The EMBO Journal (2009) 28, 1234–1245 | © 2009 European Molecular Biology Organization | All Rights Reserved 0261-4189/09
www.embojournal.org

THE EMBO JOURNAL

Architectural roles of multiple chromatin insulators at the human apolipoprotein gene cluster

Tsuyoshi Mishiro[1,2], Ko Ishihara[1], Shinjiro Hino[1], Shuichi Tsutsumi[3], Hiroyuki Aburatani[3], Katsuhiko Shirahige[4], Yoshikazu Kinoshita[2] and Mitsuyoshi Nakao[1,*]

[1]Department of Regeneration Medicine, Institute of Molecular Embryology and Genetics, Kumamoto University, Kumamoto, Japan, [2]Department of Gastroenterology and Hepatology, Shimane University School of Medicine, Izumo, Japan, [3]Genome Science Division, Research Center for Advanced Science and Technology, The University of Tokyo, Komaba, Meguro-ku, Tokyo, Japan and [4]Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Nagatsuta, Midori-ku, Yokohama City, Kanagawa, Japan

Long-range regulatory elements and higher-order chromatin structure coordinate the expression of multiple genes in cluster, and CTCF/cohesin-mediated chromatin insulator may be a key in this regulation. The human *apolipoprotein* (*APO*) *A1/C3/A4/A5* gene region, whose alterations increase the risk of dyslipidemia and atherosclerosis, is partitioned at least by three CTCF-enriched sites and three cohesin protein RAD21-enriched sites (two overlap with the CTCF sites), resulting in the formation of two transcribed chromatin loops by interactions between insulators. The *C3* enhancer and *APOC3/A4/A5* promoters reside in the same loop, where the *APOC3/A4* promoters are pointed towards the *C3* enhancer, whereas the *APOA1* promoter is present in the different loop. The depletion of either CTCF or RAD21 disrupts the chromatin loop structure, together with significant changes in the *APO* expression and the localization of transcription factor hepatocyte nuclear factor (HNF)-4α and transcriptionally active form of RNA polymerase II at the *APO* promoters. Thus, CTCF/cohesin-mediated insulators maintain the chromatin loop formation and the localization of transcriptional apparatus at the promoters, suggesting an essential role of chromatin insulation in controlling the expression of clustered genes.

The EMBO Journal (2009) **28,** 1234–1245. doi:10.1038/emboj.2009.81; Published online 26 March 2009
Subject Categories: chromatin & transcription
Keywords: apolipoprotein; cohesin; CTCF; enhancer; insulator

*Corresponding author. Department of Regeneration Medicine, Institute of Molecular Embryology and Genetics, Kumamoto University, 2-2-1 Honjo, Kumamoto 860-0811, Japan. Tel.: +81 96 373 6800; Fax: +81 96 373 6804; E-mail: mnakao@gpo.kumamoto-u.ac.jp

Received: 29 December 2008; accepted: 4 March 2009; published online: 26 March 2009

Introduction

Tissue type or developmental stage-specific expression of multiple genes in cluster on mammalian genome may require long-range regulatory elements and higher-order chromatin structure such as the chromosomal domains. In comparison with control of individual genes, chromatin-based mechanisms must have critical roles in regulating the gene clusters that have arisen by tandem duplication events (Sproul *et al*, 2005). Chromatin insulators are boundary elements that partition the genome into the chromosomal domains, through their ability to block interactions between enhancers and promoters when positioned between them (enhancer-blocking activity) and/or their ability to block repressive chromatin effects on the flanking regions (barrier activity) (Bell *et al*, 2001; Mongelard and Corces, 2001; West *et al*, 2002; Gaszner and Felsenfeld, 2006). The CCCTC-binding factor, CTCF, is known to bind insulators and exhibits the enhancer-blocking function (Ohlsson *et al*, 2001). CTCF has been also shown to mediate transcriptional repression (Lobanenkov *et al*, 1990; Klenova *et al*, 1993; Filippova *et al*, 1996; Burcin *et al*, 1997; Chernukhin *et al*, 2000; Lutz *et al*, 2000) and to activate the expression of several genes (Vostrov and Quitschke, 1997; Zhao and Dean, 2004). CTCF-mediated insulators have been particularly characterized in the chicken β-*globin* locus and the imprinted *IGF2/H19* locus in mice and humans (Bell *et al*, 1999; Bell and Felsenfeld, 2000; Hark *et al*, 2000; Saitoh *et al*, 2000). In the differentially methylated region (DMR) of the *H19* gene, CTCF binds to its binding sites in the DMR insulator, and the DMR insulator has been proposed to form a higher-order chromatin loop structure and enhance interactions between the enhancer and the promoter (Murrell *et al*, 2004). Further studies on the β-*globin* locus and the *HLA-DRB1* and *HLA-DQA1* genes have shown that these types of long-range interactions are dependent on CTCF (Splinter *et al*, 2006; Majumder *et al*, 2008). With regard to the regulatory mechanisms at the insulator sites, recent studies have shown that some CTCF sites are tethered to the nucleolus through interaction with nucleophosmin/B23 (Yusufzai *et al*, 2004b), that CTCF is associated with the poly(ADP-ribosyl)ation (Yu *et al*, 2004), with the nuclear matrix (Dunn *et al*, 2003; Yusufzai and Felsenfeld, 2004a) and with the SNF2-like chromodomain helicase protein CHD8 (Ishihara *et al*, 2006). Genome-wide analyses have then revealed the distribution of putative CTCF-binding sites and their consensus sequences (Barski *et al*, 2007; Kim *et al*, 2007; Xie *et al*, 2007). More recently, using chromatin immunoprecipitation (ChIP)-on-chip studies, we and others have further identified approximately 14 000 CTCF-binding sites on the human genome, which are frequently enriched with the cohesin complexes that mediate sister-chromatid cohesion in mitosis and gene regulation in postmitotic cells (Parelho *et al*, 2008; Stedman *et al*, 2008; Wendt *et al*, 2008).

 ©2009 European Molecular Biology Organization

However, the competence of CTCF/cohesin-binding sites for insulation, the functional relationship of CTCF and cohesins, and the implications of insulators in regulating gene clusters are not understood.

The risk of developing dyslipidemia and cardiovascular diseases is increased by high levels of circulating triglycerides in blood, which are often associated with genetic variations in the *apolipoprotein* (*APO*) genes (Watkins and Farrall, 2006; Lusis and Pajukanta, 2008; Willer *et al*, 2008). The *APOA1/C3/A4/A5* gene cluster on human chromosome 11q23.3, with the *APOA1*, *APOA4* and *APOA5* genes being transcribed in the same direction and the *APOC3* gene being transcribed in the opposite direction, is dominantly expressed in liver and intestine, and these genes are crucial for the metabolism and redistribution of lipoproteins and lipids (Lai *et al*, 2005). APOA1, APOA4 and APOA5 are the major constituents of high-density lipoprotein (HDL), and the plasma levels of these proteins are negatively correlated with the development of atherosclerotic diseases. In contrast, APOC3 contributes to the formation of very low-density lipoprotein (VLDL) and much lower amounts of HDL, thereby suggesting that expression of the *APO* genes need to be appropriately regulated. Furthermore, several single nucleotide polymorphisms (SNPs) within the *APOA1/C3/A4/A5* cluster in human populations are strongly linked to sporadic dyslipidemia and familial combined hyperlipidemia, as well as increased susceptibility to atherosclerosis (Lai *et al*, 2005). Despite the pathophysiological significance of the apolipoproteins, the epigenetic control of the *APOA1/C3/A4/A5* gene locus is largely unknown.

During the investigation of the human *APOA1/C3/A4/A5* gene cluster, we found the presence of unique insulators that are preferentially bound by CTCF and/or the cohesin protein RAD21. From the observations using chromosome conformation capture analysis, in combination with knockdown of CTCF or RAD21, we propose a mechanistic model in which CTCF/cohesin cooperatively maintains the higher-order chromatin architecture of the human *APO* gene cluster, through the formation of two long-range interactive chromatin loops *in vivo*. The loss of CTCF or RAD21 disturbs the *APO* gene expression and the occupancy of the transactivator hepatocyte nuclear factor (HNF)-4α and RNA polymerase II at the promoters, together with the significant alterations of the chromatin structure. Thus, CTCF/cohesin-mediated insulators are required for maintaining the overall structure of the *APO* gene cluster. To understand the fundamental mechanism in complex gene cluster, we propose an architectural model of the long-range assembly of the enhancer, promoter and insulator on the mammalian genome.

Results

Distribution of CTCF-enriched sites in the human APO gene region

To test the hypothesis that chromatin insulation may regulate higher-order control of the gene cluster regions, using ChIP-on-chip tiling microarray analyses (Wendt *et al*, 2008), we characterized the potential CTCF-mediated insulators in the *APOA1/C3/A4/A5* gene locus and designated the three CTCF-binding sites AC1, AC2 and AC3 (Figure 1A). The *APOA1/C3/A4* genes and *APOA5* promoter were located between AC2 and AC3, together with the *APOC3* enhancer, which may allow these *APO* genes to be expressed dominantly in hepatic cells. These AC sites in the *APO* gene region were similarly or partly detected in HeLa, retina epithelial RPE-1 and immortalized B cells (Wendt *et al*, 2008), Hep3B and HCT116 cells (data not shown), as well as IMR90 fibroblasts and CD4$^+$ T cells (Barski *et al*, 2007; Kim *et al*, 2007). To confirm CTCF-binding activity in hepatic Hep3B cells, we then performed ChIP using anti-CTCF antibodies, followed by polymerase chain reaction (PCR) with specific primers for each AC site (Figure 1B). CTCF bound AC1, AC2 and AC3, but not AR1 within the *APOC3* gene. On the basis of recent reports (Kim *et al*, 2007; Xie *et al*, 2007), we found that each AC site contained a 20-bp consensus sequence for CTCF binding (Figure 1C). To check whether these AC sequences bind directly to CTCF, we performed an electrophoretic mobility shift assay (EMSA) using radiolabelled 90-bp duplex probes for each AC site and *in vitro* transcribed/translated CTCF protein. Similar to the case for the DMR insulator of the *H19* gene as a control, the AC probes complexed with CTCF and were further supershifted by anti-CTCF antibodies. In contrast, AR1 site showed a putative CTCF-binding sequence (data not shown) but did not bind to CTCF, suggesting that CTCF preferentially binds to the AC sites in the *APO* gene region. However, our data did not exclude the possibility that the small amount of CTCF existed near the AR1 site, where cohesin protein RAD21 was dominantly enriched.

