FUNCTIONAL INTERPLAY BETWEEN MYOD AND CTCF IN REGULATING
LONG-RANGE CHROMATIN INTERACTIONS DURING DIFFERENTIATION

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SUMMARY

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 in gene regulation are poorly understood. We recently described a dynamic long-range chromatin interaction between the gene-promoter of the cdk inhibitor p57kip2 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 p57kip2 promoter and KvDMR1 and is necessary for the maintenance of their physical contact. Moreover, we show that CTCF-mediated looping is required for preventing p57kip2 expression before differentiation. Finally, we provide evidence that the induction of p57kip2 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.
INTRODUCTION

Cell differentiation involves the coordinate 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 critical 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, the physical association of co-ordinately regulated genes and their localization at functionally distinct sub-nuclear 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 which 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 beta-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 its property to interact with structural nuclear proteins such as nucleophosmin and lamins are believed to participate 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 of chromatin folding are the cause or the consequence of transcriptional dynamics.

We have previously investigated the transcriptional regulation of \(p57^{kip2}\) (\(p57\)) 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 playing a critical role in many differentiation processes and has been found 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 more than 150 kb from the \(p57\) promoter, controls \textit{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 \(p57\) promoter and a regulatory element comprised 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 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 p57 promoter in muscle cells

We had previously shown that MyoD induces p57 through an indirect mechanism that does not imply 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 sub-regions, while the third, predicted by MatInspector tool, is located in the F3 sub-region, that 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 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/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, equally competent to undergo MyoD-induced differentiation, but differentially competent regarding the binding of MyoD to KvDMR1 and the up-regulation of p57. The first, that we named as “responsive”, is normally sensitive while the second, that we named as “unresponsive”, is completely refractory to the induction of p57. These cell types represented 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).

Firstly we observed that CTCF protein is constitutively present in differentiating myoblasts and in MyoDconverted fibroblasts, regardless of their differentiation stage or their responsiveness for p57 induction (Fig. S1). Next we investigated the occurrence of in vivo CTCF binding to the consensus sequences identified within KvDMR1 and 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 embryo fibroblasts expressing exogenous MyoD. As shown in Fig. 2A, and according to a previous report (Fitzpatrick et al., 2007), we observed that CTCF binds to both the F1 and F2 sub-regions (corresponding to the CTS1
and CTS2 fragments described by Fitzpatrick and coworkers). In addition, as shown in the same figure, we found that CTCF also binds to the F3 sub-region in all the three cell types analyzed, implying the existence of a further and previously unidentified CTCF binding site within KvDMR1. The 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, while 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 like we had previously found for MyoD (Busanello et al., 2012). Notably, we detected the binding of CTCF to \( p57 \) promoter as well (Fig. 2A). Quantitative ChIP analysis confirmed that there was no significant change in CTCF binding to F3 nor to \( p57 \) promoter during differentiation (Fig. 2C).

**CTCF is involved in the formation and in the maintenance of the long-distance chromatin interaction between KvDMR1 and \( p57 \) 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 \( p57 \) promoter was suggestive of a possible role for CTCF in mediating 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 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. Cross-linked 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 deriving from the ligation of \( p57 \) promoter and KvDMR1 sequences. The results reported in Fig. 3B show the presence of the recombinant fragment, deriving from the coming together of \( p57 \) promoter and F3 sequences, in CTCF-immunoprecipitated samples. Importantly, the recombinant fragment was effectively immunoprecipitated only from undifferentiated and from unresponsive cells, that is 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 substantially similar results (Fig. S2). According to 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 (Figs. 3B and S2). Taken together, these results reveal that CTCF
participates in the long-range chromatin interaction that brings together p57 promoter and KvDMR1 sequences. Moreover, they also show that the disruption of the chromatin loop, that occurs during differentiation of responsive cells, is not accompanied by CTCF displacement from F3 nor from p57 promoter (see also below).

