to suggest the existence of dedicated transcription factories that specialize in transcribing 'similar' genes [66]. However, work by Brown *et al.* showed convincingly that co-transcribed erythroid genes do not in fact cluster within transcription factories but rather associate around common SC35-enriched splicing speckles [62,63]. Although clustering appeared to be largely stochastic, it was influenced by constraints imposed by the chromatin neighborhood of the gene as well as its transcriptional status [62,63]. Thus, in this case, interchromosomal association between co-transcribed genes appears to be a random byproduct resulting from active genes sharing common SC35-enriched splicing speckles.

Tackling the same questions, but using 4C, the de Laat group established that the active β-globin locus made intrachromosomal and interchromosomal contacts preferentially with transcribed regions of the genome [26]. Although their study confirmed the presence of previously described associations (between the β-globin locus and other erythropoietic genes), the bulk of associations involving the active β-globin locus were made with multiple active, but not necessarily tissue-specific, genes on the same chromosome, implying that specific clustering of similar genes does not occur [26].

Unanswered questions and future perspectives

What is mediating associations?

It has been suggested that multiple genes share transcription factories and, following activation, genes translocate to pre-existing factories [64,66,67]. Interestingly, RNA polymerases can generate 'pulling' forces substantially

larger than those generated by cytoskeletal motor proteins such as kinesin and myosin, providing the possibility that polymerases might play a role in shaping the genome [68]. However, 4C experiments failed to reveal genome reorganization on inhibition of RNA polymerase, implying transcription is not essential to maintain (but not necessarily shape) global genome architecture [69].

Currently, the best candidate for mediating association is the multifunctional zinc-finger protein CTCF [70]. In a number of systems, CTCF has been implicated in forming chromatin loops that bring distal regulatory elements into close proximity with promoter sequences [71–74]. CTCF is important also for interchromosomal associations seen in imprinting and X inactivation [27,44,46,52,53]. Importantly, CTCF can form dimers and maybe even oligomers, potentially providing a biophysical basis for intrachromosomal and interchromosomal associations [75]. CTCF has 13000 – 36000 possible binding sites within the genome and it is likely to play a major role in regulating global genome architecture [76–79]. However, this would imply that CTCF must collaborate with additional factors to provide specificity in intrachromosomal and interchromosomal associations. Interestingly, recent data show that CTCF is able to recruit cohesins to specific genomic locations [80–88]. Cohesins, better known for their role in mediating sister chromatid cohesion during mitosis, have established chromatin-bridging potential and are thus an obvious candidate for orchestrating spatial organization of the genome. To date, more than 15 proteins, including transcription factors, chromatin remodeling complexes and architectural proteins, have been shown to establish or maintain chromatin loops [89]. The concerted efforts of these factors (and likely unidentified members) could potentially maintain global genome architecture whilst providing sufficient flexibility to mediate specific intrachromosomal and interchromosomal associations. Interestingly, a number of loci involved in interchromosomal associations in mammals encode non-coding RNAs (for example, the *XIC* in X inactivation). In such cases, association might result simply from the process of transcribing these non-coding RNAs. Alternatively, it is tempting to speculate that non-coding RNAs themselves might serve a structural role or even function as *trans*-allelic messengers.

One final possibility that cannot be excluded is that nothing is mediating these associations directly. Instead, associations might arise by stochastic meetings afforded by constraints imposed by chromatin context and/or transcriptional status [62,63,90]. Manipulation of any factor that could influence transcription or chromatin remodeling might disrupt these stochastic associations indirectly, giving the impression that they are in fact directed, thereby complicating the analysis of interchromosomal associations. Indeed, as mentioned above, CTCF is implicated directly in binding and recruitment of Pol II [45]. Thus, deletion of CTCF and/or its binding sites might influence associations indirectly by altering rates of transcription. Caution must therefore be used when interpreting such experiments.

How dynamic are associations?