Enhancer-blocking insulator activity in the APO gene region

Earlier studies have shown that the *H19* DMR insulator contains multiple CTCF-binding sites, which are essential for the enhancer-blocking activity (Bell and Felsenfeld, 2000; Hark *et al*, 2000; Ishihara *et al*, 2006). To test whether AC1, AC2 and AC3 have enhancer-blocking effects, we performed a luciferase reporter assay in Hep3B cells (Figure 2A). The presence of AC1, AC2 or AC3 between the *APOC3* enhancer and the *APOA4* promoter reduced the luciferase activities to approximately 40–60% of the control pEALD (pEIALD1F, pEIALD2F and pEIALD3F). The AC sequences in the opposite direction showed very similar results (pEIALD1R, pEIALD2R and pEIALD3R), suggesting that the AC sites have enhancer-blocking activities that are independent of the orientation of the sequences. The use of mutant AC sites that lacked the CTCF binding lost the enhancer-blocking effect (pEMALD1F, pEMALD2F and pEMALD3F), suggesting that insulator activities of the AC sites depend on CTCF. To exclude the possibility that the AC sites exhibit silencer-like activities, the AC sequences were placed upstream of the enhancer (pIEALD1, pIEALD2 and pIEALD3). The luciferase activities were not decreased by the AC sites, but rather increased, especially for pIEALD1, suggesting that the AC sites do not possess any silencer-like functions. In addition, there was no effect of the AC sequences themselves on the promoter activities in the absence of the *C3* enhancer (pIALD1F, pIALD2F and pIALD3F), compared with the control pALD (Supplementary Figure S1). These results suggest that AC1, AC2 and AC3 are functional insulators.

Cohesins are composed of four core subunits including RAD21 (MDC1/SCC1) kleisin family protein and mediate cohesion by embracing sister chromatids (Nasmyth *et al*, 2000; Dorsett, 2007). Cohesins have been reported to frequently accumulate at CTCF-binding sites (Parelho *et al*,

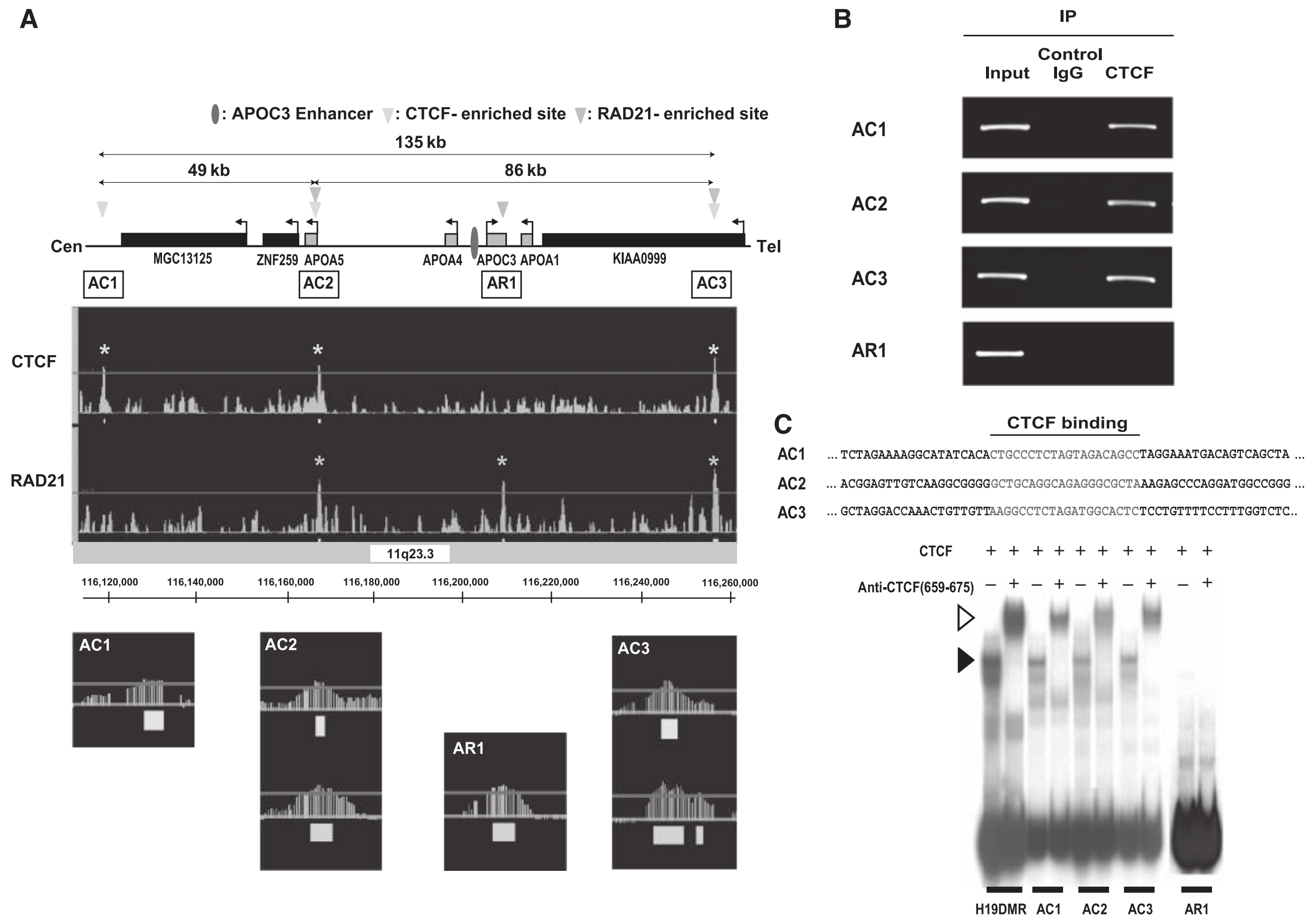

Figure 1 Distributions of CTCF/RAD21-enriched sites in the human *APO* gene region. (**A**) CTCF- and cohesin RAD21-binding sites in the *APOA1/C3/A4/A5* gene region on human chromosome 11q23.3. This chromosomal region includes seven genes (*MGC13125*, *ZNF259*, *APOA5*, *APOA4*, *APOC3*, *APOA1* and *KIAA0999*), and the *APOC3* enhancer shown in red. From the ChIP-on-chip tiling array analysis, CTCF- and RAD21-enriched sites in HeLa cells are indicated in pink and orange, respectively. The highly enriched sites are marked with asterisks, and designated AC1, AC2, AC3 and AR1. Magnifications show the enrichment of CTCF and RAD21 at AC1, AC2, AC3 and AR1. (**B**) Existence of CTCF at AC sites. Cross-linked DNA-protein complexes were immunoprecipitated with anti-CTCF and control antibodies, followed by PCR amplification with specific primers for each AC site in Hep3B cells. Genomic DNA in the 1.25% of input lysates was used as a positive control. AR1 is a control for lesser CTCF binding. (**C**) Direct binding of CTCF to AC sequences. The predicted CTCF-binding sequences are indicated within the AC1, AC2 and AC3 sites, together with the 20-bp consensus motif (shown in red) (Kim *et al*, 2007). For EMSAs, the radiolabelled 90-bp duplex probes for each AC site were incubated with CTCF protein synthesized by a coupled *in vitro* transcription/translation reaction, together with anti-CTCF antibodies. Solid and open arrowheads indicate the CTCF-DNA complexes and super-shifted complexes by the antibodies, respectively. The DMR insulator of the *H19* gene was used as a positive control. A full-colour version of this figure is available at *The EMBO Journal* Online.

2008; Stedman *et al*, 2008; Wendt *et al*, 2008). Our ChIP-on-chip analysis revealed that RAD21 was highly enriched with CTCF at AC2 and AC3, but lesser at AC1, in the *APOA1/C3/A4/A5* region (Figure 1A; Supplementary Figure S3). In addition, RAD21 dominantly bound to the AR1 site within the *APOC3* gene. It is of great interest to test how AR1 behaves in the enhancer-blocking assay. To address this, we performed the luciferase reporter assay in Hep3B cells (Figure 2B). The presence of AR1F or AR1R between the enhancer and promoter reduced the luciferase activities to about 70% of the control pEALD (pERALD1F and pERALD1R) ($P<0.01$), independent of the orientation of the sequence. The AR1 sequence upstream of the enhancer rather increased the luciferase activities (pREALD1), suggesting that the AR1 site has no silencer-like effect. Thus, AR1 has moderate enhancer-blocking activities, although an indirect involvement of CTCF may not be excluded.