In order to directly verify whether CTCF was required for the maintenance of the chromatin loop, we performed 3C assays after CTCF knock-down in unresponsive cells. Unresponsive fibroblasts, previously infected with the MyoD-encoding retrovirus, were transfected with CTCF-targeting siRNAs. 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 of the interaction frequency between p57 promoter and F3 region (Fig. 4C). This result clearly indicated 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 at p57-KvDMR1 locus and p57 repression, we analyzed the effects of CTCF knock-down on gene expression during MyoD-induced differentiation. Responsive and unresponsive fibroblasts, previously infected with the MyoD-encoding retrovirus, were transfected with the same amounts of CTCF-targeting or control siRNAs. RNA and protein extracts were collected at two differentiation time points. Western blot analysis of CTCF protein levels confirmed its efficient depletion (Fig. S3A). Remarkably, RT-PCR analysis showed that CTCF depletion correlates with p57 derepression (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 knock-down caused a partial rescue of p57-responsiveness to MyoD-dependent induction in unresponsive cells. Unlike observed for 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 that the observed rise of p57 expression is an indirect consequence of a generally increased differentiation. These results strongly support the conclusion that CTCF, by mediating 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 recently reported, 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 keeping p57 under control, we asked whether kcnq1 was also subject to
CTCF-dependent regulation. The results of qRT-PCR reported in Fig. 5B indicate that kcnq1, expressed at very low levels in unresponsive cells, was appreciably up-regulated 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 takes part in the co-regulation of some genes of the p57 imprinting domain during muscle differentiation.

Since CTCF is known to participate in the molecular mechanisms driving allele-specific expression from Igf2/H19 and other imprinted loci (Hikichi et al., 2003; Singh et al., 2012; Yoon et al., 2005), we asked whether the increase of p57 and kcnq1 expression observed after CTCF depletion resulted from 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 the up-regulation from 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 siRNA as above and tested for the efficient reduction of CTCF levels (Fig. S3B). p57 and kcnq1 RNAs were then examined by combining RT-PCR with Restriction Fragment Length Polymorphism (RFLP) analysis. The results reported in Fig. 6, in addition to confirm that even in this cell type p57 and kcnq1 are already expressed in the undifferentiated state after CTCF depletion, also show that both genes are up-regulated exclusively from the respective maternal, non-imprinted, alleles. This finding allows us to exclude that the de-repression of the two genes, consequent to CTCF depletion, depends on the release of imprinting, thus highlighting the complexity of CTCF function at KvDMR1 (see also 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 critical 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 (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. For this purpose we performed sequential ChIP (Re-ChIP) assays using C2.7 myoblasts, a cell type which is responsive to MyoD binding, loop disruption and p57 expression 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 is not detectable before differentiation,
when MyoD has not yet bound to the target region. In contrast, after the shift to differentiation
medium, the two factors do co-occupy F3.

These results denote the occurrence of a physical interaction of MyoD with 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 in concomitance with MyoD binding, we wondered whether the
disruption of the chromatin loop was associated with some alteration involving the cohesin
complex, 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 and differentiated C2.7 cells (Fig. 7A).
Moreover, in line with the extensive co-localization of cohesin and CTCF-binding sites observed
throughout the genome (Parelho et al., 2008; Rubio et al., 2008), the Re-ChIP assays reported 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 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 cohesin
detachment 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 reported in Fig. 7C indicated that Rad21, like CTCF, binds not only to F3 but also to p57
promoter and, most importantly, that exogenous MyoD expression results in a significant reduction
of its binding to both regions. Taken together, these results indicate that the MyoD ability to disrupt
the repressive chromatin loop that restricts p57 expression involves its 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 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 involved in the formation and stabilization of chromatin contacts. However, in most cases, their functional relevance in gene regulation is not completely clear (Sexton et al., 2009).

We have recently reported that a long-distance chromatin interaction between KvDMR1 and p57 promoter correlates with p57 silencing in undifferentiated and in unresponsive muscle cells (Busanello et al., 2012). In that work we have 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 provided evidence for a functional role of its dynamics in the regulation of p57 expression during myogenesis.

