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RESEARCH ARTICLE

Functional interplay between MyoD and CTCF in regulating long-range chromatin interactions during differentiation

Cecilia Battistelli, Anna Busanello* and Rossella Maione‡

ABSTRACT

Higher-order chromatin structures appear to be dynamically arranged during development and differentiation. However, the molecular mechanism underlying their maintenance or disruption and their functional relevance to gene regulation are poorly understood. We recently described a dynamic long-range chromatin interaction between the gene promoter of the cdk inhibitor *p57*^{kip2} (also known as *Cdkn1c*) and the imprinting control region KvDMR1 in muscle cells. Here, we show that CTCF, the best characterized organizer of long-range chromatin interactions, binds to both the *p57*^{kip2} promoter and KvDMR1 and is necessary for the maintenance of their physical contact. Moreover, we show that CTCF-mediated looping is required to prevent *p57*^{kip2} expression before differentiation. Finally, we provide evidence that the induction of *p57*^{kip2} during myogenesis involves the physical interaction of the muscle-regulatory factor MyoD with CTCF at KvDMR1, the displacement of the cohesin complex subunit Rad21 and the destabilization of the chromatin loop. The finding that MyoD affects chromatin looping at CTCF-binding sites represents the first evidence that a differentiation factor regulates chromatin-loop dynamics and provides a useful paradigm for gaining insights into the developmental regulation of long-range chromatin contacts.

KEY WORDS: MyoD, CTCF, chromatin loops, p57^{kip2}, KvDMR1

INTRODUCTION

Cell differentiation involves the coordinated activation of specific sets of genes and the repression of others, through the cooperation of several molecular mechanisms. In addition to the activity of specific transcription factors and chromatin modifying enzymes, which target specific regulatory sequences, the three-dimensional organization of genes and their regulatory elements is recognized to play a crucial role in transcriptional regulation (Dekker, 2008; Felsenfeld and Dekker, 2012; Kadauke and Blobel, 2009). Long-range interactions allow communication between promoters and distant regulatory elements, as well as the physical association of coordinately regulated genes and their localization at functionally distinct subnuclear compartments (Van Bortle and Corces, 2012). Despite the longstanding recognition of the existence and of the functional relevance of chromatin loops (Zlatanova and van Holde, 1992), the molecular mechanisms responsible for the establishment of chromatin contacts and their impact on gene expression have begun to be elucidated only recently.

A number of chromatin proteins, transcription factors and co-factors have been reported to participate in chromatin looping in different genomic contexts (Holwerda and de Laat, 2012). In this regard, the CCCTC-binding factor (CTCF) is the best characterized organizer of chromatin architecture. CTCF is a highly conserved 11-zinc-finger protein that plays multiple roles in transcriptional regulation, ranging from direct effects on promoters or enhancers to long-distance effects, including intra- and inter-chromosomal interactions (Phillips and Corces, 2009; Splinter et al., 2006; Zlatanova and Caiafa, 2009a). CTCF binds genome-wide to variant DNA sequences located in numerous genic and intergenic regions (Barski et al., 2007; Kim et al., 2007; Shen et al., 2012). The involvement of CTCF in mediating chromatin contacts has been analyzed in detail for the β-globin locus, where the protein participates in the formation of an active chromatin hub (Splinter et al., 2006), and for the *Igf2/H19* imprinting domain, where CTCF determines allele-specific looping and enhancer blocking (Kurukuti et al., 2006). The ability of the protein to form dimers or multimers and to interact with structural nuclear proteins, such as nucleophosmin and lamins, is believed to be involved in the mechanism by which CTCF brings together distant elements (Pant et al., 2004; Yusufzai et al., 2004). More recently, it has been revealed that the cohesin complex is co-recruited genome-wide with CTCF and plays a crucial role in maintaining chromatin interactions between CTCF-binding sites (Hadjur et al., 2009; Hou et al., 2010; Mishiro et al., 2009; Nativio et al., 2009).

Dynamic changes of higher-order chromatin structures have been described for specific loci during developmental processes (Holwerda and de Laat, 2012; Misteli, 2007). However, little is known about the signalling pathways and the regulatory factors that functionally interact with chromatin complexes involved in the formation and/or maintenance of chromatin loops. Moreover, it is still unclear whether the observed changes in chromatin folding are the cause or the consequence of transcriptional dynamics.

We have previously investigated the transcriptional regulation of *p57*^{kip2} (*p57*, also known as *Cdkn1c*) in differentiating muscle cells (Busanello et al., 2012; Figliola et al., 2008; Figliola and Maione, 2004; Vaccarello et al., 2006). *p57* codes for a cdk inhibitor that plays a crucial role in many differentiation processes and has been found to be mutated or silenced in several developmental pathologies and in cancer (Pateras et al., 2009). *p57* is a paternally imprinted gene and is regarded as subject to elaborate epigenetic mechanisms. *p57* is located within the *p57/kcnq1* imprinting domain, a conserved gene cluster arranged on distal chromosome 7 in mouse and on a syntenic segment of chromosome 11p15 in human. A distant regulatory region, KvDMR1, located >150 kb from the *p57*

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promoter, controls in cis the allele-specific silencing of a group of genes belonging to the imprinting domain (Fitzpatrick et al., 2002; Horike et al., 2000). Although the molecular mechanisms have not been completely clarified, it has been suggested that both CTCF-mediated insulation and long-noncoding-RNA-mediated silencing participate in the function of this imprinting control region (Shin et al., 2008). We have recently reported that, during skeletal muscle differentiation, the induction of *p57* correlates with the disruption of a chromatin loop formed by a long-range chromatin interaction between the *p57* promoter and a regulatory element contained within KvDMR1 (Busanello et al., 2012). We also showed that the removal of such a loop ensues from the binding of the bHLH myogenic factor MyoD to an E-box-like sequence within KvDMR1. In the present work, we focused on the molecular mechanism participating in the formation and in the disruption of the higher-order chromatin structure involved in *p57* regulation. We report that CTCF mediates the formation of the repressive chromatin loop that constrains *p57* expression and that this constraint is relieved by a functional interaction between MyoD and CTCF at KvDMR1.

RESULTS

CTCF binds to KvDMR1 and the *p57* promoter in muscle cells

We have previously shown that MyoD induces *p57* through an indirect mechanism that does not involve its binding to the promoter region of the gene (Figliola et al., 2008). More recently, we reported that MyoD binds *in vivo* to multiple sites within KvDMR1 (Busanello et al., 2012). Then we noticed that the MyoD-binding sites are overlapping or adjacent to three CTCF-binding sites (Fig. 1). Two of these sites, previously demonstrated to be bound by CTCF in mouse fibroblasts (Fitzpatrick et al., 2007), are located in the F1 and F2 subregions, whereas the third, predicted by MatInspector tool, is located in the F3 subregion, which we had previously demonstrated to be functionally relevant for MyoD-induced rearrangement (Busanello et al., 2012). We also noticed the presence of at least one putative CTCF-binding site within the *p57* promoter (Fig. 1). A similar distribution of CTCF recognition sequences is also present in the syntenic region of human chromosome 11p15, thus supporting their functional role in the regulation of this locus.

To address the involvement of CTCF in MyoD- and KvDMR1-dependent regulation of *p57*, we employed two different cell systems undergoing MyoD-induced differentiation. One consists of spontaneously differentiating C2.7 myoblasts, driven by endogenous MyoD. The other one consists of fibroblast cells expressing exogenous MyoD and undergoing myogenic conversion, a well-established system for the analysis of MyoD-dependent regulation (Bergstrom et al., 2002; Cao et al., 2010). In particular, we have previously characterized two different fibroblast cell types, both equally competent to undergo MyoD-induced differentiation, but differentially competent regarding the binding of MyoD to KvDMR1 and the upregulation of *p57* expression. The first, which we term 'responsive', is normally sensitive, whereas the second, which we term 'unresponsive', is completely refractory to the induction of *p57*. These cell types represent a valuable experimental tool to highlight the existence of cis-acting constraints restricting *p57* expression and the functional role of the interaction between MyoD and KvDMR1 (Busanello et al., 2012; Figliola et al., 2008; Figliola and Maione, 2004; Vaccarello et al., 2006).

First, we observed that CTCF protein is constitutively present in differentiating myoblasts and in MyoD-converted fibroblasts, regardless of their differentiation stage or their responsiveness with respect to *p57* induction (supplementary material Fig. S1). Next, we investigated the occurrence of *in vivo* CTCF binding to the consensus sequences identified within KvDMR1 and the *p57* promoter. For this purpose, we performed chromatin immunoprecipitation (ChIP) assays in spontaneously differentiating C2.7 myoblasts and in responsive (C57BL/6) and unresponsive (C3H10T1/2) mouse embryonic fibroblasts expressing exogenous MyoD. As shown in Fig. 2A, and in agreement with a previous report (Fitzpatrick et al., 2007), we observed that CTCF binds to both the F1 and F2 subregions (corresponding to the CTS1 and CTS2 fragments described by Fitzpatrick and co-workers). In addition, as shown in the same figure, we found that CTCF also binds to the F3 subregion in all three cell types analyzed, implying the existence of a further and previously unidentified CTCF-binding site within KvDMR1. The