Further, HNF4α is known to be a key regulator of the *APOA1/C3/A4/A5* genes in hepatic cells, and to potentiate the *C3* enhancer rather than the *APO* gene promoters (Zannis *et al*, 2001; Prieur *et al*, 2005). The *APOA4* promoter alone showed lower luciferase activities than the coexistence of the *APOA4* promoter and *C3* enhancer in Hep3B cells (pALD and pEALD) (Figure 2A). To test the crucial role of the *C3* enhancer, the luciferase analysis was done in the presence of overexpression of HNF4α in HeLa cells that do not express *APOA1/C3/A4/A5* or *HNF4α* (HNF4α/pALD and HNF4α/pEALD) (Figure 2C). Compared with the controls (mock/pALD and mock/pEALD), exogenous HNF4α increased transcription from the *A4* promoter by 4-folds, and by 10-folds in the coexistence of the *APOA4* promoter and *C3* enhancer. Thus, HNF4α can enhance the *APO4* promoter through the *C3* enhancer. In addition, we examined whether exogenous HNF4α can affect the *APO* genes and their neighbouring genes in HeLa cells (Supplementary Figure S2). A quantitative reverse transcription (RT)–PCR analysis showed that overexpression of HNF4α markedly enhanced the expression of the *APOA1/C3/A4/A5* genes, but not the

©2009 European Molecular Biology Organization

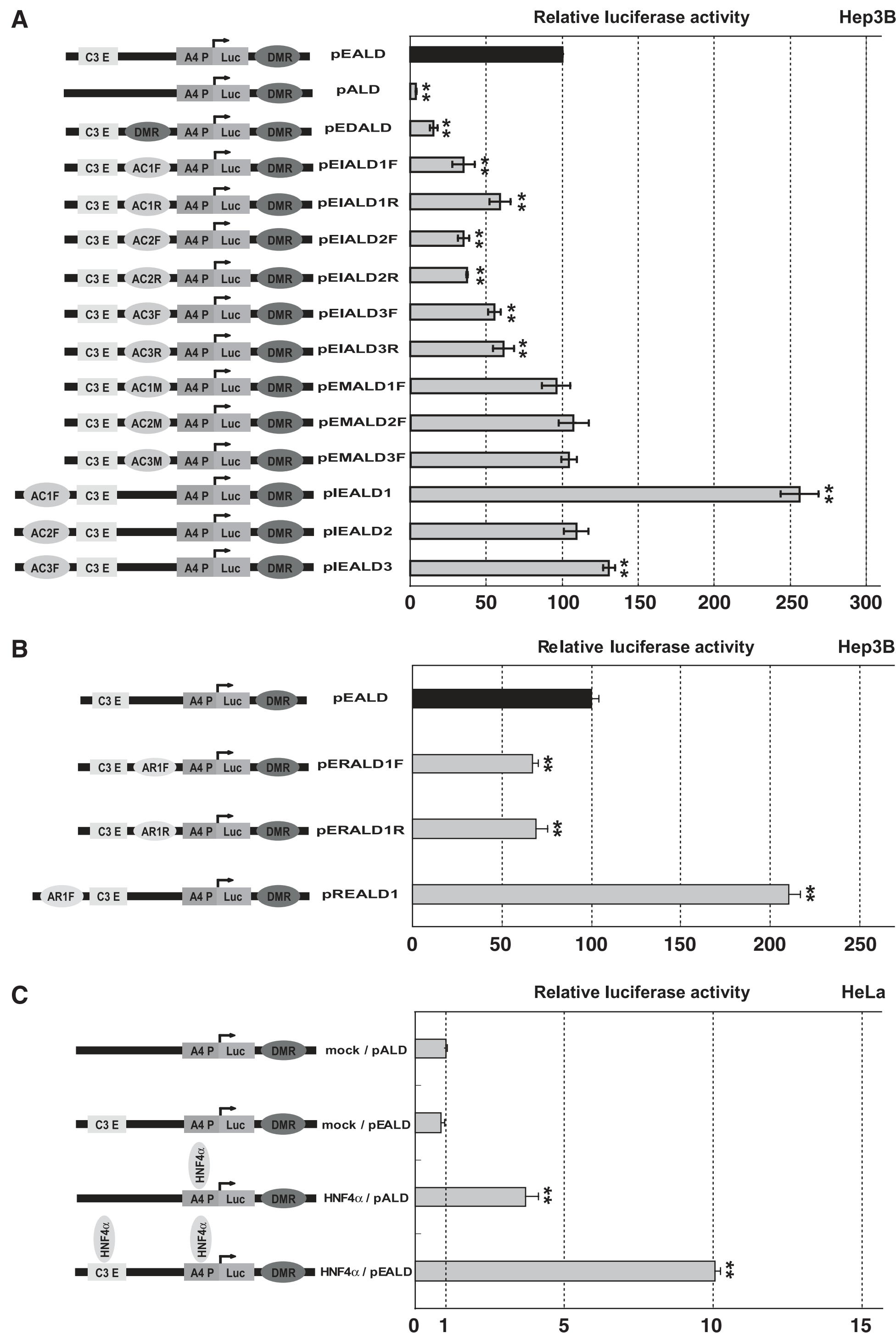

Figure 2 CTCF/cohesin-mediated enhancer-blocking activity in the *APO* gene region. (**A**, **B**) Enhancer-blocking activities. The reporter constructs contained the *C3* enhancer (C3 E) and *APOA4* promoter (A4 P) upstream of the *luciferase* gene. The indicated reporter constructs were introduced into Hep3B cells to examine the enhancer–promoter associations. The luciferase activities from pEALD were normalized to 100. The *H19* DMR insulator was used as a control. The values are given as means and standard deviations of the results from more than three independent experiments. pEMALD plasmids had the mutant AC1, AC2 and AC3 fragments that lacked CTCF binding (pEMALD1F, pEMALD2F and pEMALD3F). Luc, *luciferase* gene; A4 P, human *APOA4* promoter; C3 E, *APOC3* enhancer; DMR, *H19* DMR insulator; AC1-AC3, CTCF-enriched sites (A); AR1, RAD21-enriched site (B); F, forward orientation; R, reverse orientation. $**P<0.01$. (**C**) Effect of HNF4α on enhancer and promoter. The indicated reporter constructs (pALD and pEALD) were introduced into HeLa cells, together with overexpression of HNF4α. The luciferase activities from pALD with mock plasmid were normalized to 1. HNF4α enhances the *APOA4* promoter through the *C3* enhancer. A full-colour version of this figure is available at *The EMBO Journal* Online.

MGC13125, *ZNF259* and *KIAA0999* genes (Supplementary Figure S2A). Using the ChIP-quantitative PCR method, overexpressed HNF4α was found to bind the *APO* gene promoters and *C3* enhancer in HeLa cells (Supplementary Figure S2B). Collectively, our data suggest that HNF4α effectively activates the *APO* genes, which are localized

©2009 European Molecular Biology Organization

between the AC2 and AC3 insulators, through the enhancer–promoter cooperation.

Role of CTCF and cohesins in transcriptional control of the APO gene cluster

To investigate the role of insulators in the *APO* locus, we used RNA interference-mediated knockdown of CTCF and RAD21 in Hep3B cells (Figure 3). Western blot and quantitative RT–PCR analyses showed that CTCF and RAD21 were depleted at both the protein and mRNA levels (Figure 3A and data not shown). Using the ChIP-quantitative PCR method, we confirmed the amount of CTCF and RAD21 at the AC sites and AR1 under knockdown of either protein (Supplementary Figure S3). CTCF knockdown reduced the localization of CTCF at the AC1, AC2 and AC3 sites (left panel). Under CTCF knockdown, the amount of RAD21 was also decreased at the AC1, AC2 and AC3 sites. On the other hand, RAD21 knockdown remarkably decreased RAD21 localization at the AC1, AC2, AC3 and AR1 sites (right panel). In addition, it was also observed that the amount of CTCF at AC3 was reduced to 57.6% by the loss of RAD21 (See the Discussion).

We then checked the expression levels in the *APO* gene region, using the quantitative RT–PCR analysis. As shown earlier, the *APOA1/C3* genes were dominantly expressed in hepatic cells, compared with the *APOA4/A5* genes. Under these conditions, the loss of CTCF substantially decreased the transcript of the *APOC3* to about 41.6% of the control (Figure 3B, left). In contrast, the expression levels of the *APOA1* and neighbouring transcripts tended to increase probably due to deregulation of the locus. As analogous to the CTCF knockdown, RAD21 depletion markedly reduced the expression level of the *APOC3* transcript to about 64.1% of the control (Figure 3B, right). The neighbouring genes showed no significant changes in their expression levels under the loss of RAD21. Especially, the *APOA1* gene was induced about three-fold by loss of CTCF and was not