The results we obtained through ChIP, ChIP-3C and depletion assays demonstrated that the chromatin contact between KvDMR1 and p57 promoter, associated with p57 repression, is mediated by CTCF.

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 H19/Igf2 locus is required for both chromatin folding and imprinted expression (Kurukuti et al., 2006). In 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 of CTCF depletion during MyoD-induced differentiation showed that p57 expression was anticipated at earlier times during differentiation and, remarkably, was at least in part rescued in unresponsive cells. This finding strongly suggests that CTCF-mediated looping between KvDMR1 and p57 promoter plays a critical role in the control of p57 transcription in muscle cells. Currently, we still cannot exclude 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 events would require new and more sophisticated approaches, based for example on the genome modification at CTCF-binding sites.

We have recently reported that kcnq1 (also named kvlqt1), a gene 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 about 200 kb
far from KvDMR1, on the opposite side respect to p57 promoter (Mancini-DiNardo et al., 2003). A
suggestive hypothesis is that KvDMR1 may establish a long-range interaction also with kcnq1
promoter and that the two genes participate in the same repressive chromatin hub that is perturbed
by MyoD binding. kcnq1, coding for 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 quite restricted
expression pattern in vivo, in large part 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, i.e. MyoD and E47, during muscle and
neural differentiation respectively (Busanello et al., 2012; Rothschild et al., 2006), we advance the
hypothesis that the co-ordinate 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 point to the
suggestion that chromatin folding mediated by KvDMR1 and CTCF may 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
eexpression of many imprinted genes. However, although the monoallelic binding of CTCF to the F1
sub-region 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 that additional mechanisms, such as chromating 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 came out from a 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 sub-region 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 may also affect the developmental regulation of a wider range of imprinted genes.
CTCF is an 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 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 5′ β-globin insulator, is capable to relief CTCF enhancer-blocking activity, in episomal assays (Defossez et al., 2005). Similarly, the thyroid hormone abrogates the enhancer-blocking activity of CTCF-TR composite elements associated with chicken Lysozyme and human c-myc genes (Lutz et al., 2003). However, the chromatin dynamics linked 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 p57 promoter. What is more, we show that MyoD recruitment relates with the disruption of the chromatin contact between the two CTCF-binding sites present within F3 and p57 promoter.

Regarding how MyoD affects CTCF function, our results of 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 p57 promoter after MyoD binding. A simple explanation of how MyoD alters CTCF activity even in the absence of its 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 recently established that the cohesin multiprotein complex plays a critical 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 Strom, 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 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 remodelling. We have observed that p68 is constitutively bound to both p57 promoter and F3, regardless of cell differentiation and responsiveness (unpublished data). At this moment 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, may 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 previously reported (Delgado-Olguin et al., 2011). In that work the authors showed that CTCF promotes the ability of MyoD to trans-activate some muscle-specific genes and that CTCF depletion impairs myogenic differentiation of C2C12 cells. Accordingly we observed that while CTCF depletion releases the repression of p57 expression, at the same time it 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 while 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 unable to elucidate, at this moment, 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 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 may represent a more widespread phenomenon than was 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 cultures

C3H10T1/2, C57BL/6 and (C57BL/6 X SD7)F1 mouse embryo fibroblasts and C2.7 mouse myoblasts were grown in DMEM supplemented with 10% fetal calf serum (FCS). (C57BL/6 X SD7)F1 fibroblasts, carrying single nucleotide polymorphisms in the p57/\textit{kcnq1} locus, were kindly provided by Andrea Riccio (University of Naples 2). To trigger differentiation, myoblasts and MyoD-expressing fibroblasts were grown to confluence and shifted to differentiation medium (DMEM-0.5% FCS). Cells were collected before the addition of differentiation medium (GM: growth medium) or 24 hours after the shift to differentiation medium (DM: differentiation medium).