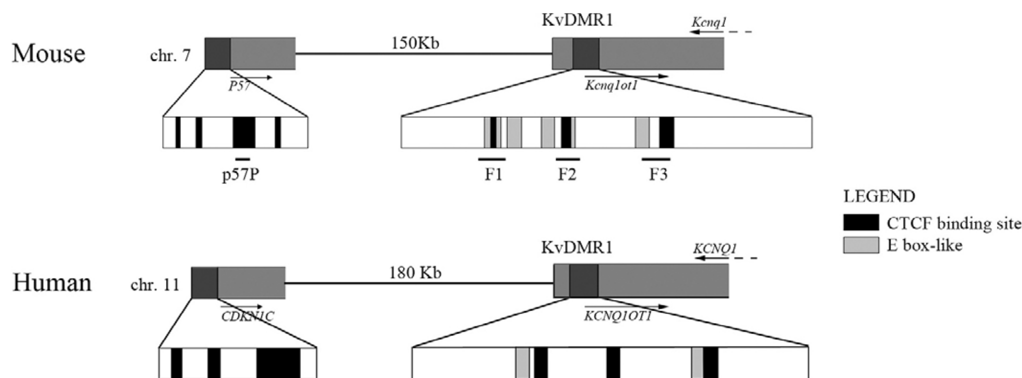

Fig. 1. The genomic organization of the *p57*/KvDMR1 locus. Diagram showing the location of *p57*, *Kcnq1* and *Kcnq1ot1* genes and KvDMR1 regions in mouse and human. The positions of the putative CTCF-binding sites and of the E-box-like sequences, predicted by the MatInspector tool, are also indicated. The enlarged regions represent: the mouse *p57* promoter extending from nucleotide +1 to +1099 of the AF160190 sequence (upper left); mouse KvDMR1 extending from nucleotide +1561 to +3420 of the AF119385 sequence (upper right); the human *p57* promoter extending from nucleotide +2882218 to +2883217 of the NC_000011.10 sequence (lower left); and the human KvDMR1 element extending from nucleotide +2699081 to +2700857 of the NC_000011.10 sequence (lower right). Thick black bars within promoters represent multiple and contiguous CTCF recognition sequences. Black lines represent the amplicons of ChIP assays.

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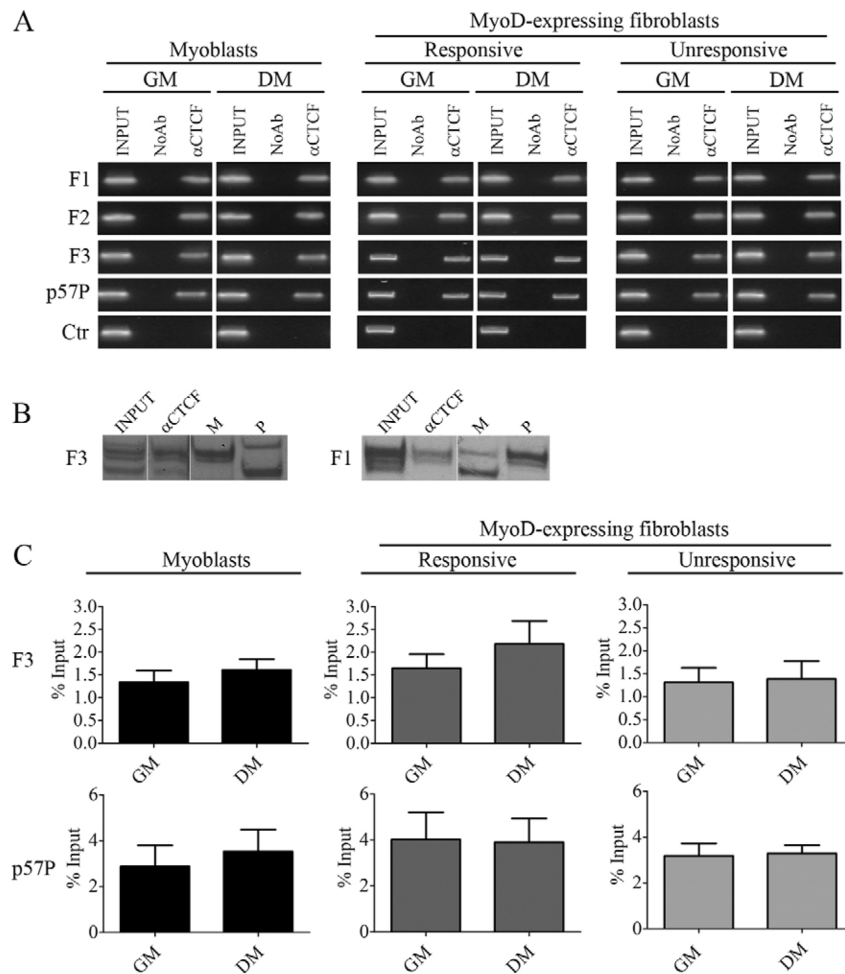

Fig. 2. CTCF binds to KvDMR1 in differentiating muscle cells. (A) *In vivo* binding activity of CTCF during differentiation. Chromatin from C2.7 myoblasts and responsive (C57BL/6) and unresponsive (C3H10T1/2) mouse embryonic fibroblasts expressing exogenous MyoD, kept either in growth (GM) or in differentiation medium for 24 hours (DM), was immunoprecipitated using a specific antibody against CTCF or in the absence of antibody (NoAb). Input represents non-immunoprecipitated crosslinked chromatin. F1, F2, F3 and *p57* promoter (p57P) fragments were amplified by PCR as described in Materials and Methods. An unrelated fragment, mapping 313 nucleotides downstream of F3 and not containing CTCF recognition sites, was used as a negative control (Ctr). The results are representative of three independent experiments. (B) Allele-specific ChIP of CTCF. (C57BL/6×SD7) F1 hybrid mouse embryonic fibroblasts, carrying single nucleotide polymorphisms in the *p57/kcnq1* locus, were infected with the MyoD-expressing retrovirus, incubated for 24 hours in differentiation medium and treated for ChIP assay as reported in Materials and Methods. Chromatin that was immunoprecipitated with an antibody against CTCF was then analyzed by PCR with primers specific for either F1 or F3, followed by single strand conformation polymorphism (SSCP) analysis. 'M' and 'P' show the electrophoretic mobility of maternal- and paternal-specific bands, respectively. The parental alleles were distinguished by SSCP analysis using primer pairs amplifying F1 (Fitzpatrick et al., 2007) or F3 (Busanello et al., 2012) regions. After denaturation, PCR products were resolved by non-denaturing acrylamide gel electrophoresis. (C) qPCR analysis of ChIP assays for CTCF binding to F3 and the *p57* promoter. Values derived from three independent experiments are expressed as the percentage of input chromatin (% Input). Data are shown as the mean±s.e.m.

inspection of ChIP-seq data from ENCODE/Caltech for CTCF and MyoD in mouse C2C12 muscle cells (a cell line related to C2.7) showed weak but detectable signals for both factors along KvDMR1, further supporting our ChIP results. Interestingly, whereas CTCF binding to F1 is specific for the paternal allele (Fitzpatrick et al., 2007), we observed that CTCF binds biallelically to F3 (Fig. 2B), just as we had previously found for MyoD (Busanello et al., 2012). Notably, we detected the binding of CTCF to the *p57* promoter as well (Fig. 2A). Quantitative ChIP analysis confirmed that there was no significant change in CTCF binding to F3 nor to the *p57* promoter during differentiation (Fig. 2C).

CTCF is involved in the formation and in the maintenance of the long-distance chromatin interaction between KvDMR1 and the *p57* promoter

CTCF participates in long-distance gene regulation by orchestrating chromatin contacts, at least in part through its ability to form homodimers and multimers (Pant et al., 2004). The observation that CTCF binds to both F3 and the *p57* promoter was suggestive of a possible role for CTCF in mediating the formation of the chromatin loop that constrains *p57* expression. To verify whether this factor participates in the architecture of the chromatin loop, we employed the ChIP-loop method, a technique combining ChIP and chromatin conformation capture (3C) methods and allowing analysis of chromatin loops anchored to a specific chromatin-bound protein. Differentiating C2.7 myoblasts and MyoD-converted fibroblasts, both responsive and unresponsive, were collected before and after the induction of differentiation. Crosslinked chromatin was subjected to digestion, immunoprecipitation with antibodies specific for CTCF or MyoD and then ligation, as described in Materials and Methods. qPCR reactions were performed, as outlined in Fig. 3A, in order to amplify hybrid fragments derived from the ligation of the *p57* promoter and KvDMR1 sequences. The results in Fig. 3B show the presence of the recombinant fragment derived from the physical proximity of the *p57* promoter and F3 sequences in CTCF-immunoprecipitated samples. Importantly, the recombinant fragment was effectively immunoprecipitated only from undifferentiated and from unresponsive cells, i.e. under the same conditions in which we had previously detected the occurrence of the long-range chromatin interaction (Busanello et al., 2012). PCR amplification with a different primer pair gave very similar results (supplementary material Fig. S2). In agreement with our previous finding that MyoD binding to F3 was accompanied by the release of the chromatin loop, we did not detect the recombinant fragment in MyoD immunoprecipitates (Fig. 3B; supplementary material Fig. S2). Taken together, these results reveal that CTCF participates in the long-range chromatin interaction that brings together the *p57* promoter and KvDMR1 sequences. Moreover, they also show that the disruption of the chromatin loop, which occurs during the differentiation of responsive cells, is not accompanied by CTCF displacement from F3 nor from the *p57* promoter (see also below).