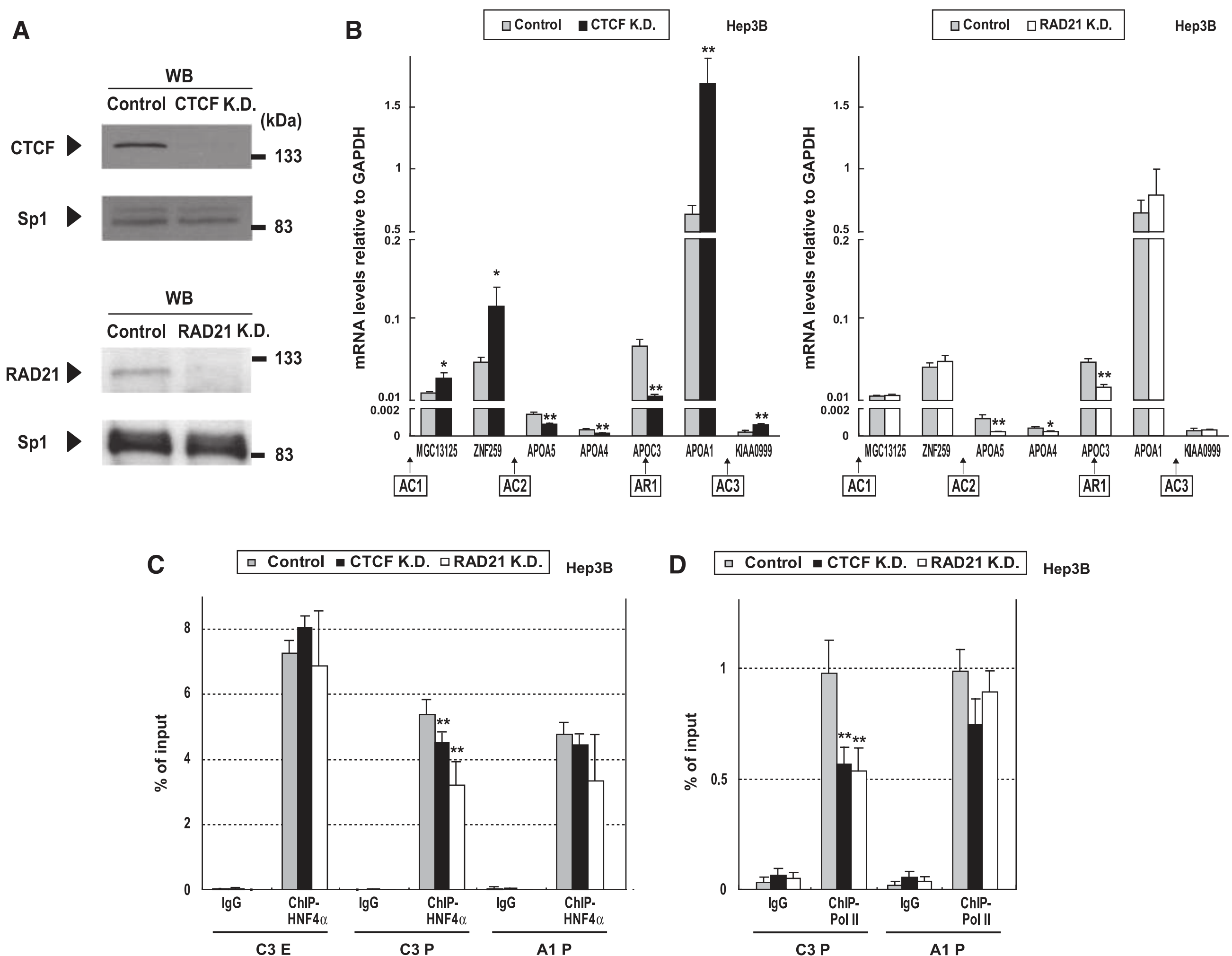

Figure 3 CTCF and cohesins are involved in transcriptional control of the *APO* gene region. (**A**) RNA interference-mediated knockdown of CTCF and RAD21. Specific depletion of CTCF and RAD21 was achieved by more than two distinct small-interfering RNAs (siRNAs) (Ishihara *et al*, 2006). Western blot analysis was carried out using whole-cell extracts of Hep3B cells. Specific siRNAs and control siRNAs (GL3) were used for the assay. K.D., knockdown. (**B**) Effects of CTCF and RAD21 knockdown on the transcriptional status of the *APO* gene cluster. Using quantitative real-time PCR, the transcriptional levels were analyzed relative to *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) and control GL3. The values are given as means and standard deviations of the results from more than three independent experiments. $*P<0.05$, $**P<0.01$. (**C**, **D**) Effects of CTCF and RAD21 on the enrichment of HNF4α (C) and RNA polymerase II (D). The amount of the indicated proteins was shown at the *APOC3* enhancer, *APOC3* and *APOA1* promoters. Using quantitative ChIP analyses, the values are given as means and standard deviations of the results from more than three independent experiments.

 ©2009 European Molecular Biology Organization

changed on RAD21 depletion, suggesting that CTCF and cohesins are actively involved in transcriptional regulation of the *APO* gene cluster through partially overlapping but distinct mechanisms. The distinct effects on the *APO* genes may be explained by our proposed model that the *C3* enhancer and *APOC3/A4/A5* promoters reside in the same loop, where the *APOA5* promoter is distal from the enhancer, whereas the *APOA1* promoter is present in the different loop (Figure 5B). Throughout this study, the knockdown experiments were strictly carried out under the condition of no significant cell damages or cell cycle defects (Supplementary Figure S4).

As depletion of CTCF or RAD21 particularly affected the *APOA1/C3* genes, we checked the existence of HNF4α in the *APO* gene promoters and the *C3* enhancer (Figure 3C; Supplementary Figure S5A). Using a ChIP analysis followed by quantitative PCR, knockdown of either CTCF or RAD21 decreased the enrichment of HNF4α in the *APOC3* promoter ($P<0.01$), whereas the levels of HNF4α in the *APOA1* promoter and *APOC3* enhancer seemed to be unaffected. We then examined the existence of transcriptionally active form of RNA polymerase II at the *APO* gene promoters in Hep3B cells (Figure 3D; Supplementary Figure S5B). The loss of either CTCF or RAD21 reduced the enrichment of RNA polymerase II at the *APOC3* promoter but not the *APOA1* promoter. Thus, compared with the *APOC3* gene, the *APOA1* induction under the CTCF knockdown (Figure 3B) was not paralleled by an increase of HNF4α or RNA polymerase II binding at the promoter (see the Discussion). These results suggest that CTCF and cohesins cooperate for chromatin insulation and may regulate the occupancy of HNF4α and RNA polymerase II at the *APO* gene locus.

Role of CTCF and cohesins in overall structure of the APO gene cluster

To clarify the long-range effects of the insulators on the *APOA1/C3/A4/A5* region, we performed a chromosome conformation capture (3C) assay (Splinter *et al*, 2004; Hagege *et al*, 2007) in Hep3B cells (Figure 4). First, we measured the ligation frequencies of the *APOC3* enhancer (close to the AR1), as a reference, with 15 distinct *Bgl*II sites in the *APO* region. The efficiency of *Bgl*II digestion of individual sites was quantitatively >80% (data not shown). No ligation samples after the digestion were used as a negative control. The *APOC3* enhancer/AR1 was colocalized with the AC2 (*APOA5* promoter) and AC3 (Figure 4A, control), whereas *MGC13125* gene was associated with the *C3* enhancer/AR1 to lesser extent. We next tested the frequencies of AC3 ligation with other *Bgl*II sites within the *APO* region and found that AC3 interacted with the *APOC3* enhancer/AR1 and AC2/*APOA5* promoter (Figure 4B, control). Further, AC2/*APOA5* promoter was found to be colocalized with *APOC3* enhancer/AR1 and AC3 (Figure 4C, control). These results indicate that, the AC2/*APOA5* promoter, *APOC3* enhancer/AR1 and AC3 are closely localized in the nuclei, suggesting the possible formation of two chromatin loops in the *APO* gene cluster (see the model in Figure 5B).

We further examined whether CTCF knockdown affects these spatial interactions in the nuclei. Interestingly, CTCF depletion decreased the colocalization between the AC2/*APOA5* promoter, *APOC3* enhancer/AR1, and AC3 ($P<0.01$), whereas the *APOA1* promoter and AC1 did not

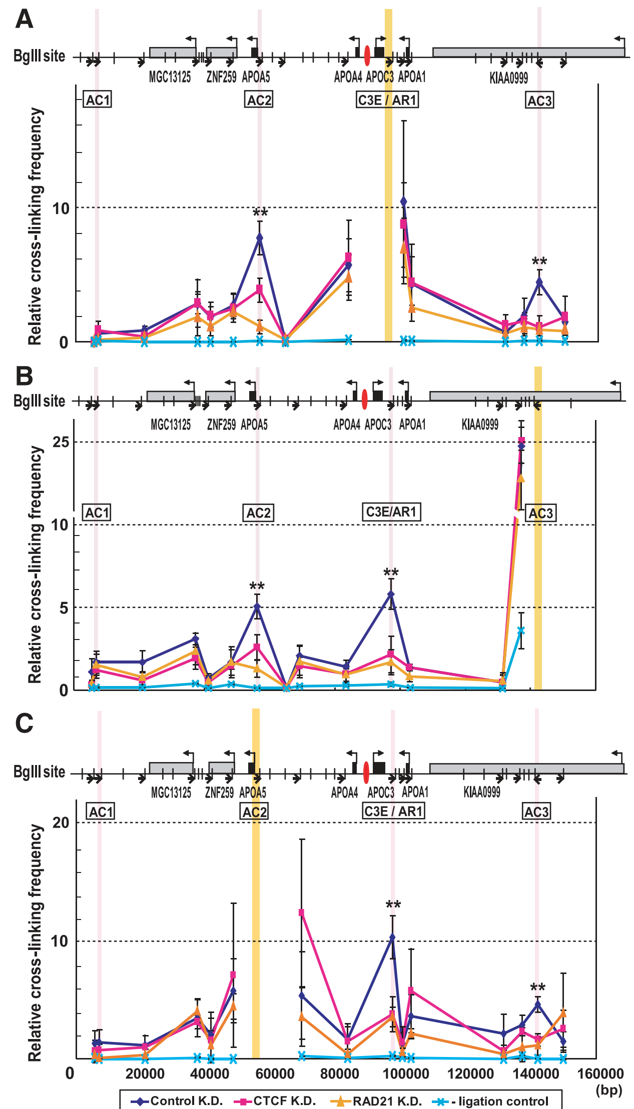

Figure 4 CTCF and cohesins are involved in chromosomal conformation of the *APO* gene region. The positions of the *Bgl*II sites indicated in the *APO* locus were used to design a 3C analysis in Hep3B cells. The relative cross-linking frequency between the reference *APOC3* enhancer fragment (yellow bar) and other individual *Bgl*II fragments (**A**) was determined by quantitative PCR measurement of three different samples from control and knockdown Hep3B cells (control K.D., CTCF K.D., and RAD21 K.D.). Similarly, the relative cross-linking frequency between the reference AC3 fragment (yellow bar) and other *Bgl*II fragments (**B**), and between the reference AC2 fragment (yellow bar) and other *Bgl*II fragments (**C**) is shown. To normalize the cross-linking and ligation efficiency between two restriction fragments, the *GAPDH* gene locus was used as a loading control for quantitative PCR. No ligation samples after the *Bgl*II digestion are shown as a negative control (-ligation control). Primers are indicated by small arrows. $^{**}P<0.01$.

change their positions relative to the reference sites. These data suggest that the spatial colocalization of the insulator sites is dependent on CTCF. Similarly, RAD21 depletion had significant effects on long-range chromatin conformation in the *APO* locus. As was the case for CTCF knockdown, the colocalization of the AC2/*APOA5* promoter, *APOC3* enhancer/AR1 and AC3 was disturbed under the loss of RAD21 ($P<0.01$). The RAD21 depletion did not significantly affect the position of the *APOA1* promoter and AC1 relative to the