The 3C-based methods require a very high number of PCR cycles to reveal interchromosomal associations and it is difficult to interpret such data quantitatively. Furthermore, due to the nature of the technique, it is theoretically possible to generate a 3C product from any two regions of the genome by chance. This is most apparent in 4C experiments, where specific interchromosomal associations appear to be embedded in a sea of thousands of low-level, non-specific (presumably) background interchromosomal associations [26]. Thus, 3C-based experiments must be extremely well controlled and interpreted with care. Importantly, these methods are routinely supported by microscopy-based techniques, such as fluorescent *in situ* hybridization (FISH), which although limited in resolution (250–500 nm), allow quantification of association frequency at the single-cell level. Interestingly, the most striking observation from microscopic analysis is that associations are detected in only a limited proportion of cells at any one time; frequencies of association in the range 5–15% are generally reported, but frequencies as high as 30–60% have been noted [26,27 36,37,41,43,44,46,50,51,57,60,62–64,67]. Can associations present in a minority of cells within a population be physiologically relevant? Unfortunately, as current methodology relies on the fixation of cells before analysis, creating a snapshot of a single moment in time, we know little regarding the dynamics of association. What do associations caught *'in flagrante'* by 3C and FISH actually represent? Do they correspond to stable associations present in only a few cells or are they dynamic, transient events that occur stochastically in all cells? Assuming that associations are functional, these two extreme possibilities obviously have very different biological implications (Figure 4). For example, if an association is stable, but present in only a

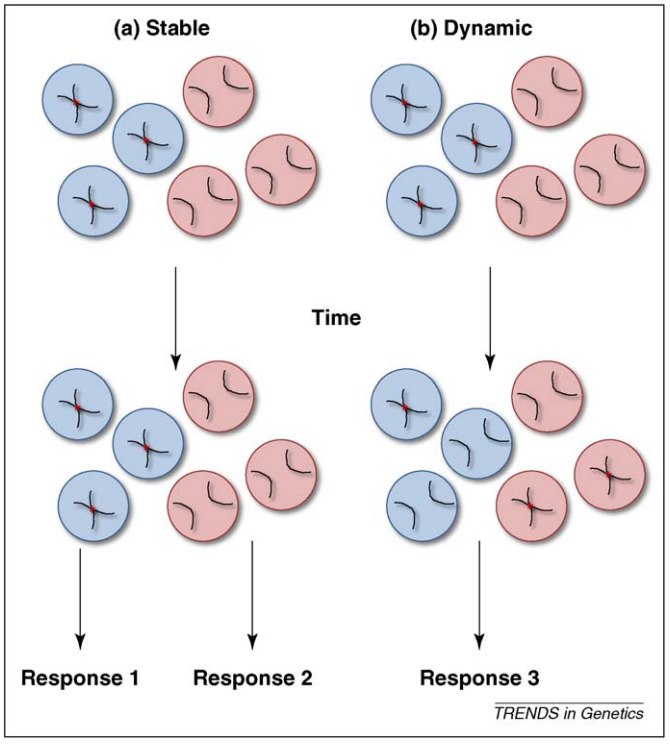

Figure 4. Dynamics of chromosomal association. (**a**) If associations are stable but present in only a proportion of cells, these cells might generate different responses (response 1 or 2). (**b**) If associations are dynamic, all cells within a population have the potential to form associations. Most probably, each cell would generate a similar response (response 3). However, it is possible that cells with or without associations at the moment of stimulation might respond differently, as in **a**.

proportion of cells, these cells might generate a different response to cells without the association. In contrast, if an association is dynamic, most probably each cell would generate a similar response. Thus, it will be particularly informative to track associations by microscopy using transgenic arrays of Lac operator repeats tagged *in vivo* with GFP–Lac repressor fusion proteins [91–94]. Such methodologies have already been utilized to study transvection in *Drosophila*, revealing associations to be stable for hours rather than minutes [95]. The presence of such stable associations is more in line with our current understanding of chromosome dynamics; as mentioned previously, loci are constrained within a small (<1 μm) diffusion corral [13]. However, the possibility that associations can result from specific dynamic and large-scale chromatin movements has yet to be excluded.

How general are functional interchromosomal associations?

Do co-transcribed genes generally coalesce? Although anecdotal evidence suggests that this could be the case, emerging 4C data suggest that this might be a rare event. Most likely, chromatin folding will be directed by self-organizing principles [26]. Thus, positioning of a locus might be probabilistic, determined by the sum of properties of neighboring sequences and the chromosome as a whole [96,97]. It is important to note that some sequences (such as nucleolar organizer regions) might be dominant in shaping the genome, and transgenes can sometimes override endogenous positioning of the site of integration [49,51].