Production of MyoD-expressing retrovirus, retroviral infections and MyoD-induced differentiation were performed as previously described (Figliola et al., 2008)

Chromatin immunoprecipitation (ChIP), and Sequential ChIP (Re-ChIP)

ChIP assays were carried out as described (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 \textit{p57} promoter) were amplified using the same DNA quantity for each sample (Input, No Antibody and IP), the primer pairs and the protocol previously reported (Busanello et al., 2012). The genomic region not-bound by CTCF (negative control, Ctr) was amplified using the primers:

Fw 5’-GCCAGCACCAAGGTAAGTGAG-3’ and Rev 5’-CTCGGAATAAGCAGCCTCCC-3’.

Quantitative PCR (qPCR) analysis of immunoprecipitated F3 fragment and \textit{p57} promoter was performed in triplicate using 5 ng of DNA, Go Taq qPCR Master Mix (Promega) and the primer pairs specific for F3 fragment, previously reported (Busanello et al., 2012) and for \textit{p57} promoter: Fw 5’-GTGTCACGTATACCCGGCA-3’ and Rev 5’-CTCATTGCTGCGGACAGCAG-3’ at the final concentration of 150 nM. The reaction was performed in the termocycler “MiniOpticon Real-Time PCR detection system” (Bio-Rad). The primer pair efficiency, the relative quantity of each immunoprecipitated (ΔC(t)) respect to 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 normalised respect to the quantities of each Input sample.

The parental alleles were distinguished by SSCP-PCR using F1 or F3 primers labeled at their 5’ ends with \textsuperscript{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 anti-MyoD ([sc-760] Santa Cruz Biotechnology, Inc.), anti-CTCF ([07-729] Millipore) or anti-Rad21 ([A300-080A] Bethyl Laboratories) antibodies and extensive washing (3 washes with 0.1% SDS, 2 mM EDTA, 20 mM Tris-HCl pH 8, 1% Triton X-100, 150 mM NaCl; 2 washes with 0.1% SDS, 2 mM EDTA, 20 mM Tris-HCl pH 8, 1% Triton X-100, 500 mM NaCl, 1 wash with 10 mM Tris-HCl pH 8, 1 mM EDTA), complexes were eluted by twice incubation for 30 min at 37°C in 25 μl of 10 mM 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 anti-CTCF, anti-MyoD or anti-Rad21 antibodies), washing, elution and reverse formaldehyde cross-linking as for conventional ChIP. After double immunoprecipitation, F3 fragment was amplified using the same DNA quantity for each sample and the primer pairs and the protocol previously reported (Busanello et al., 2012).

**Immunoprecipitation and Western blot analyses**

Immunoprecipitations were carried out as described (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 carried out as described (Figliola and Maione, 2004). The primary antibodies used were anti-MyoD ([clone 5.8A] Dako), anti-CTCF ([07-729] Millipore), anti-RAD21 ([A300-080A] Bethyl Laboratories), and anti-Tubulin ([sc-8035] Santa Cruz Biotechnologies), used as a loading control.

**Gene expression analysis**

Total cellular RNA was extracted with “High Pure RNA Isolation Kit” according to the manufacturer’s instructions (Roche Diagnostics). 1 μg of total RNA was reverse-transcribed with 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 RT product, 0.2 mM dNTP mix, 1.5 mM MgCl₂, 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 previously reported (Busanello et al., 2012). The amplification consisted of one cycle at 94°C for 4 min followed by 30 cycles at 94°C for 30 s, 65°C for 30 s and 72°C 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 have been previously reported (Busanello et al., 2012). The primer sequences for myogenin are: Fw 5’-GTCTCTTCCTGAAGCCAGTTGCG-3’ and Rev 5’-TGCAAATGCTTGGCCCCAGAG-3’. The reactions were performed in the termocycler “MiniOpticon Real-Time PCR detection system” (Bio-Rad). The primer pair efficiency, the normalized expression (ΔΔC(t)) and the standard error of the mean (SEM) were determined with CFX Manager™ software (Bio-Rad). 18S rRNA was used as a reference gene. Experiments were performed in triplicate and expressed as mean ± SEM. For statistical analysis comparisons were done 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 was determined as p < 0.05 (*) or p ≤ 0.01(**). To assay the allelic expression status of p57 and kcnq1, cDNAs obtained from RNA extracted from MyoD-infected (C57BL/6 x SD7)F1 fibroblasts were amplified by RT-PCR using the primers reported before (Busanello et al., 2012). Maternal and paternal RT-PCR products were distinguished by RFLP analysis of previously described polymorphic restriction sites (Casparry et al., 1998)