©2009 European Molecular Biology Organization

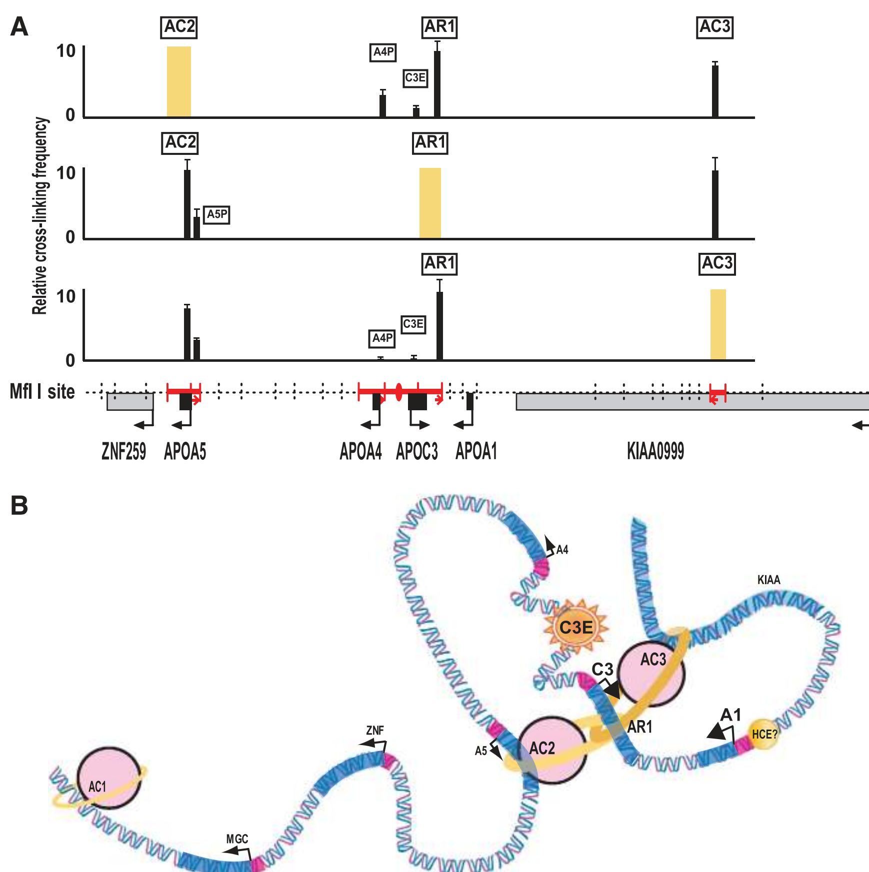

Figure 5 Spatial localization of the enhancer, promoter and insulator in the *APO* gene region. (**A**) The higher resolution 3C analysis of the *APO* locus. The experiment was performed in Hep3B cells, using the *Mfl*I digestion, which cut the fragments between AC2 and *APOA5* promoter, and between *APOA4* promoter, *APOC3* enhancer/promoter and AR1. The relative cross-linking frequencies of the references AC2, AR1 and AC3 (yellow bar) to other individual *Mfl*I fragments were determined by quantitative PCR measurement more than three times. To normalize the cross-linking and ligation efficiency between two restriction fragments, the *GAPDH* gene locus was used as a loading control for quantitative PCR. Primers are indicated by small arrows. (**B**) CTCF/cohesin-mediated insulators form chromatin loops to maintain interactions between enhancer and promoters. The 3C assays indicate that the insulators AC2, AR1 and AC3 are closely colocalized in the nuclei. The *C3* enhancer (C3 E) and *APOC3/A4/A5* promoters reside in the same loop, where the *APOC3/A4* promoters are pointed towards the *C3* enhancer, whereas *APOA1* promoter is present in the different loop. Cohesins at the AR1 may connect AC2 with AC3, and create the two chromatin loops. In this model, CTCF and cohesins maintain the enhancer–promoter association, which may facilitate the occupancy of HNF4α and RNA polymerase II at the *APO* promoters. *APOA1* gene may be induced by additional hepatic cis-element (HCE).

reference sites. Collectively, our results suggest that CTCF/cohesins-mediated insulators maintain the overall conformation in the locus, which impact regulation of the *APO* genes (Figures 3 and 4).

CTCF/cohesin-mediated insulators form chromatin loop structure that facilitates association of the enhancer and APO gene promoters

There are several functional elements in the *APOA1/C3/A4/A5* gene region, including the promoters, enhancers and CTCF/cohesin-mediated insulators (Figure 1A). To investigate what elements can form the chromatin loops and drive these interactions between distinct sites, we further performed a higher resolution 3C experiment in Hep3B cells (Figure 5). To analyze the interactions between the three reference fragments shown in Figure 4, we used the *Mfl*I digestion, to cut the fragments between AC2 and *APOA5* promoter, and between *APOA4* promoter, *APOC3* enhancer/promoter and AR1. This experiment enabled us to examine possible interactions between insulator elements, between insulators and enhancer/promoters, and between enhancer and promoters. The efficiency of *Mfl*I digestion of individual sites was >80% (data not shown). When the insulators AC2, AR1 and AC3 were used for a reference (yellow), they were frequently colocalized with each other (Figure 5A), suggesting that insulators themselves coexist in the nuclei. In addition, these insulator sites were not associated with the *C3* enhancer, *APOA4* promoter or *APOA5* promoter. We then checked the cross-linking frequencies of the *APOA5* promoter, *APOA4* promoter and *C3* enhancer with other *Mfl*I sites (Supplementary Figure S6). *APOA5* promoter was not colocalized with the *APOA4* promoter, the *C3* enhancer or the *APOA1* promoter. Thus, higher-order chromatin structure in the *APO* locus depends on interactions between the insulator elements (AC2, AC3 and AR1), leading to formation of two transcribed loops of the *APO* gene cluster (Figure 5B).

Chromatin insulators maintain cellular homeostasis

Among the apolipoproteins, APOA5 is uniquely low expressed in hepatic cells but is known to be a crucial regulator of plasma triglyceride concentrations and lipid homeostasis (Pennacchio *et al*, 2001; Willer *et al*, 2008). In addition,

©2009 European Molecular Biology Organization

APOA5 expression was some but significantly downregulated by the loss of CTCF-mediated insulation (Figure 3B). As AC2 is present near the *APOA5* promoter region, we checked whether CTCF binding to AC2 affects the promoter activity (Supplementary Figure S7). Our results indicated that binding of CTCF to AC2 did not affect transcription from the *APOA5* promoter. To examine the impact of CTCF on cellular function, we investigated the formation of lipid droplets in Hep3B cells, using Oil Red O staining (Figure 6A). Multiple droplets consisting of triglycerides were stained red and accumulated under knockdown of CTCF as well as APOA5. More than three repeated experiments showed that the use of control small-interfering RNAs (siRNAs) induced little such changes. Quantification of the Oil Red O also revealed that knockdown of CTCF or APOA5 markedly augmented the lipid accumulation ($P<0.01$), suggesting that CTCF insulators are involved in hepatic lipid dynamics, at least in part, by regulating the *APOA1/C3/A4/A5* gene cluster.

In humans, several important SNPs within the *APOA1/C3/A4/A5* cluster genes are strongly linked to dyslipidemia and increased susceptibility to atherosclerotic diseases (Lai *et al*, 2005). The AC2 site includes two significant SNPs, rs3135506 and rs2072560 (Qi *et al*, 2007). To confirm the effects of these SNPs on the AC2 insulator activity, we finally performed a luciferase reporter assay using modified reporter constructs shown in Figure 6B. Plasmids containing the SNPs were transiently introduced into Hep3B cells to quantify the enhancer–promoter associations. The luciferase activities for the modified reporters pESALD2F (SNP types) were comparable to those for pEIALD2F (wild type). In contrast, the use of AC2 mutant that lacked the CTCF binding lost the enhancer-blocking effect (pEMALD2F) (Figure 2). The results suggest that these SNPs are unlikely to affect the enhancer-blocking insulator activity of AC2, although a possible influence of other SNPs on the insulator function was not excluded.

Discussion

The present study has investigated the role of chromatin-based mechanisms in regulating the expression and higher-order structure of clustered genes in mammalian cells. CTCF/cohesin-mediated insulators play an essential role in the