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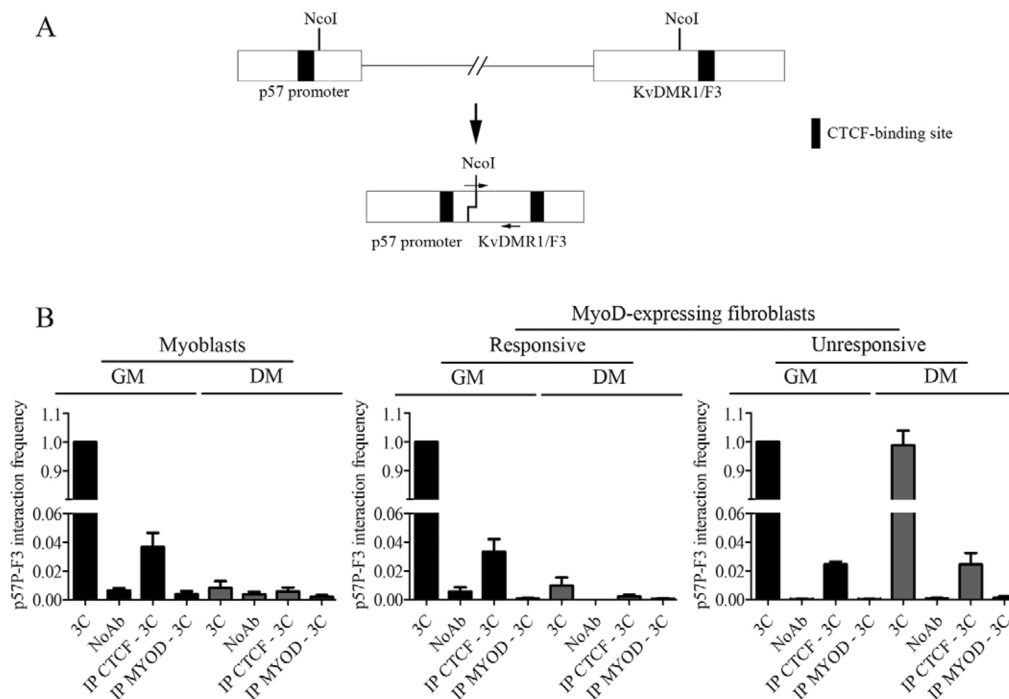

Fig. 3. CTCF is present at the chromatin contact between F3 and the *p57* promoter. (A) Diagram showing the locations of the *Nco*I restriction sites and of the PCR primers (horizontal arrows) used for ChIP-loop analysis. (B) qPCR analysis of ChIP-loop templates. Crosslinked chromatin obtained from the same cells under the same conditions as in Fig. 2A was digested and either directly ligated ('3C') or immunoprecipitated with the indicated antibodies (IP CTCF - 3C and IP MYOD - 3C) or without antibody (NoAb) before ligation. ChIP-loop templates were analyzed for the presence of the recombinant fragment deriving from the ligation of the *p57* promoter and F3 sequences by qPCR amplification with primer pair 1, as reported in Materials and Methods. For each cell type, values are expressed as relative to the interaction frequency in undifferentiated cells (3C in growth medium). GM, growth medium; DM, differentiation medium. Data show the mean±s.e.m. (three independent experiments).

In order to directly verify whether CTCF was required for the maintenance of the chromatin loop, we performed 3C assays after CTCF knockdown in unresponsive cells. Unresponsive fibroblasts, previously infected with a MyoD-encoding retrovirus, were transfected with CTCF-targeting small interfering (si)RNAs. Western blot analysis of CTCF protein levels confirmed its efficient depletion (Fig. 4A,B). More importantly, quantitative 3C analysis of the *p57*-KvDMR1 locus showed a significant reduction in the interaction frequency between the *p57* promoter and the F3 region (Fig. 4C). This result clearly indicates that the observed long-range chromatin interaction is CTCF dependent.

CTCF is required for *p57* repression in undifferentiated and in unresponsive cells

To demonstrate the existence of a causal link between CTCF-mediated looping between *p57* and KvDMR1 and *p57* repression, we analyzed the effects of CTCF knockdown on gene expression during MyoD-induced differentiation. Responsive and unresponsive fibroblasts, previously infected with a MyoD-encoding retrovirus, were transfected with the same amounts of CTCF-targeting or control siRNAs. RNA and proteins were collected at two time-points during differentiation. Western blot analysis of CTCF protein levels confirmed its efficient depletion (supplementary material Fig. S3A). Remarkably, RT-PCR analysis showed that CTCF depletion correlated with *p57* de-repression (Fig. 5A,B). In particular, the expression of *p57* was detectable even before differentiation in responsive cells and appeared to be further increased after differentiation. Moreover, and notably, CTCF knockdown caused a partial rescue of *p57* responsiveness to MyoD-dependent induction in unresponsive cells. Unlike that of *p57*, the expression of the muscle differentiation marker myogenin did not increase after CTCF depletion – rather it appeared to slightly decrease both in responsive and in unresponsive cells (Fig. 5B). This allows us to exclude the possibility that the observed increase in *p57* expression is an indirect consequence of a generally increased extent of differentiation. These results strongly support the conclusion that CTCF, by mediating the formation of a repressive chromatin loop, plays a primary role in restricting *p57* expression both before the onset of differentiation and in unresponsive muscle cell types.

As we reported recently, *p57* is co-regulated with the co-imprinted gene *kcnq1* in muscle cells (Busanello et al., 2012). In particular, the two genes are co-induced by MyoD in responsive cells and are similarly refractory to MyoD-dependent activation in unresponsive cells. In light of the observed role of CTCF in controlling the expression of *p57*, we asked whether *kcnq1* was also subject to CTCF-dependent regulation. The qRT-PCR results shown in Fig. 5B indicate that *kcnq1*, expressed at very low levels in unresponsive cells, was appreciably upregulated after CTCF depletion. Moreover, as observed for *p57*, the already high levels of *kcnq1* RNA present in responsive cells were further increased. These data suggest that the relief of CTCF-mediated repression is involved in the co-regulation of some genes of the *p57* imprinting domain during muscle differentiation.

Because CTCF is known to participate in the molecular mechanisms driving allele-specific expression from the *Igf2/H19* locus and other imprinted loci (Hikichi et al., 2003; Singh et al., 2012; Yoon et al., 2005), we asked whether the increase in *p57* and *kcnq1* expression observed after CTCF depletion resulted

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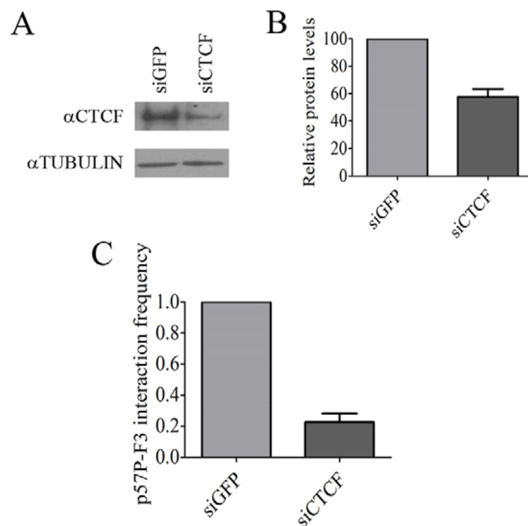

Fig. 4. CTCF is required for the chromatin contact between F3 and the *p57* promoter. (A) Western blot analysis showing the siRNA-mediated reduction in CTCF protein levels. C3H10T1/2 unresponsive fibroblasts were infected with the MyoD retroviral vector and, 48 hours later, were transfected with either control siRNA (siGFP) or CTCF-targeting siRNA (siCTCF). At 48 hours after transfection, cells were shifted to differentiation medium for a further 24 hours and were then collected for the analysis of protein levels. Tubulin was used as a loading control. The results are representative of three independent experiments. (B) Densitometric analysis of the western blot results. The protein levels are expressed as a percentage relative to the control sample. Data show the mean±s.e.m. (three independent experiments). (C) Quantitative 3C analysis of the *p57*-promoter–F3 interaction following CTCF depletion. Chromatin was extracted in parallel with protein from cells treated as indicated in A. 3C templates were analyzed by qPCR amplification with primer pair 1, as reported in Materials and Methods. Data show the interaction frequency relative to that of the control sample and are expressed as the mean±s.e.m. (three independent experiments). Almost identical results were obtained by qPCR amplification with primer pair 2 (data not shown).

from a loss of imprinting. Taking advantage of responsive fibroblasts carrying single nucleotide polymorphisms in the *p57*/*kcnq1* locus, we have already demonstrated that the MyoD-dependent induction of *p57* involves upregulation of the normally active maternal allele and not the de-repression of the normally silent paternal allele (Busanello et al., 2012). We used the same fibroblast cell type to determine the allele-specific induction of *p57* and *kcnq1* after CTCF depletion. MyoD-converted fibroblasts were transfected with CTCF-targeting siRNAs as above and tested for the efficient reduction of CTCF levels (supplementary material Fig. S3B). *p57* and *kcnq1* RNAs were then examined by combining RT-PCR with restriction fragment length polymorphism (RFLP) analysis. The results, shown in Fig. 6, confirm that, even in this cell type, *p57* and *kcnq1* are already expressed in the undifferentiated state after CTCF depletion and also show that both genes are upregulated exclusively from the respective maternal non-imprinted alleles. This finding allows us to exclude the possibility that the de-repression of the two genes subsequent to CTCF depletion depends on the release of imprinting, thus highlighting the complexity of CTCF function at KvDMR1 (see Discussion).

MyoD functionally interacts with CTCF at KvDMR1

As mentioned above, the CTCF-binding site located within F3 lies close to the E-box-like element that is crucial for the disruption of the chromatin loop caused by MyoD binding at the onset of differentiation. In agreement with a previous report (Delgado-Olguín et al., 2011), we found that MyoD and CTCF interact with each other in C2.7 cell extracts, both before and after the induction of differentiation (supplementary material Fig. S4). To explore the molecular mechanism by which MyoD interferes with the maintenance of the CTCF-mediated loop, we determined whether the two proteins participated in the same regulatory complex bound to F3. To do this, we performed sequential ChIP (re-ChIP) assays using C2.7 myoblasts, a cell type that is responsive to MyoD binding and loop disruption, and that expresses *p57* after the induction of differentiation (Busanello et al., 2012). The immunoprecipitation of MyoD-bound chromatin with anti-CTCF antibody and the reciprocal immunoprecipitation were performed in both undifferentiated and differentiated cells (Fig. 7B). As expected, the presence of a MyoD–CTCF complex at F3 was not detectable before differentiation, when MyoD has not yet bound to the target region. By contrast, after the shift to differentiation medium, the two factors do co-occupy F3. These results indicate the occurrence of a physical interaction between MyoD and CTCF within KvDMR1.