3C and ChIP-loop assays

3C and ChIP-loop assays were performed as previously described (Hagege et al., 2007). Briefly, after chromatin cross-link and nuclei isolation, DNA was digested with 400 units of NcoI restriction enzyme and ligated in 1X ligation buffer (NEB). 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 were used for qPCR or PCR analysis, performed in triplicate. Primers used for 3C sample amplifications were: Pair 1: FW 5’-CCTTCGACCATGGTGAGGTC-3’ and REV 5’-GTGCTGAAACGATCCACACG-3’ and Pair 2: FW 5’-CCGCGTTTGCACAGCCTTCG-3’ and REV 5’-CGACCGCGCTGGAGTGATCC-3’.

ChIP-loop assays were performed as previously described (Cai et al., 2006). Briefly, chromatin was crosslinked and nuclei were isolated. Chromatin was digested with NcoI restriction enzyme, immunoprecipitated with anti-CTCF [07-729] (Millipore) or anti-MyoD [sc-760] (Santa Cruz Biotechnology, Inc.) and ligated in 1X ligation buffer (NEB). Ligation products were treated as for 3C assays and analyzed by PCR or qPCR performed in triplicate with the same primers as in 3C.
RNA interference

C57BL/6, C3H10T1/2 and (C57BL/6 X SD7)F1 fibroblasts, grown in 100-mm dishes, were infected with a MyoD-expressing retrovirus. Forty-eight hours after infection 8x10^5 cells were transfected with 5 μl of 100 μM siRNA duplexes in 800 μl 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 1000μF. Following electroporation cells were kept 48 hours in DMEM–10% FCS and analysed after a further 0 or 24 h in differentiation medium (DMEM-0.5% FCS) by Western blotting, RT-PCR and 3C. A mixture of four chemically synthesized siRNA duplexes (GENE CTCF Mouse SMART POOL, Dharmacon) was used to target CTCF and an equal amount of siGFP (5'-GGC UAC GUC CAG GAG CGC ACC-3') was used as a control.
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REFERENCES


FIGURE LEGENDS

Fig. 1

The genomic organization of the p57/KvDMR1 locus. Schematic diagram showing the reciprocal location of p57, Kcnq1, 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 MatInspector tool, are also indicated. Enlarged boxes represent: mouse p57 promoter extending from nt +1 to +1099 of AF160190 sequence; mouse KvDMR1 extending from nt +1561 to +3420 of AF119385 sequence; human p57 promoter extending from nt +2882218 to +2883217 of NC_000011.10 sequence; human KvDMR1 element extending from nt +2699081 to +2700857 of NC_000011.10 sequence. Large black bars within promoters represent multiple and contiguous CTCF recognition sequences. Black lines represent the amplicons of ChIP assays.

Fig. 2

CTCF binds to KvDMR1 in differentiating muscle cells.

(A) In vivo binding activity of CTCF during differentiation. Chromatin from C2.7 myoblasts, responsive (C57BL/6) and unresponsive (C3H10T1/2) mouse embryo fibroblasts expressing exogenous MyoD, kept either in growth (GM) or in differentiation medium for 24 hours (DM) was immunoprecipitated using a specific antibody to CTCF or in the absence of antibody (NoAb). Input represents non-immunoprecipitated cross-linked 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 shown are representative of three independent experiments.