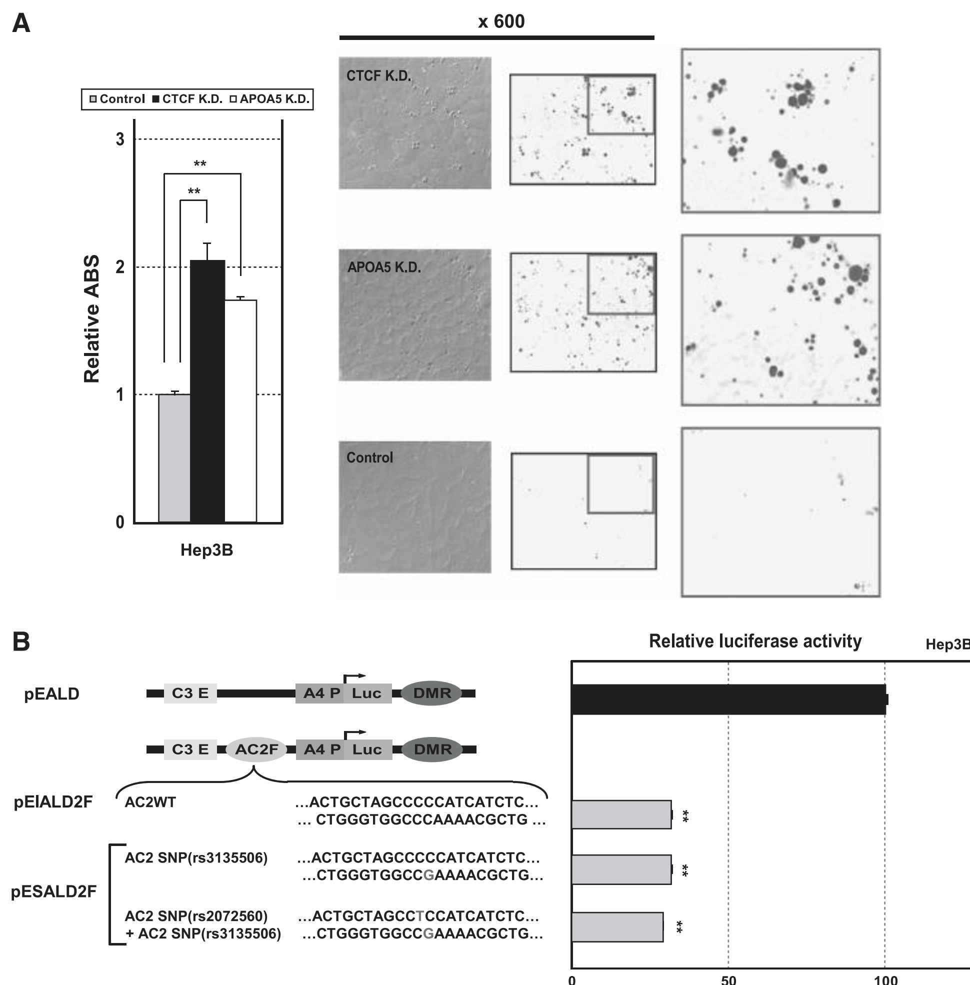

Figure 6 Chromatin insulators maintain cellular homeostasis. (**A**) Accumulation of cytoplasmic lipid droplets in CTCF- and APOA5-knockdown cells. Hep3B cells were transfected with siRNAs against *CTCF* and *APOA5* transcripts, and with control GL3. At 60 h after transfection, knockdown of CTCF as well as APOA5 induced accumulation of cytoplasmic triglyceride droplets that are positively stained with Oil Red O. Magnifications show the accumulation of triglyceride droplets. ABS, absorbance at 500 nm for quantification of Oil Red O. $^{**}P<0.01$. (**B**) Effect of the single nucleotide polymorphisms (SNPs) at AC2 on the enhancer-blocking activity. Luciferase reporter assay using pGL3 reporter plasmids containing the two different SNPs, rs3135506 and rs2072560 (Qi *et al*, 2007). The modified reporters pESALD2F (SNP types) did not alter the enhancer-blocking activities, which are comparable to those for pEIALD2F (wild type). A full-colour version of this figure is available at *The EMBO Journal* Online.

©2009 European Molecular Biology Organization

long-range control of the *APOA1/C3/A4/A5* gene cluster through topologically maintaining the chromatin loops and enhancer–promoter association, which is framed by AC2, AR1 and AC3. As shown in Figure 5B, we propose the possible mechanistic model in the *APO* gene locus: (1) insulators form two transcriptionally active chromatin loops that facilitate the cooperation of the enhancer and *APO* promoters; (2) the *C3* enhancer and *APOC3/A4/A5* promoters reside in the same chromatin loop, where the *APOC3/A4* promoters are pointed towards the enhancer, whereas the *APOA1* promoter is present in the different loop; (3) insulator-mediated chromatin formation is required for coordinating gene expression in the entire *APO* locus and (4) HNF4α and RNA polymerase II are loaded to the *APO* promoters in the higher-order chromatin structure. In addition, it is noted that CTCF/cohesin-mediated insulators may be heterogeneous with respect to function and composition. For instance, AC2 and AC3, but not the distal AC1, are cooperatively involved in the loop formation. The localization of RAD21 is sensitive to the reduction of CTCF, whereas both CTCF and RAD21 may codependently exist at the AC3 (Supplementary Figure S3). We predicted that cohesins at the AR1 may connect AC2 with AC3, and create the two chromatin loops. In agreement with this model, the UCSC Genome Browser on Human (http://genome.ucsc.edu/ENCODE/encode.hg18.html) shows that, in hepatic cells, the enhancer–promoter association unit in the *APO* region has highly acetylated histone H3, compared with the outside of the unit, and that HNF4α accumulates in the hyperacetylated *APO* locus (Rada-Iglesias *et al*, 2005). Thus, independently of the neighbouring genes, higher-order chromatin formation may maintain expression of the entire *APO* genes, through gathering HNF4α, acetylated histones and RNA polymerase II for the enhancer–promoter cooperation. In our study, there were close correlations between the *APO* gene expression, the localization of HNF4α and RNA polymerase II, and the higher-order chromatin structure. These correlated states were simultaneously affected by the loss of either CTCF or RAD21, suggesting that insulators are required for the overall control of the entire *APO* locus.

It is interesting that CTCF or RAD21 uniquely affected the *APOA5* gene and *APOA1* gene (Figure 3). As shown in Figure 5B, *APOA5* promoter is most distal from the *C3* enhancer in the same chromatin loop, and the effect of the CTCF or RAD21 knockdown on the *APOA5* expression was significant but small. This may be consistent with a earlier report that the *C3* enhancer acts on *APOA4/C3/A1* genes rather than *APOA5* gene, using transgenic mice carrying the human gene cluster with or without the *C3* enhancer (Gao *et al*, 2005; Li *et al*, 2008). On the other hand, only *APOA1* gene is present in the distinct loop, which may include alternative regulatory elements. The *APOA1* gene had higher expression than the adjacent *APOC3* and other *APO* genes in the locus, and it was more induced under the loss of CTCF but not by the RAD21 depletion (Figure 3B). This may be explained by the earlier report that the hepatic cis-acting elements are present just upstream the transcription start site of the *APOA1* gene (Harnish *et al*, 1996). Further, under the knockdown of CTCF or RAD21, the recruitment of HNF4α was found at the *C3* enhancer and *APOA1* promoter, but it was inhibited at the *APOA5/A4/C3* promoters within the same loop (Figure 3C; Supplementary Figure S5A), suggesting the possibility that HNF4α may be loaded to the *APOA5/A4/C3* promoters through the *C3* enhancer association. Taken together, our present study provides fundamental mechanisms that the spatial positioning of the enhancer, promoter and insulator plays essential roles in higher-order regulation of the entire gene cluster. What is the biological significance of the cohesin-mediated AR1 between the *APOA1* and *APOC3* genes? Both genes are expressed at high levels in hepatocytes, and the APOA1 and APOC3 are the major component of HDL and VLDL, respectively, which have entirely opposite roles in the metabolism and redistribution of lipoproteins and cholesterol. These two *APO* genes may be necessary to be distinctly regulated within the gene cluster. In addition, moderate insulator function at the AR1 may allow the *APOA1* gene to be partly controlled by the *C3* enhancer.

Considering the pathophysiological involvements of insulation, insulators may be affected by CpG methylation at the CTCF-binding sites on the genome. In the DMR of the *H19* gene, CpG methylation blocks the localization of CTCF to its binding sites in the DMR insulator (Bell and Felsenfeld, 2000; Hark *et al*, 2000), and CTCF protects the adjacent sequences against *de novo* CpG methylation (Butcher *et al*, 2004; Filippova *et al*, 2005). The EMSAs showed that CTCF bound CpG-methylated as well as unmethylated AC2 (Supplementary Figure S8), suggesting no effect of CpG-methylation on CTCF binding to AC2. In addition, *IGF2* imprinting is altered in aged and senescent human epithelial cells, probably due to reduced expression of CTCF (Fu *et al*, 2004). The age-dependent increase of dyslipidemia may be associated with a reduction in CTCF-mediated insulation. Finally, it was reported that a homozygous proband died of coronary artery atherosclerosis and had undetectable levels of plasma APOA1, APOC3 and HDL (Ordovas *et al*, 1989). The deletion breakpoints in the *APO* locus were mapped close to the AC2 site in which CTCF and cohesins coexist. Further studies are required for investigating implications of the higher-order chromatin formation in human diseases. In conclusion, our study at the human *APO* locus shed light on the importance of a long-range architecture of the enhancer, promoter and insulator in multiple genes in cluster.

Materials and methods

Cell culture

Hep3B and HeLa cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's minimum essential medium and Ham's F-12 nutrient medium (Sigma) supplemented with 10% (v/v) fetal bovine serum and penicillin/streptomycin.

ChIP microarray analysis

Immunoprecipitated DNA (ChIP) and a control for nonenriched DNA (whole-cell extract; WCE) were amplified by *in vitro* transcription, labelled with biotin and hybridized to high-density oligonucleotide tiling arrays (Affymetrix) (Wendt *et al*, 2008). Briefly, after treatment of cultured human cells with formaldehyde to cross-link proteins with genomic DNA, the protein–DNA complexes were fragmented by sonication and immunoprecipitated with anti-CTCF and anti-RAD21 antibodies. The ChIP DNA samples were analyzed on Affymetrix arrays representing all nonrepetitive elements of the human genome with 35-bp resolution. After scanning and data extraction, enrichment values (ChIP/WCE) were calculated by the MAT algorithm to normalize probe-specific biases under the hybridization conditions. The resulting MAT scores were proportional to the logarithm of the fold-enrichment of the ChIP-on-chip sample. We mapped the MAT scores to positions in the human genome assembly Hg 18 (NCBI Build 36). The bandwidth, MaxGap

 ©2009 European Molecular Biology Organization

and MinProbe parameters were set to 250, 1000 and 12, respectively. The cut-off threshold *P*-values were set to 1.0310210, 1.031028 and 1.031027.5 for the ENCODE 1.0, ENCODE 2.0 and Human Tiling 1.0R arrays, respectively. These *P*-values were equivalent to MAT scores of >4.85. The false-discovery rates were also calculated by the MAT program. For all experiments, the false-detection rates were $<2\%$.

ChIP and quantitative PCR analysis

Hep3B cells (1×10^7) were cross-linked with 1% formaldehyde at 37°C for 10 min. Crude cell lysates were sonicated to generate DNA fragments of 200–1000 bp. ChIP was performed with anti-CTCF, anti-RAD21, anti-HNF4α and anti-RNA polymerase II antibodies as well as control IgG (Ishihara *et al*, 2006). PCR amplification was carried out for 33 cycles under conditions of 95°C for 30 s, 58°C for 30 s and 72°C for 30 s. The DNA enrichment in the ChIP samples was determined by agarose gel electrophoresis and real-time PCR analysis using an ABI Prism 7500 (PE Applied Biosystems) and SYBR green fluorescence. The threshold was set to cross a point at which PCR amplification was linear, and the number of cycles (Ct) required to reach the threshold was collected and analyzed using Microsoft Excel. The PCR amplifications were performed using precipitated DNA samples and the input DNA. Primer sequences are listed in Supplementary Table I.