In light of the above observations from ChIP, ChIP-loop and re-ChIP assays, indicating that CTCF is not displaced from F3 concomitant with MyoD binding, we wondered whether the disruption of the chromatin loop was associated with some alteration involving the cohesin complex, which is known to be required for the stabilization of a number of CTCF-mediated chromatin contacts (Dorsett, 2011). According to previous findings showing that cohesin complex subunits copurify with CTCF (Xiao et al., 2011), we observed that CTCF and the Rad21 cohesin subunit physically interact with each other in both undifferentiated an differentiated C2.7 cells (Fig. 7A). Moreover, in line with the extensive colocalization of cohesin and CTCF-binding sites observed throughout the genome (Parelho et al., 2008; Rubio et al., 2008), the re-ChIP assays shown in Fig. 7B indicated that Rad21 is present in a protein complex with CTCF at F3. Intriguingly, the CTCF–Rad21 and CTCF–MyoD complexes appeared to be mutually exclusive on this region, the first being present in undifferentiated cells and the second in differentiated cells. However, the observation that the two different complexes are both present in cell extracts throughout differentiation suggests that the observed exchange does not represent a differentiation-dependent change in CTCF protein–protein interactions but, rather, the displacement of cohesin from F3 upon MyoD binding. To further support the hypothesis that MyoD plays a causal role in the detachment of cohesin from the CTCF-mediated loop, we analyzed the *in vivo* binding of Rad21 to F3 in the presence and in the absence of MyoD. Responsive fibroblasts were infected with either the MyoD-expressing retroviral vector or the empty vector and induced to differentiate. The results of the ChIP assays shown in Fig. 7C indicated that Rad21, like CTCF, binds not only to F3 but also to the *p57* promoter and, most importantly, that exogenous MyoD expression results in a significant reduction in its binding to both regions. Taken together, these results indicate that the ability of MyoD to disrupt the repressive chromatin loop that restricts *p57* expression involves it binding close to a CTCF-occupied site and the consequent displacement of cohesin complex subunits.

DISCUSSION

An increasing number of studies, based on both single-locus and genome-wide analyses, indicate that looped chromatin structures

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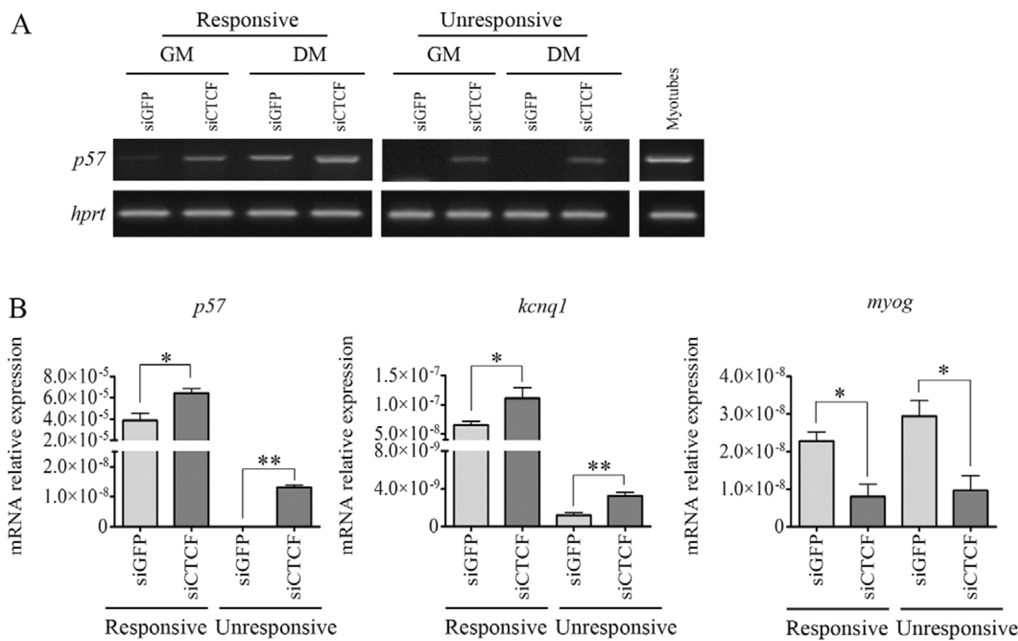

Fig. 5. CTCF depletion causes *p57* and *kcnq1* de-repression. C57BL/6 mouse embryonic fibroblasts (responsive) and C3H10T1/2 mouse embryonic fibroblasts (unresponsive) expressing exogenous MyoD were transfected with the indicated siRNAs as described for Fig. 4A and collected before (GM, growth medium) and after (DM, differentiation medium) the shift to differentiation medium. (A) RT-PCR analysis of *p57* mRNA levels performed in parallel with the western blot analysis shown in supplementary material Fig. S3A. Cell extracts from differentiated C2.7 cells (myotubes) were used as a positive control for *p57* expression. *hprt* expression was used as an internal control for the amount of mRNA. A total of 30 amplification cycles were performed. The results are representative of three independent experiments. (B) qPCR analysis of *p57*, *kcnq1* and myogenin (*myog*) expression performed after the shift to differentiation medium. Data are expressed relative to the expression of 18S RNA, and show the mean±s.e.m. (three independent experiments); *$P<0.05$, **$P\leq0.01$.

represent a general feature of chromatin organization. Chromatin loops are generally formed through long-range interactions between regulatory elements. Several transcription factors and chromatin regulatory proteins have been reported to be involved in the formation and stabilization of chromatin contacts. However, in most cases, their functional relevance to gene regulation is not completely clear (Sexton et al., 2009).

We have recently reported that a long-distance chromatin interaction between KvDMR1 and the *p57* promoter correlates with *p57* silencing in undifferentiated and in unresponsive muscle cells (Busanello et al., 2012). In that work, we also highlighted the dynamic nature of the observed three-dimensional structure during MyoD-induced differentiation. In the present work, we have characterized the molecular mechanism underlying the formation and the disruption of such a chromatin loop and have provided evidence for a functional role of its dynamics in the regulation of *p57* expression during myogenesis. The results, which we obtained through ChIP, ChIP-3C and depletion assays, demonstrated that the chromatin contact between KvDMR1 and the *p57* promoter, associated with *p57* repression, is mediated by CTCF.

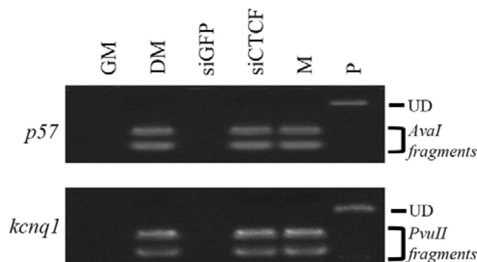

Fig. 6. CTCF depletion does not cause loss of imprinting of *p57* and *kcnq1*. RT-PCR analysis of allele-specific expression of *p57* and *kcnq1*. (C57BL/6×SD7) F1 hybrid mouse embryonic fibroblasts expressing MyoD were kept either in growth medium (GM) or in differentiation medium for 24 hours (DM), or transfected with siRNAs and collected before the shift to differentiation medium as in Fig. 5A. Maternal and paternal alleles were distinguished by RFLP analysis as described in Materials and Methods. UD indicates the electrophoretic mobility of the undigested paternal-specific fragments (P), whereas *Ava*I and *Pvu*II fragments indicate that of digested maternal-specific fragments (M).

Even though rearrangements of higher-order chromatin structures have long been related to transcriptional activation or repression, several aspects of this issue still remain unclear. Regarding CTCF-mediated loops, their functional roles seem to depend on the specific locus. For example, CTCF binding to the imprinting control region of the *Igf2/H19* locus is required for both chromatin folding and imprinted expression (Kurukuti et al., 2006). By contrast, CTCF-mediated looping is dispensable for the activation of the β-globin locus by the locus control region (LCR) element (Splinter et al., 2006). Our experiments involving CTCF depletion during MyoD-induced differentiation showed that *p57* expression was induced at earlier times during differentiation and, remarkably, was at least partly rescued in unresponsive cells. This finding strongly suggests that CTCF-mediated looping between KvDMR1 and the *p57* promoter plays a crucial role in the control of *p57* transcription in muscle cells. Currently, we still cannot exclude the possibility that *p57* de-repression is an indirect effect of CTCF depletion and/or that the disruption of the chromatin loop is a consequence of *p57* transcription. A direct demonstration of the cause-effect relationships between these

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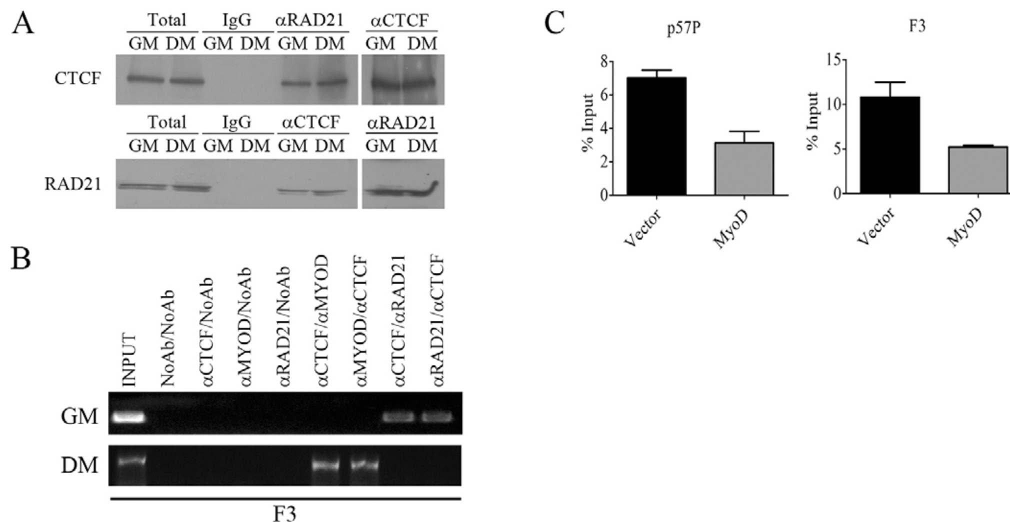