Following early examples of regulatory interchromosomal associations only a few cases have been identified. Does this imply that such associations are rare, or are we just looking in the wrong places? It is difficult to draw decisive conclusions because very few loci have been studied in detail. These are questions that will need to be addressed by the next generation of open-ended high-throughput 3C-based methods (Figure 1). Indeed, a recent publication reported whole genome-wide associations at a resolution of 1 megabase [25]. As the resolution increases and the cost decreases, these immensely powerful methods will allow interrogation of genome-wide associations in different cell types and under different conditions. This might reveal a plethora of new associations and potentially provide an estimate of the global frequency of such events.

Do associations represent functional interactions?

Perhaps the most pressing question is whether associations are functionally significant? Although appealing, it is erroneous to suppose that coalescing of similar genes implies co-regulation and that association between genes and regulatory sequences on other chromosomes gives evidence for *trans*-regulation. It is more probable that the bulk of associations simply reflect stochastic encounters due to the constraints of sharing limited space and resources within the nucleus (Figure 5). Proving associations are functionally relevant is technically difficult, and a number of criteria should be addressed, such as those described by Brown *et al.* [62]. (1) Are associations conserved across species? If not, it is less likely that they represent functional interactions. (2) Does the mutation of alleles affect association and/or gene expression *in trans*? Potential effects from loss of gene products (protein or non-coding RNA) from modified alleles must be taken into account. Furthermore, mutation of candidate binding sites is preferable to ablation of protein mediators, which is often accompanied by pleiotropic effects. (3) Do transgenes form the same associations? If not, are they regulated appropriately? Does their presence affect endogenous associations and/or gene regulation? (4) Are there other sequences (such as NORs) on the same chromosome, proximal to the region of interest, which might instead be responsible for driving association?

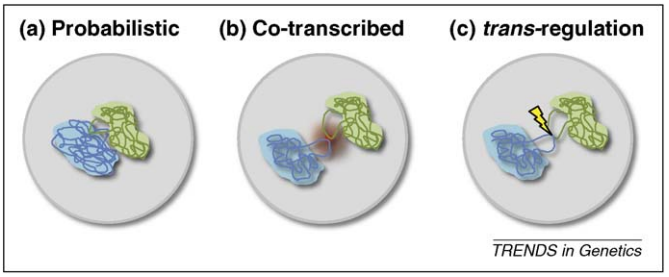

Figure 5. Three types of interchromosomal association. (**a**) Positioning of loci is probabilistic and is determined by the sum of properties of neighboring sequences and the chromosome as a whole. Associations have no obvious functional significance. (**b**) Co-transcribed genes coalesce in and around nuclear bodies such as transcription factories and splicing speckles. As such bodies might be specialized in transcribing similar genes, these associations could help in coordinating gene expression and increase efficiency of transcription. Alternatively, these associations might be probabilistic as in **a** and have no functional significance. (**c**) Sequences regulate gene expression *in trans* through interchromosomal contacts.

Concluding remarks

Identification of interchromosomal associations in mammalian cells has initiated a new and exciting field in mammalian biology. Currently, the physiological significance of these associations has been studied in only a handful of cases, and although it is now well accepted that genomic regions can associate *in trans*, it is still controversial whether these associations represent the basis of a mammalian equivalent of *Drosophila* transvection. Fueled by recent technological developments we are beginning to build a detailed three-dimensional picture of genome organization. We must now focus on how such ordering is achieved and what the implications are for regulating gene expression. Importantly, a more thorough interrogation of how chromatin context and transcriptional status affects interchromosomal association is urgently required. It is likely that the nucleus is predominantly governed by self-organizing principles; however, within a sea of probabilistic intrachromosomal and interchromosomal associations, identification of specific and functional interactions should continue to reveal exciting aspects of nuclear biology and gene regulation. An understanding of the mechanisms governing these functional interactions might establish a more complete paradigm of gene regulation in mammals.

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Conflict of interest

The authors declare that they have no financial conflict of interest.

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