Electrophoretic mobility shift assay

CTCF protein was synthesized by a coupled *in vitro* transcription/translation reaction using a TNT T7 Quick system (Promega) according to the manufacturer's protocol. For super-shift assays, the reaction mixture was combined with 1 μl of anti-CTCF antibodies (Ishihara and Sasaki, 2002). The sequences of the probes were as follows: *H19* DMR, 5′-TGGCACGGAATTGGTTGTAGTTGTGGAATCGGAAGTGGCCGCGCGGCGGCAGTGCAGGCTCACACATCACAGCCCGAGCCCGCCCCAACT-3′; AC1, 5′-GGGCTCCGCTTTCGCCAGTCTAGAAAAGGCATATCACACTGCCCTCTAGTAGACAGCCTAGGAAATGACAGTCAGCTAGGGACTGGACAG-3′; AC2, 5′-GGGGGCAACAGCTACGGAGTTGTCAAGGCGGGGGCTGCAGGCAGAGGGCGCTAAAGAGCCCAGGATGGCCGGGATCTGCAGACAGAGCTA-3′; AC3, 5′-TGTGTAGGGAGAAGGCTAGGACCAAACTGTTGTTAAGGCCTCTAGATGGCACTCTCCTGTTTTCCTTTGGTCTCCACACACAATTTAGTG-3′; AR1, 5′-TTTGTGCCTCAGGCCCAGGGGCATAAACATCTGAGGTGACCTGGAGATGGCAGGGTTTGACTTGTGCTGGGGTTCCTGCAAGGATATCTC-3′. Methylation of the probes was carried out using *Sss*I methylase (New England Biolabs) according to the manufacturer's instructions. The methylation reaction was monitored by digestion of the probes with the methylation-sensitive restriction enzyme *Hha*I.

Luciferase reporter assay

The reporter plasmid pEALD had a *luciferase* (*Luc*) gene driven by the human *APOA4* promoter (-700 to $+10$ from the transcription start site), the *APOC3* enhancer (-900 to -400 from the transcription start site) and a 1.8-kb *Aat*II-*Hin*dIII fragment containing the *H19* DMR insulator downstream of the *Luc* gene. pEDALD was constructed by placing the *H19* DMR fragment between the *Luc* gene and the enhancer. The pEIALD plasmids were constructed by inserting the AC1, AC2 and AC3 fragments instead of the *H19* DMR in the forward direction (pEIALD1F, pEIALD2F and pEIALD3F) or reverse direction (pEIALD1R, pEIALD2R and pEIALD3R), respectively. The pEMALD plasmids were prepared by inserting the mutant AC1, AC2 and AC3 fragments, which lacked CTCF binding (pEMALD1F, pEMALD2F and pEMALD3F). To prepare pIEALD plasmids (pIEALD1, pIEALD2 and pIEALD3), the AC fragments were inserted into the upstream of the enhancer in pEALD. To test the effects of SNPs on the insulation, we constructed pEMALD2F through the introduction of base substitutions in the CTCF consensus sequences in the AC2 region of pEIALD2F. For dual luciferase activities (Ishihara *et al*, 2006), values are shown as means and standard deviations of the results from at least three independent experiments.

siRNA-mediated knockdown

siRNA duplexes were designed to target specific mRNAs (Japan Bioservice) as follows: human CTCF, 5′-GUGUCUAAAGAGGGCCUUGTT-3′ and 5′-CAAGGCCCUCUUUAGACAC-3′, and 5′-AGUGAACCCAUGAUAUGCCTT-3′ and 5′-GGCAUAUCAUGGGUUCACUTT-3′; human RAD21, 5′-UGAGCAAAGCUAGGCCUGATT-3′ and 5′-UCAGGCCUAGCUUUGCUCATT-3′, and 5′-GGUGAAAAUGGCAUUACGGTT-3′ and 5′-CCGUAAUGCCAUUUUCACCTT-3′. The siRNAs for GL3 were reported earlier (Ishihara *et al*, 2006). The siRNAs were transfected into the cells using Lipofectamine RNAiMAX (Invitrogen) for 48 h (RAD21) and 96 h (CTCF).

3C assay and quantitative PCR analysis

For the 3C assay (Splinter *et al*, 2004; Hagege *et al*, 2007), formaldehyde-cross-linked chromatin from Hep3B cells was digested with *Bgl*II or *Mfl*I restriction enzyme overnight, followed by ligation with T4 DNA ligase at 16°C for 4 h. To prepare control templates for standard curves, BAC spanning the *APO* locus RP11-442E11 was digested with *Bgl*II or *Mfl*I, followed by random religation. After reversing the cross-links, genomic DNA was purified by phenol extraction and ethanol precipitation. The DNA samples were further purified with a MinElute Reaction Cleanup Kit (Qiagen). Assessment of the ligated products was performed by real-time PCR using an ABI Prism 7500 (PE Applied Biosystems) and SYBR green fluorescence. All PCR products were cloned and sequenced to confirm the ligated products. We evaluated the efficiency of *Bgl*II or *Mfl*I digestion after the entire 3C treatment, by real-time PCR to amplify uncut fragments spanning *Bgl*II or *Mfl*I site. More than 80% of the individual restriction sites were digested in these experiments (data not shown). 3C-quantitative PCR data were normalized towards a loading control, using internal primers located in the *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) gene, to normalize the amount of template DNAs. Statistical analysis was performed by Student's *t*-test using more than three independent experiments. Primer sequences are listed in Supplementary Table I.

Oil Red O staining

To quantify cytoplasmic triglyceride droplets in Hep3B cells, the cells were fixed in 3.7% formaldehyde for 10 min, washed with 60% isopropanol for 30 s, stained with Oil Red O in 60% isopropanol for 20 min, washed with 60% isopropanol for 30 s, washed in cold water and viewed under DIC microscope (IX-71; Olympus) equipped with 60× NA1.0 Plan Apo objective lens. After the microscopic observations, the Oil Red O stain was extracted with 100% isopropanol for 20 min and quantified by the absorbance at 500 nm.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Acknowledgements

We appreciate Dr Jan-Michael Peters (Research Institute of Molecular Pathology) for collaboration using ChIP-on-chip analyses, and the members in our laboratory for helpful discussions. This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology (MN), by a Grant-in-Aid for the Global Center of Excellence (COE) 'Cell Fate Regulation Research and Education Unit', Kumamoto University, and in part by a research grant from the Uehara Memorial Foundation (MN).

References

Barski A, Cuddapah S, Cui K, Roh TY, Schones DE, Wang Z, Wei G, Chepelev I, Zhao K (2007) High-resolution profiling of histone methylations in the human genome. *Cell* **129:** 823–837

Bell AC, Felsenfeld G (2000) Methylation of a CTCF-dependent boundary controls imprinted expression of the Igf2 gene. *Nature* **405:** 482–485

©2009 European Molecular Biology Organization

Bell AC, West AG, Felsenfeld G (1999) The protein CTCF is required for the enhancer blocking activity of vertebrate insulators. *Cell* **98:** 387–396

Bell AC, West AG, Felsenfeld G (2001) Insulators and boundaries: versatile regulatory elements in the eukaryotic. *Science* **291:** 447–450

Burcin M, Arnold R, Lutz M, Kaiser B, Runge D, Lottspeich F, Filippova GN, Lobanenkov VV, Renkawitz R (1997) Negative protein 1, which is required for function of the chicken lysozyme gene silencer in conjunction with hormone receptors, is identical to the multivalent zinc finger repressor CTCF. *Mol Cell Biol* **17:** 1281–1288

Butcher DT, Mancini-DiNardo DN, Archer TK, Rodenhiser DI (2004) DNA binding sites for putative methylation boundaries in the unmethylated region of the BRCA1 promoter. *Int J Cancer* **111:** 669–678

Chernukhin IV, Shamsuddin S, Robinson AF, Carne AF, Paul A, El-Kady AI, Lobanenkov VV, Klenova EM (2000) Physical and functional interaction between two pluripotent proteins, the Y-box DNA/RNA-binding factor, YB-1, and the multivalent zinc finger factor, CTCF. *J Biol Chem* **275:** 29915–29921

Dorsett D (2007) Roles of the sister chromatid cohesion apparatus in gene expression, development, and human syndromes. *Chromosoma* **116:** 1–13

Dunn KL, Zhao H, Davie JR (2003) The insulator binding protein CTCF associates with the nuclear matrix. *Exp Cell Res* **288:** 218–223

Filippova GN, Cheng MK, Moore JM, Truong JP, Hu YJ, Nguyen DK, Tsuchiya KD, Disteche CM (2005) Boundaries between chromosomal domains of X inactivation and escape bind CTCF and lack CpG methylation during early development. *Dev Cell* **8:** 31–42

Filippova GN, Fagerlie S, Klenova EM, Myers C, Dehner Y, Goodwin G, Neiman PE, Collins SJ, Lobanenkov VV (1996) An exceptionally conserved transcriptional repressor, CTCF, employs different combinations of zinc fingers to bind diverged promoter sequences of avian and mammalian c-myc oncogenes. *Mol Cell Biol* **16:** 2802–2813

Fu VX, Schwarze SR, Kenowski ML, Leblanc S, Svaren J, Jarrard DF (2004) A loss of insulin-like growth factor-2 imprinting is modulated by CCCTC-binding factor down-regulation at senescence in human epithelial cells. *J Biol Chem* **279:** 52218–52226

Gao J, Wei Y, Huang Y, Liu D, Liu G, Wu M, Wu L, Zhang Q, Zhang Z, Zhang R, Liang C (2005) The expression of intact and mutant human apoAI/CIII/AIV/AV gene cluster in transgenic mice. *J Biol Chem* **280:** 12559–12566

Gaszner M, Felsenfeld G (2006) Insulators: exploiting transcriptional and epigenetic mechanisms. *Nat Rev Genet* **7:** 703–713

Hagege H, Klous P, Braem C, Splinter E, Dekker J, Cathala G, de Laat W, Forne T (2007) Quantitative analysis of chromosome conformation capture assays (3C-qPCR). *Nat Protoc* **2:** 1722–1733