Fig. 7. MyoD interacts with CTCF at KvDMR1 and causes Rad21 displacement. (A) Immunoprecipitation followed by western blot analysis. Total proteins were extracted from C2.7 cells that were maintained either in growth medium (GM) or in differentiation medium for 24 hours (DM). Protein samples were immunoprecipitated with the indicated antibodies. Total extracts and immune complexes were then analyzed by western blotting using antibodies specific for the indicated proteins. The last two lanes of each western blot (IP CTCF/western blot CTCF and IP RAD21/western blot RAD21) were spliced to the remaining ones, for visualization purposes, after removing intervening empty lanes. (B) Re-ChIP assays performed in C2.7 cells cultured as in A. Protein complexes from the first immunoprecipitation were subjected to a second immunoprecipitation with the indicated antibodies. The presence of the F3 fragment was detected by PCR as for conventional ChIP. NoAb, no antibody. (C) ChIP assays for Rad21 were performed in (C57BL/6×SD7) F1 hybrid mouse embryonic fibroblasts that were infected with retrovirus carrying either the empty vector or the MyoD-expressing vector and incubated for 24 hours in differentiation medium. Rad21 binding to the *p57* promoter (p57P) and F3 fragment was measured by qPCR analysis. Values are shown as percentages relative to the Input chromatin (% Input). Data show the mean±s.e.m. (two independent experiments).

events would require new and more sophisticated approaches, based, for example, on genome modification at CTCF-binding sites.

We have recently reported that *kcnq1* (also known as *kvlqt1*), a gene that is co-imprinted with *p57*, is co-induced with *p57* during MyoD-induced differentiation and co-repressed with *p57* in unresponsive cells (Busanello et al., 2012). Here, we report that *kcnq1*, like *p57*, is also subject to CTCF-dependent regulation. The *kcnq1* gene is transcribed from a promoter located ∼200 kb away from KvDMR1, in the opposite direction with respect to the *p57* promoter (Mancini-DiNardo et al., 2003). One hypothesis is that KvDMR1 might also establish a long-range interaction with the *kcnq1* promoter, and the two genes might participate in the same repressive chromatin hub that is perturbed by MyoD binding. *kcnq1*, which encodes a subunit of a voltage-dependent potassium channel, plays an important role in the physiology of several tissues and is involved in the hearth rhythm disorder ‘long QT syndrome’ (Wang et al., 1996; Yang et al., 1997). *kcnq1* displays a restricted expression pattern *in vivo*, largely overlapping with that of *p57*, in tissues and organs such as skeletal muscle, heart, brain, kidney and lung (Mancini-DiNardo et al., 2003; Matsuoka et al., 1995; Rothschild et al., 2006; Wang et al., 1996; Yang et al., 1997). In light of the finding that the two genes are co-induced by two different bHLH proteins, MyoD and E47, during muscle and neural differentiation, respectively (Busanello et al., 2012; Rothschild et al., 2006), we advance the hypothesis that the coordinated expression and silencing of *kcnq1* and *p57* could be controlled by a conserved epigenetic mechanism, shared by various differentiation processes and based on the functional interaction between bHLH factors and CTCF at KvDMR1. Our results also suggest that chromatin folding mediated by KvDMR1 and CTCF might account for the restricted expression of *p57* and *kcnq1* during development and, possibly, for their aberrant silencing in some pathologies.

CTCF is known to participate in the molecular mechanisms driving allele-specific expression of many imprinted genes. However, although the monoallelic binding of CTCF to the F1 subregion of paternal KvDMR1 is supposed to mediate the silencing in cis of several genes of the imprinting domain, we did not observe loss of imprinting of *p57* and *kcnq1* after CTCF depletion. This finding probably reflects the fact that additional mechanisms, such as chromatin silencing by the long non-coding RNA *kcnq1ot1* (Shin et al., 2008), participate in maintaining the imprinted chromatin state. Our results also highlight the existence of a repressive function of CTCF at KvDMR1, distinct from the imprinting control. A related finding was reported in recent work showing a non-allelic repressive role of CTCF at the *Igf2* locus (Lin et al., 2011). In the case of KvDMR1, the imprinting-independent role of CTCF would involve its biallelic binding to the F3 subregion and would be required for preventing *p57* and *kcnq1* expression from the maternal non-imprinted alleles until the occurrence of an activating signal. Considering that the presence of multiple CTCF-binding sites is a feature of several imprinting control regions (Kim, 2008), it is tempting to speculate that similar mechanisms might also affect the developmental regulation of a wider range of imprinted genes.

CTCF is a ubiquitously expressed factor and binds genome-wide in a conserved fashion in different cell types (Barski et al., 2007; Kim et al., 2007; Shen et al., 2012). However, the analysis of the CTCF-chromatin interactome in embryonic stem cells revealed that only a small percentage of the genomic sites bound by this factor participate in chromatin loops (Handoko et al., 2011). This finding calls attention to the importance of CTCF-interacting partners in regulating the arrangement of CTCF-mediated chromatin contacts. CTCF associates with chromatin

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proteins, including histones, histone-modifying enzymes, poly(ADP-ribose)polymerase-1 and cohesin (Phillips and Corces, 2009; Zlatanova and Caiafa, 2009b). Moreover, CTCF has been recognized to functionally interact with some transcription factors bound to adjacent sites on DNA, which seem to modulate CTCF function in diverse regulatory contexts (Weth and Renkawitz, 2011). For example, the ubiquitous zinc-finger protein Kaiso, that binds close to a CTCF site in the 5′ β-globin insulator, is capable of relieving the enhancer-blocking activity of CTCF in episomal assays (Defossez et al., 2005). Similarly, the thyroid hormone abrogates the enhancer-blocking activity of CTCF-TR (thyroid hormone receptor) composite elements associated with chicken lysozyme and human c-myc genes (Lutz et al., 2003). However, the chromatin dynamics associated with the observed functional interactions were not addressed in those studies. Our work reveals that MyoD is an additional transcription factor that functionally interacts with CTCF. In light of our previous and present results, we claim that MyoD, by interacting with CTCF within F3, is capable of relieving the repressive activity that this region exerts on the *p57* promoter. Furthermore, we show that MyoD recruitment is associated with the disruption of the chromatin contact between the two CTCF-binding sites present within F3 and the *p57* promoter.

Regarding the mechanism by which MyoD affects CTCF function, our results from co-immunoprecipitation and sequential ChIP assays indicate that the two factors physically interact within KvDMR1 chromatin. As inferred from ChIP and ChIP-3C assays, CTCF is not displaced from KvDMR1 nor from the *p57* promoter after MyoD binding. A simple explanation of how MyoD alters CTCF activity even in the absence of CTCF displacement could be that MyoD association hinders some CTCF domain involved in dimerization or in other protein–protein interactions required for chromatin looping. Remarkably, we observed that the binding of MyoD and the consequent disruption of the chromatin loop correlate with the displacement of the cohesin protein Rad21. It has been established recently that the cohesin multiprotein complex plays a crucial role in the formation of CTCF-mediated chromatin contacts, by directly interacting with CTCF and facilitating looping between CTCF-binding sites (Dorsett, 2011; Dorsett and Ström, 2012; Hadjur et al., 2009; Hou et al., 2010; Nativio et al., 2009). In some cases, cohesin binding to genomic sites appeared to change in a cell-type-specific manner (Chien et al., 2011; Kim et al., 2011). However, the factors underlying this phenomenon are not yet clear. A potential regulator of cohesin interaction with CTCF-bound sites, and hence of CTCF function, is the DEAD-box RNA helicase p68 (also known as DDX5) with its associated non-coding RNA, steroid receptor RNA activator (SRA). In fact, depletion of either p68 or SRA reduces cohesin binding to CTCF at the *IGF2*/*H19* imprinting control region, causes rearrangements of chromatin contacts at this locus and leads to increased *IGF2* expression (Yao et al., 2010). Intriguingly, MyoD has been found to physically and functionally interact with p68–SRA (Caretti et al., 2006). In differentiating muscle cells, p68–SRA is co-recruited with MyoD to muscle gene promoters and facilitates the formation of the transcription initiation complex and chromatin remodeling. We have observed that p68 is constitutively bound to both the *p57* promoter and F3, regardless of cell differentiation and responsiveness (data not shown). Currently, we are unable to say whether the probable interaction of MyoD with p68 plays any role in the *p57* regulatory context. However, one possibility is that MyoD, by interacting with the CTCF–cohesin–p68 complex, might induce some structural alteration of such a complex, leading to the detachment of cohesin and hence to the destabilization of the chromatin loop (see model depicted in Fig. 8).

A physical interaction between MyoD and CTCF has been reported previously (Delgado-Olguín et al., 2011). In that work, the authors showed that CTCF promotes the ability of MyoD to transactivate some muscle-specific genes and that CTCF depletion impairs myogenic differentiation of C2C12 cells. Accordingly, we observed that although CTCF depletion relieves the repression of *p57* expression, at the same time it

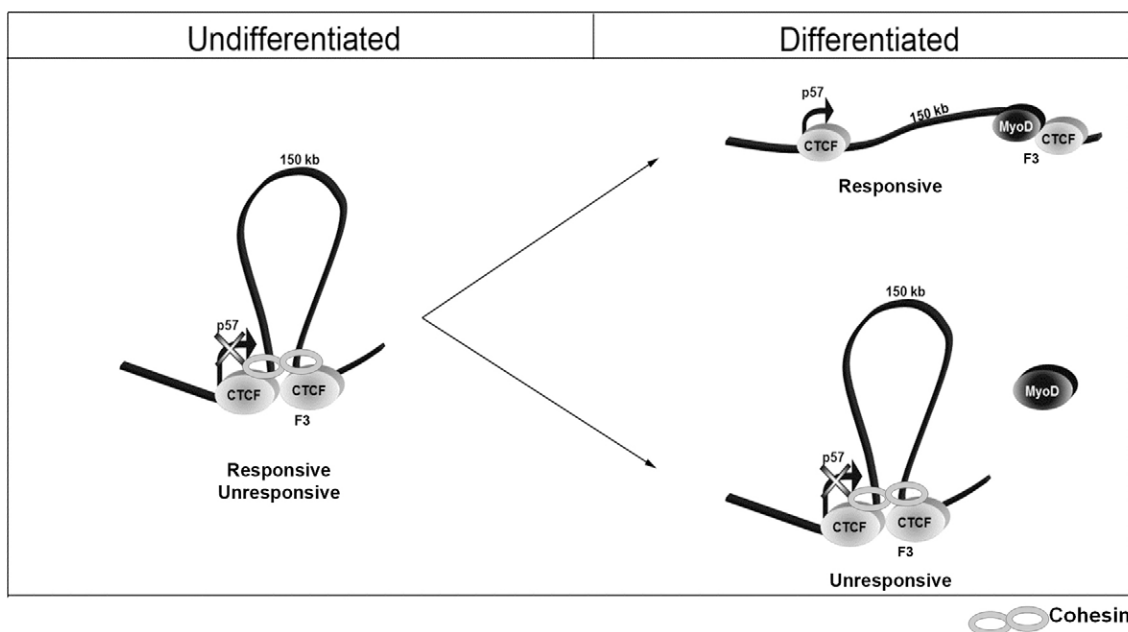

Fig. 8. Schematic model of the functional interaction between MyoD, CTCF and the cohesin complex at the chromatin loop between the F3 and *p57* promoter. In undifferentiated cells, the chromatin loop, mediated by CTCF and cohesin, prevents *p57* expression. Upon the induction of differentiation, MyoD binds to the F3 in responsive but not in unresponsive cells. The interaction of MyoD with CTCF within F3 causes the displacement of cohesin and the destabilization of the chromatin loop.

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inhibits the expression of the muscle-specific gene myogenin (see Fig. 5). This suggests that CTCF is involved in at least two distinct types of functional interaction with MyoD. At muscle-specific gene promoters it behaves as a transcriptional co-activator of this myogenic factor, whereas at the *p57* locus, where it acts as a negative regulator of chromatin conformation, CTCF is counteracted by MyoD. Interestingly, we found that MyoD and CTCF can be co-immunoprecipitated even before differentiation. We are currently unable to elucidate the molecular mechanisms and the functional role of CTCF–MyoD interaction in undifferentiated myoblasts. However, it is worth mentioning that the myogenic factor, in addition to binding to differentiation-induced genes, also binds to thousands of intergenic sites in both myoblasts and myotubes, where it induces histone modifications (Cao et al., 2010). It is intriguing to imagine that a functional interaction between MyoD and CTCF might represent a more widespread phenomenon than that revealed by our results, playing an important role in the changes of the genome architecture during myogenesis.

We believe that this is the first evidence that a differentiation factor regulates chromatin looping at CTCF-binding sites. This finding not only reveals a novel regulatory strategy exploited by MyoD, the master regulator of myogenesis, but also provides a paradigm for better understanding the differentiation-stage- and cell-type-specific regulation of CTCF-mediated chromatin contacts.

MATERIALS AND METHODS

Cell culture

C3H10T1/2, C57BL/6 and (C57BL/6×SD7)F1 mouse embryonic fibroblasts and C2.7 mouse myoblasts were grown in DMEM supplemented with 10% fetal calf serum (FCS). (C57BL/6×SD7)F1 fibroblasts, carrying single nucleotide polymorphisms in the *p57*/*kcnq1* locus, were kindly provided by Andrea Riccio (the Second University of Naples, Italy). To trigger differentiation, myoblasts and MyoD-expressing fibroblasts were grown to confluence and shifted to differentiation medium (DMEM containing 0.5% FCS). Cells were collected before the addition of differentiation medium (growth medium samples, GM) or 24 hours after the shift to differentiation medium (differentiation medium samples, DM). Production of MyoD-expressing retrovirus, retroviral infections and MyoD-induced differentiation were performed as described previously (Figliola et al., 2008).

ChIP and re-ChIP

ChIP assays were performed as described previously (Figliola et al., 2008). Chromatin was immunoprecipitated with anti-CTCF (07-729; Millipore) or with anti-Rad21 (A300-080A; Bethyl Laboratories) antibodies. After immunoprecipitation, the genomic regions of interest (F1, F2, F3 fragments and the *p57* promoter) were amplified using the same DNA quantity for each sample (input, no antibody and IP). The primer pairs and the protocol were as reported previously (Busanello et al., 2012). The genomic region that was not bound by CTCF (negative control, Ctr) was amplified using the primers Fw, 5′-GCCAGCACC-AAGGTAGTGAG-3′ and Rev, 5′-CTCCGAATAAGCAGCCTCCC-3′.

Quantitative PCR (qPCR) analysis of the immunoprecipitated F3 fragment and *p57* promoter was performed in triplicate using 5 ng of DNA, Go Taq qPCR Master Mix (Promega) and the previously reported primer pairs specific for the F3 fragment (Busanello et al., 2012) and the *p57*-promoter-specific primers Fw, 5′-GTGTCACGTTACCGCCCGCA-3′ and Rev, 5′-CTCATTGGCTGCGCGCACACA-3′ at a final concentration of 150 nM. The reaction was performed in the MiniOpticon Real-Time PCR detection system thermocycler (Bio-Rad). The primer pair efficiency, the relative quantity of each immunoprecipitated fragment [ΔC(t)] with respect to the input sample and the standard deviations of the relative quantities were determined with CFX Manager™ software (Bio-Rad). The percentages of the relative quantities of each immunoprecipitated sample were normalized with respect to the quantities of each input sample.

The parental alleles were distinguished by single-strand conformation polymorphism (SSCP)-PCR using F1 or F3 primers labeled at their 5′ ends with [^{32}P]-γATP. After denaturation, PCR products were resolved by non-denaturing acrylamide gel electrophoresis and detected by autoradiography.

In re-ChIP experiments, after the first immunoprecipitation with antibodies against MyoD (sc-760; Santa Cruz Biotechnology), CTCF (07-729; Millipore) or Rad21 (A300-080A; Bethyl Laboratories) and extensive washing (three washes with 0.1% SDS, 2 mM EDTA, 20 mM Tris-HCl pH 8, 1% Triton X-100, 150 mM NaCl; two washes with 0.1% SDS, 2 mM EDTA, 20 mM Tris-HCl pH 8, 1% Triton X-100, 500 mM NaCl, one wash with 10 mM Tris-HCl pH 8, 1 mM EDTA), complexes were eluted by two 30-min incubations at 37℃ in 25 µl of 10 mM dithiothreitol (DTT). After centrifugation, the supernatant was diluted 1:20 with re-ChIP buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl pH 8 supplemented with protease inhibitors) and subjected to pre-clearing, second immunoprecipitation (with antibodies against CTCF, MyoD or Rad21), washing, elution and reverse formaldehyde crosslinking as for conventional ChIP. After double immunoprecipitation, the F3 fragment was amplified using the same DNA quantity for each sample. The primer pairs and the protocol were as reported previously (Busanello et al., 2012).

Immunoprecipitation and western blot analyses

Immunoprecipitations were performed as described previously (Figliola and Maione, 2004). The following primary antibodies were used: anti-RAD21 (A300-080A; Bethyl Laboratories), anti-CTCF (07-729; Millipore), anti-MyoD (sc-760; Santa Cruz Biotechnologies) and normal rabbit IgG (12-370; Millipore; used as a negative control).

Western blotting analyses were performed as described previously (Figliola and Maione, 2004). The primary antibodies used were against MyoD (clone 5.8A; Dako), CTCF (07-729; Millipore), RAD21 (A300-080A; Bethyl Laboratories) and tubulin (sc-8035 Santa Cruz Biotechnologies; used as a loading control).

Gene expression analysis

Total cellular RNA was extracted by using the High Pure RNA Isolation Kit according to the manufacturer's instructions (Roche Diagnostics). 1 µg of total RNA was reverse transcribed with the iScript cDNA Synthesis Kit (Bio-Rad).