Hark AT, Schoenherr CJ, Katz DJ, Ingram RS, Levorse JM, Tilghman SM (2000) CTCF mediates methylation-sensitive enhancer-blocking activity at the H19/Igf2 locus. *Nature* **405:** 486–489

Harnish DC, Malik S, Kilbourne E, Costa R, Karathanasis SK (1996) Control of apolipoprotein AI gene expression through synergistic interactions between hepatocyte nuclear factors 3 and 4. *J Biol Chem* **271:** 13621–13628

Ishihara K, Oshimura M, Nakao M (2006) CTCF-dependent chromatin insulator is linked to epigenetic remodeling. *Mol Cell* **23:** 733–742

Ishihara K, Sasaki H (2002) An evolutionarily conserved putative insulator element near the 3′ boundary of the imprinted Igf2/H19 domain. *Hum Mol Genet* **11:** 1627–1636

Kim TH, Abdullaev ZK, Smith AD, Ching KA, Loukinov DI, Green RD, Zhang MQ, Lobanenkov VV, Ren B (2007) Analysis of the vertebrate insulator protein CTCF-binding sites in the human genome. *Cell* **128:** 1231–1245

Klenova EM, Nicolas RH, Paterson HF, Carne AF, Heath CM, Goodwin GH, Neiman PE, Lobanenkov VV (1993) CTCF, a conserved nuclear factor required for optimal transcriptional activity of the chicken c-myc gene, is an 11-Zn-finger protein differentially expressed in multiple forms. *Mol Cell Biol* **13:** 7612–7624

Lai CQ, Parnell LD, Ordovas JM (2005) The APOA1/C3/A4/A5 gene cluster, lipid metabolism and cardiovascular disease risk. *Curr Opin Lipidol* **16:** 153–166

Li YJ, Wei YS, Fu XH, Hao DL, Xue Z, Gong H, Zhang ZQ, Liu DP, Liang CC (2008) The apolipoprotein CIII enhancer regulates both extensive histone modification and intergenic transcription of human apolipoprotein AI/CIII/AIV genes but not apolipoprotein AV. *J Biol Chem* **283:** 28436–28444

Lobanenkov VV, Nicolas RH, Adler VV, Paterson H, Klenova EM, Polotskaja AV, Goodwin GH (1990) A novel sequence-specific DNA binding protein which interacts with three regularly spaced direct repeats of the CCCTC-motif in the 5′-flanking sequence of the chicken c-myc gene. *Oncogene* **5:** 1743–1753

Lusis AJ, Pajukanta P (2008) A treasure trove for lipoprotein biology. *Nat Genet* **40:** 129–130

Lutz M, Burke LJ, Barreto G, Goeman F, Greb H, Arnold R, Schultheiss H, Brehm A, Kouzarides T, Lobanenkov V, Renkawitz R (2000) Transcriptional repression by the insulator protein CTCF involves histone deacetylases. *Nucleic Acids Res* **28:** 1707–1713

Majumder P, Gomez JA, Chadwick BP, Boss JM (2008) The insulator factor CTCF controls MHC class II gene expression and is required for the formation of long-distance chromatin interactions. *J Exp Med* **205:** 785–798

Mongelard F, Corces VG (2001) Two insulators are not better than one. *Nat Struct Biol* **8:** 192–194

Murrell A, Heeson S, Reik W (2004) Interaction between differentially methylated regions partitions the imprinted genes Igf2 and H19 into parent-specific chromatin loops. *Nat Genet* **36:** 889–893

Nasmyth K, Peters JM, Uhlmann F (2000) Splitting the chromosome: cutting the ties that bind sister chromatids. *Science* **288:** 1379–1385

Ohlsson R, Renkawitz R, Lobanenkov V (2001) CTCF is a uniquely versatile transcription regulator linked to epigenetics and disease. *Trends Genet* **17:** 520–527

Ordovas JM, Cassidy DK, Civeira F, Bisgaier CL, Schaefer EJ (1989) Familial apolipoprotein A-I, C-III, and A-IV deficiency and premature atherosclerosis due to deletion of a gene complex on chromosome 11. *J Biol Chem* **264:** 16339–16342

Parelho V, Hadjur S, Spivakov M, Leleu M, Sauer S, Gregson HC, Jarmuz A, Canzonetta C, Webster Z, Nesterova T, Cobb BS, Yokomori K, Dillon N, Aragon L, Fisher AG, Merkenschlager M (2008) Cohesins functionally associate with CTCF on mammalian chromosome arms. *Cell* **132:** 422–433

Pennacchio LA, Olivier M, Hubacek JA, Cohen JC, Cox DR, Fruchart JC, Krauss RM, Rubin EM (2001) An apolipoprotein influencing triglycerides in humans and mice revealed by comparative sequencing. *Science* **294:** 169–173

Prieur X, Schaap FG, Coste H, Rodriguez JC (2005) Hepatocyte nuclear factor-4α regulates the human apolipoprotein AV gene: identification of a novel response element and involvement in the control by peroxisome proliferator-activated receptor-γ coactivator-1α, AMP-activated protein kinase, and mitogen-activated protein kinase pathway. *Mol Endocrinol* **19:** 3107–3125

Qi L, Liu S, Rifai N, Hunter D, Hu FB (2007) Associations of the apolipoprotein A1/C3/A4/A5 gene cluster with triglyceride and HDL cholesterol levels in women with type 2 diabetes. *Atherosclerosis* **192:** 204–210

Rada-Iglesias A, Wallerman O, Koch C, Ameur A, Enroth S, Clelland G, Wester K, Wilcox S, Dovey OM, Ellis PD, Wraight VL, James K, Andrews R, Langford C, Dhami P, Carter N, Vetrie D, Ponten F, Komorowski J, Dunham I *et al* (2005) Binding sites for metabolic disease related transcription factors inferred at base pair resolution by chromatin immunoprecipitation and genomic microarrays. *Hum Mol Genet* **14:** 3435–3447

Saitoh N, Bell AC, Recillas-Targa F, West AG, Simpson M, Pikaart M, Felsenfeld G (2000) Structural and functional conservation at the boundaries of the chicken beta-globin domain. *EMBO J* **19:** 2315–2322

Splinter E, Grosveld F, de Laat W (2004) 3C technology: analyzing the spatial organization of genomic loci *in vivo*. *Methods Enzymol* **375:** 493–507

Splinter E, Heath H, Kooren J, Palstra RJ, Klous P, Grosveld F, Galjart N, de Laat W (2006) CTCF mediates long-range chromatin looping and local histone modification in the beta-globin locus. *Genes Dev* **20:** 2349–2354

Sproul D, Gilbert N, Bickmore WA (2005) The role of chromatin structure in regulating the expression of clustered genes. *Nat Rev Genet* **6:** 775–781

©2009 European Molecular Biology Organization

Stedman W, Kang H, Lin S, Kissil JL, Bartolomei MS, Lieberman PM (2008) Cohesins localize with CTCF at the KSHV latency control region and at cellular c-myc and H19/Igf2 insulators. *EMBO J* **27:** 654–666

Vostrov AA, Quitschke WW (1997) The zinc finger protein CTCF binds to the APBbeta domain of the amyloid beta-protein precursor promoter. Evidence for a role in transcriptional activation. *J Biol Chem* **272:** 33353–33359

Watkins H, Farrall M (2006) Genetic susceptibility to coronary artery disease: from promise to progress. *Nat Rev Genet* **7:** 163–173

Wendt KS, Yoshida K, Itoh T, Bando M, Koch B, Schirghuber E, Tsutsumi S, Nagae G, Ishihara K, Mishiro T, Yahata K, Imamoto F, Aburatani H, Nakao M, Imamoto N, Maeshima K, Shirahige K, Peters JM (2008) Cohesin mediates transcriptional insulation by CCCTC-binding factor. *Nature* **451:** 796–801

West AG, Gaszner M, Felsenfeld G (2002) Insulators: many functions, many mechanisms. *Genes Dev* **16:** 271–288

Willer CJ, Sanna S, Jackson AU, Scuteri A, Bonnycastle LL, Clarke R, Heath SC, Timpson NJ, Najjar SS, Stringham HM, Strait J, Duren WL, Maschio A, Busonero F, Mulas A, Albai G, Swift AJ, Morken MA, Narisu N, Bennett D *et al* (2008) Newly identified loci that influence lipid concentrations and risk of coronary artery disease. *Nat Genet* **40:** 161–169

Xie X, Mikkelsen TS, Gnirke A, Lindblad-Toh K, Kellis M, Lander ES (2007) Systematic discovery of regulatory motifs in conserved regions of the human genome, including thousands of CTCF insulator sites. *Proc Natl Acad Sci USA* **104:** 7145–7150

Yu W, Ginjala V, Pant V, Chernukhin I, Whitehead J, Docquier F, Farrar D, Tavoosidana G, Mukhopadhyay R, Kanduri C, Oshimura M, Feinberg AP, Lobanenkov V, Klenova E, Ohlsson R (2004) Poly(ADP-ribosyl)ation regulates CTCF-dependent chromatin insulation. *Nat Genet* **36:** 1105–1110

Yusufzai TM, Felsenfeld G (2004a) The 5′-HS4 chicken beta-globin insulator is a CTCF-dependent nuclear matrix-associated element. *Proc Natl Acad Sci USA* **101:** 8620–8624

Yusufzai TM, Tagami H, Nakatani Y, Felsenfeld G (2004b) CTCF tethers an insulator to subnuclear sites, suggesting shared insulator mechanisms across species. *Mol Cell* **13:** 291–298

Zannis VI, Kan HY, Kritis A, Zanni E, Kardassis D (2001) Transcriptional regulation of the human apolipoprotein genes. *Front Biosci* **6:** D456–D504

Zhao H, Dean A (2004) An insulator blocks spreading of histone acetylation and interferes with RNA polymerase II transfer between an enhancer and gene. *Nucleic Acids Res* **32:** 4903–4919

©2009 European Molecular Biology Organization