For RT-PCR analyses, the reactions were performed in 50 µl of reaction buffer containing 2 µl of the reverse transcription product, 0.2 mM dNTP mix, 1.5 mM $MgCl_2$, 2.5 U of Taq DNA polymerase (Promega) and 0.5 µM of each primer. *p57* and *hprt* (used as a reference gene) transcripts were amplified using the primers reported previously (Busanello et al., 2012). The amplification consisted of one cycle at 94℃ for 4 min followed by 30 cycles at 94℃ for 30 s, 65℃ for 30 s and 72℃ for 60 s.

qRT-PCR reactions were performed in 20 µl of reaction buffer containing 1 µl of diluted cDNA, 10 µl of Go Taq qPCR Master Mix (Promega) and each primer at the optimized final concentration. The primer sequences specific for *18S*, *p57* and *kcnq1* were as reported previously (Busanello et al., 2012). The primer sequences for myogenin were Fw, 5′-GTCTCTTCCTGAAGCCAGTTGCG-3′ and Rev, 5′-TGCAAATGCTTGGCCCCCAGAG-3′. The reactions were performed in the MiniOpticon Real-Time PCR detection system thermocycler (Bio-Rad). The primer pair efficiency, the normalized expression [ΔΔC(t)] and the standard error of the mean (s.e.m.) were determined with CFX Manager™ software (Bio-Rad). *18S* rRNA was used as a reference gene. Experiments were performed in triplicate and data are expressed as the mean±s.e.m. For statistical analysis, comparisons were performed using parametric Student's *t*-test analysis or non-parametric Mann–Whitney U-tests, depending on the normality of the distribution, as assessed using the Kolmogorov–Smirnov statistics. Statistical significance is shown as *$P<0.05$ or **$P\leq0.01$. To assay the allelic expression status of *p57* and *kcnq1*, cDNAs obtained from RNA extracted from MyoD-infected (C57BL/6×SD7)F1 fibroblasts were amplified by RT-PCR using the primers reported previously (Busanello et al., 2012). Maternal and

paternal RT-PCR products were distinguished by RFLP analysis of previously described polymorphic restriction sites (Caspary et al., 1998).

3C and ChIP-loop assays

3C assays were performed as described previously (Hagège et al., 2007). Briefly, after chromatin crosslinking and nuclei isolation, DNA was digested with 400 U of *Nco*I restriction enzyme and ligated in 1× ligation buffer (New England Biolabs). Ligation products were extracted with phenol-chloroform, precipitated with sodium acetate and ethanol, washed with 70% (v/v) ethanol and resuspended in 150 μl of distilled water. 10 ng of each sample was used for qPCR or PCR analysis, performed in triplicate. The primers used for 3C sample amplifications were as follows: pair 1; Fw, 5′-CCTTCGACCATGGTGAGGTC-3′ and Rev, 5′-GTGCTGAAACGATCCACACG-3′ and pair 2; Fw, 5′-CCGCGTTTG-CACAGCCTTCG-3′ and Rev, 5′-CGACCGCGCTGGAGTGATCC-3′.

ChIP-loop assays were performed as described previously (Cai et al., 2006). Briefly, chromatin was crosslinked and nuclei were isolated. Chromatin was digested with *Nco*I restriction enzyme, immunoprecipitated with antibody against CTCF (07-729; Millipore) or MyoD (sc-760; Santa Cruz Biotechnology) and ligated in 1× ligation buffer (New England Biolabs). Ligation products were treated as for 3C assays and analyzed by PCR or qPCR performed in triplicate with the same primers as used for the 3C assay.

RNA interference

C57BL/6, C3H10T1/2 and (C57BL/6×SD7)F1 fibroblasts, grown in 100-mm dishes, were infected with a MyoD-expressing retrovirus. At 48 hours after infection, 8×10^5 cells were transfected with 5 μl of 100 μM siRNA duplexes in 800 μl of HEPES-buffered saline (HBS) buffer pH 7.05 in a 4-mm-gap cuvette (Bio-Rad Laboratories) by using a Gene Pulser II (Bio-Rad Laboratories) at 300 mV and 1000 μF. Following electroporation, cells were kept for 48 hours in DMEM containing 10% FCS and were analyzed after a further 0 or 24 hours in differentiation medium (DMEM containing 0.5% FCS) by western blotting, RT-PCR and 3C. A mixture of four chemically synthesized siRNA duplexes (CTCF Mouse SMARTpool, Dharmacon) was used to target CTCF, and an equal amount of siGFP (5′-GGCUACGUCCAG-GAGCGCACC-3′) was used as a control.

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Competing interests
The authors declare no competing interests.

Author contributions
A.B. initiated the study of chromatin looping at the *p57/kvDMR1* locus in muscle cells and performed the allele-specific analysis of chromatin binding and gene expression. C.B. extended these studies and performed most of the experimental work. R.M. directed the research and wrote the paper. All authors participated in the discussion of the results and commented on the manuscript.

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Supplementary material
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References
Barski, A., Cuddapah, S., Cui, K., Roh, T. Y., Schones, D. E., Wang, Z., Wei, G., Chepelev, I. and Zhao, K. (2007). High-resolution profiling of histone methylations in the human genome. *Cell* **129**, 823-837.
Bergstrom, D. A., Penn, B. H., Strand, A., Perry, R. L., Rudnicki, M. A. and Tapscott, S. J. (2002). Promoter-specific regulation of MyoD binding and signal transduction cooperate to pattern gene expression. *Mol. Cell* **9**, 587-600.
Busanello, A., Battistelli, C., Carbone, M., Mostocotto, C. and Maione, R. (2012). MyoD regulates p57kip2 expression by interacting with a distant cis-element and modifying a higher order chromatin structure. *Nucleic Acids Res.* **40**, 8266-8275.
Cai, S., Lee, C. C. and Kohwi-Shigematsu, T. (2006). SATB1 packages densely looped, transcriptionally active chromatin for coordinated expression of cytokine genes. *Nat. Genet.* **38**, 1278-1288.
Cao, Y., Yao, Z., Sarkar, D., Lawrence, M., Sanchez, G. J., Parker, M. H., MacQuarrie, K. L., Davison, J., Morgan, M. T., Ruzzo, W. L. et al. (2010). Genome-wide MyoD binding in skeletal muscle cells: a potential for broad cellular reprogramming. *Dev. Cell* **18**, 662-674.
Caretti, G., Schiltz, R. L., Dilworth, F. J., Di Padova, M., Zhao, P., Ogryzko, V., Fuller-Pace, F. V., Hoffman, E. P., Tapscott, S. J. and Sartorelli, V. (2006). The RNA helicases p68/p72 and the noncoding RNA SRA are coregulators of MyoD and skeletal muscle differentiation. *Dev. Cell* **11**, 547-560.
Caspary, T., Cleary, M. A., Baker, C. C., Guan, X. J. and Tilghman, S. M. (1998). Multiple mechanisms regulate imprinting of the mouse distal chromosome 7 gene cluster. *Mol. Cell. Biol.* **18**, 3466-3474.
Chien, R., Zeng, W., Kawauchi, S., Bender, M. A., Santos, R., Gregson, H. C., Schmiesing, J. A., Newkirk, D. A., Kong, X., Ball, A. R., Jr et al. (2011). Cohesin mediates chromatin interactions that regulate mammalian β-globin expression. *J. Biol. Chem.* **286**, 17870-17878.
Defossez, P. A., Kelly, K. F., Filion, G. J., Pérez-Torrado, R., Magdinier, F., Menoni, H., Nordgaard, C. L., Daniel, J. M. and Gilson, E. (2005). The human enhancer blocker CTC-binding factor interacts with the transcription factor Kaiso. *J. Biol. Chem.* **280**, 43017-43023.
Dekker, J. (2008). Mapping in vivo chromatin interactions in yeast suggests an extended chromatin fiber with regional variation in compaction. *J. Biol. Chem.* **283**, 34532-34540.
Delgado-Olguín, P., Brand-Arzamendi, K., Scott, I. C., Jungblut, B., Stainier, D. Y., Bruneau, B. G. and Recillas-Targa, F. (2011). CTCF promotes muscle differentiation by modulating the activity of myogenic regulatory factors. *J. Biol. Chem.* **286**, 12483-12494.
Dorsett, D. (2011). Cohesin: genomic insights into controlling gene transcription and development. *Curr. Opin. Genet. Dev.* **21**, 199-206.
Dorsett, D. and Ström, L. (2012). The ancient and evolving roles of cohesin in gene expression and DNA repair. *Curr. Biol.* **22**, R240-R250.
Felsenfeld, G. and Dekker, J. (2012). Genome architecture and expression. *Curr. Opin. Genet. Dev.* **22**, 59-61.
Figliola, R. and Maione, R. (2004). MyoD induces the expression of p57Kip2 in cells lacking p21Cip1/Waf1: overlapping and distinct functions of the two cdk inhibitors. *J. Cell. Physiol.* **200**, 468-475.
Figliola, R., Busanello, A., Vaccarello, G. and Maione, R. (2008). Regulation of p57(KIP2) during muscle differentiation: role of Egr1, Sp1 and DNA hypomethylation. *J. Mol. Biol.* **380**, 265-277.
Fitzpatrick, G. V., Soloway, P. D. and Higgins, M. J. (2002). Regional loss of imprinting and growth deficiency in mice with a targeted deletion of KvDMR1. *Nat. Genet.* **32**, 426-431.
Fitzpatrick, G. V., Pugacheva, E. M., Shin, J. Y., Abdullaev, Z., Yang, Y., Khatod, K., Lobanenkov, V. V. and Higgins, M. J. (2007). Allele-specific binding of CTCF to the multipartite imprinting control region KvDMR1. *Mol. Cell. Biol.* **27**, 2636-2647.
Hadjur, S., Williams, L. M., Ryan, N. K., Cobb, B. S., Sexton, T., Fraser, P., Fisher, A. G. and Merkenschlager, M. (2009). Cohesins form chromosomal cis-interactions at the developmentally regulated IFNG locus. *Nature* **460**, 410-413.
Hagège, H., Klous, P., Braem, C., Splinter, E., Dekker, J., Cathala, G., de Laat, W. and Forné, T. (2007). Quantitative analysis of chromosome conformation capture assays (3C-qPCR). *Nat. Protoc.* **2**, 1722-1733.
Handoko, L., Xu, H., Li, G., Ngan, C. Y., Chew, E., Schnapp, M., Lee, C. W., Ye, C., Ping, J. L., Mulawadi, F. et al. (2011). CTCF-mediated functional chromatin interactome in pluripotent cells. *Nat. Genet.* **43**, 630-638.
Hikichi, T., Kohda, T., Kaneko-Ishino, T. and Ishino, F. (2003). Imprinting regulation of the murine Meg1/Grb10 and human GRB10 genes; roles of brain-specific promoters and mouse-specific CTCF-binding sites. *Nucleic Acids Res.* **31**, 1398-1406.
Holwerda, S. and de Laat, W. (2012). Chromatin loops, gene positioning, and gene expression. *Front. Genet.* **3**, 217.
Horike, S., Mitsuya, K., Meguro, M., Kotobuki, N., Kashiwagi, A., Notsu, T., Schulz, T. C., Shirayoshi, Y. and Oshimura, M. (2000). Targeted disruption of the human LIT1 locus defines a putative imprinting control element playing an essential role in Beckwith-Wiedemann syndrome. *Hum. Mol. Genet.* **9**, 2075-2083.
Hou, C., Dale, R. and Dean, A. (2010). Cell type specificity of chromatin organization mediated by CTCF and cohesin. *Proc. Natl. Acad. Sci. USA* **107**, 3651-3656.
Kadauke, S. and Blobel, G. A. (2009). Chromatin loops in gene regulation. *Biochim. Biophys. Acta* **1789**, 17-25.
Kim, J. (2008). Multiple YY1 and CTCF binding sites in imprinting control regions. *Epigenetics* **3**, 115-118.
Kim, T. H., Abdullaev, Z. K., Smith, A. D., Ching, K. A., Loukinov, D. I., Green, R. D., Zhang, M. Q., Lobanenkov, V. V. and Ren, B. (2007). Analysis of the

Journal of Cell Science

vertebrate insulator protein CTCF-binding sites in the human genome. *Cell* **128**, 1231-1245.

Kim, Y. J., Cecchini, K. R. and Kim, T. H. (2011). Conserved, developmentally regulated mechanism couples chromosomal looping and heterochromatin barrier activity at the homeobox gene A locus. *Proc. Natl. Acad. Sci. USA* **108**, 7391-7396.

Kurukuti, S., Tiwari, V. K., Tavoosidana, G., Pugacheva, E., Murrell, A., Zhao, Z., Lobanenkov, V., Reik, W. and Ohlsson, R. (2006). CTCF binding at the H19 imprinting control region mediates maternally inherited higher-order chromatin conformation to restrict enhancer access to Igf2. *Proc. Natl. Acad. Sci. USA* **103**, 10684-10689.

Lin, S., Ferguson-Smith, A. C., Schultz, R. M. and Bartolomei, M. S. (2011). Nonallelic transcriptional roles of CTCF and cohesins at imprinted loci. *Mol. Cell. Biol.* **31**, 3094-3104.

Lutz, M., Burke, L. J., LeFevre, P., Myers, F. A., Thorne, A. W., Crane-Robinson, C., Bonifer, C., Filippova, G. N., Lobanenkov, V. and Renkawitz, R. (2003). Thyroid hormone-regulated enhancer blocking: cooperation of CTCF and thyroid hormone receptor. *EMBO J.* **22**, 1579-1587.

Mancini-DiNardo, D., Steele, S. J., Ingram, R. S. and Tilghman, S. M. (2003). A differentially methylated region within the gene Kcnq1 functions as an imprinted promoter and silencer. *Hum. Mol. Genet.* **12**, 283-294.

Matsuoka, S., Edwards, M. C., Bai, C., Parker, S., Zhang, P., Baldini, A., Harper, J. W. and Elledge, S. J. (1995). p57KIP2, a structurally distinct member of the p21CIP1 Cdk inhibitor family, is a candidate tumor suppressor gene. *Genes Dev.* **9**, 650-662.

Mishiro, T., Ishihara, K., Hino, S., Tsutsumi, S., Aburatani, H., Shirahige, K., Kinoshita, Y. and Nakao, M. (2009). Architectural roles of multiple chromatin insulators at the human apolipoprotein gene cluster. *EMBO J.* **28**, 1234-1245.

Misteli, T. (2007). Beyond the sequence: cellular organization of genome function. *Cell* **128**, 787-800.

Nativio, R., Wendt, K. S., Ito, Y., Huddleston, J. E., Uribe-Lewis, S., Woodfine, K., Krueger, C., Reik, W., Peters, J. M. and Murrell, A. (2009). Cohesin is required for higher-order chromatin conformation at the imprinted IGF2-H19 locus. *PLoS Genet.* **5**, e1000739.

Pant, V., Kurukuti, S., Pugacheva, E., Shamsuddin, S., Mariano, P., Renkawitz, R., Klenova, E., Lobanenkov, V. and Ohlsson, R. (2004). Mutation of a single CTCF target site within the H19 imprinting control region leads to loss of Igf2 imprinting and complex patterns of de novo methylation upon maternal inheritance. *Mol. Cell. Biol.* **24**, 3497-3504.

Parelho, V., Hadjur, S., Spivakov, M., Leleu, M., Sauer, S., Gregson, H. C., Jarmuz, A., Canzonetta, C., Webster, Z., Nesterova, T. et al. (2008). Cohesins functionally associate with CTCF on mammalian chromosome arms. *Cell* **132**, 422-433.

Pateras, I. S., Apostolopoulou, K., Niforou, K., Kotsinas, A. and Gorgoulis, V. G. (2009). p57KIP2: ''Kip''ing the cell under control. *Mol. Cancer Res.* **7**, 1902-1919.

Phillips, J. E. and Corces, V. G. (2009). CTCF: master weaver of the genome. *Cell* **137**, 1194-1211.

Rothschild, G., Zhao, X., Iavarone, A. and Lasorella, A. (2006). E Proteins and Id2 converge on p57Kip2 to regulate cell cycle in neural cells. *Mol. Cell. Biol.* **26**, 4351-4361.

Rubio, E. D., Reiss, D. J., Welcsh, P. L., Disteche, C. M., Filippova, G. N., Baliga, N. S., Aebersold, R., Ranish, J. A. and Krumm, A. (2008). CTCF physically links cohesin to chromatin. *Proc. Natl. Acad. Sci. USA* **105**, 8309-8314.

Sexton, T., Bantignies, F. and Cavalli, G. (2009). Genomic interactions: chromatin loops and gene meeting points in transcriptional regulation. *Semin. Cell Dev. Biol.* **20**, 849-855.

Shen, Y., Yue, F., McCleary, D. F., Ye, Z., Edsall, L., Kuan, S., Wagner, U., Dixon, J., Lee, L., Lobanenkov, V. V. et al. (2012). A map of the cis-regulatory sequences in the mouse genome. *Nature* **488**, 116-120.

Shin, J. Y., Fitzpatrick, G. V. and Higgins, M. J. (2008). Two distinct mechanisms of silencing by the KvDMR1 imprinting control region. *EMBO J.* **27**, 168-178.

Singh, P., Lee, D. H. and Szabó, P. E. (2012). More than insulator: multiple roles of CTCF at the H19-Igf2 imprinted domain. *Front. Genet.* **3**, 214.

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

Vaccarello, G., Figliola, R., Cramerotti, S., Novelli, F. and Maione, R. (2006). p57Kip2 is induced by MyoD through a p73-dependent pathway. *J. Mol. Biol.* **356**, 578-588.

Van Bortle, K. and Corces, V. G. (2012). Nuclear organization and genome function. *Annu. Rev. Cell Dev. Biol.* **28**, 163-187.

Wang, Q., Curran, M. E., Splawski, I., Burn, T. C., Millholland, J. M., VanRaay, T. J., Shen, J., Timothy, K. W., Vincent, G. M., de Jager, T. et al. (1996). Positional cloning of a novel potassium channel gene: KVLQT1 mutations cause cardiac arrhythmias. *Nat. Genet.* **12**, 17-23.

Weth, O. and Renkawitz, R. (2011). CTCF function is modulated by neighboring DNA binding factors. *Biochem. Cell Biol.* **89**, 459-468.

Xiao, T., Wallace, J. and Felsenfeld, G. (2011). Specific sites in the C terminus of CTCF interact with the SA2 subunit of the cohesin complex and are required for cohesin-dependent insulation activity. *Mol. Cell. Biol.* **31**, 2174-2183.

Yang, W. P., Levesque, P. C., Little, W. A., Conder, M. L., Shalaby, F. Y. and Blanar, M. A. (1997). KvLQT1, a voltage-gated potassium channel responsible for human cardiac arrhythmias. *Proc. Natl. Acad. Sci. USA* **94**, 4017-4021.

Yao, H., Brick, K., Evrard, Y., Xiao, T., Camerini-Otero, R. D. and Felsenfeld, G. (2010). Mediation of CTCF transcriptional insulation by DEAD-box RNA-binding protein p68 and steroid receptor RNA activator SRA. *Genes Dev.* **24**, 2543-2555.

Yoon, B., Herman, H., Hu, B., Park, Y. J., Lindroth, A., Bell, A., West, A. G., Chang, Y., Stablewski, A., Piel, J. C. et al. (2005). Rasgrf1 imprinting is regulated by a CTCF-dependent methylation-sensitive enhancer blocker. *Mol. Cell. Biol.* **25**, 11184-11190.

Yusufzai, T. M., Tagami, H., Nakatani, Y. and Felsenfeld, G. (2004). CTCF tethers an insulator to subnuclear sites, suggesting shared insulator mechanisms across species. *Mol. Cell* **13**, 291-298.

Zlatanova, J. and Caiafa, P. (2009a). CCCTC-binding factor: to loop or to bridge. *Cell. Mol. Life Sci.* **66**, 1647-1660.

Zlatanova, J. and Caiafa, P. (2009b). CTCF and its protein partners: divide and rule? *J. Cell Sci.* **122**, 1275-1284.

Zlatanova, J. S. and van Holde, K. E. (1992). Chromatin loops and transcriptional regulation. *Crit. Rev. Eukaryot. Gene Expr.* **2**, 211-224.