(B) Allele-specific chromatin immunoprecipitation of CTCF. (C57BL/6 x SD7) F1 hybrid mouse embryo fibroblasts, carrying single nucleotide polymorphisms in the p57/kcnq1 locus, were infected with the MyoD-expressing, kept 24 hours in differentiation medium and treated for ChIP assay as reported in Materials and Methods. Anti-CTCF-immunoprecipitated chromatin were then analyzed by PCR with specific primers for either F1 or F3, followed by SSCP analysis. M and P show the electrophoretic mobility of maternal- and paternal- specific bands, respectively. The parental alleles were distinguished by Single Strand Conformation Polymorphism 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 p57 promoter. Values derived from three independent experiments are expressed as percentage of input chromatin (% Input). Data are shown as mean ± SEM.
**Fig. 3**
CTCF is present at the chromatin contact between F3 and p57 promoter.  
(A) Locations of the NcoI restriction sites and of the PCR primers (horizontal arrows) used for ChIP-loop analysis. (B) qPCR analysis of ChIP-loop templates. Cross-linked chromatin obtained from the same cells under the same conditions as in Fig. 2A were digested and either directly ligated (3C samples) or immunoprecipitated with the indicated antibodies (IP CTCF – 3C and IP MYOD - 3C samples) or without antibody (NoAb samples) before ligation. ChIP-loop templates were analyzed for the presence of the recombinant fragment deriving from the ligation of 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 GM). Data are shown as mean ± SEM of three independent experiments.

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

**Fig. 5**
CTCF depletion causes p57 and kcnq1 de-repression.  
C57BL/6 mouse embryo fibroblasts (responsive) and C3H10T1/2 mouse embryo fibroblasts (unresponsive), expressing exogenous MyoD, were transfected with the indicated siRNAs as
described in Fig. 4A and collected before (GM) and after (DM) the shift to differentiation medium. (A) RT-PCR analysis of p57 mRNA levels performed in parallel with the western blot analysis of 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 mRNA amounts. The results of 30 amplification cycles, representative of three independent experiments, are shown. (B) qPCR analysis of p57, kcnq1 and myogenin expression performed after the shift to differentiation medium. Values, expressed as relative to those of 18S RNA, are the mean of three independent experiments ±SEM. Statistical significance: p < 0.05 (*) or p ≤ 0.01(**)

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 x SD7) F1 hybrid mouse embryo fibroblasts expressing MyoD were kept either in growth (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). AvaI and PvuII fragments indicate that of digested maternal-specific fragments (M).

Fig. 7
MyoD interacts with CTCF at KvDMR1 and causes Rad21 displacement.
(A) Immunoprecipitation-western blot analysis. Total proteins extracted from C2.7 cells, kept either in growth medium (GM) or in differentiation medium for 24 hours (DM), were immunoprecipitated with the indicated antibodies. Total extracts and immune complexes were then analyzed by western blot 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 IP were subjected to the second IP, with the indicated antibodies. The presence of F3 fragment was detected by PCR as for conventional ChIP. (C) ChIP assays for Rad21 were performed in (C57BL/6 x SD7) F1 hybrid mouse embryo fibroblasts, infected with either the empty vector or the MyoD-expressing vector and kept 24 hours in differentiation medium. Rad21 binding to p57 promoter (p57P) and F3 fragment was measured by qPCR analysis. Values derived from two independent experiments are expressed as mean of the percentages respect to the Input chromatin (% Input) ± SEM.
Fig. 8
Schematic model of the functional interaction between MyoD, CTCF and cohesin complex at the chromatin loop involving F3 and p57 promoter.
Fig. 3
Fig. 4
Fig. 5

A

Responsive

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B

Responsive

- p57
- kcnq1
- myog

Unresponsive

- p57
- kcnq1
- myog

Mature of mRNA relative expression

Responsive Unresponsive

- p57
- kcnq1
- myog

Mature of mRNA relative expression
Fig. 6
Fig. 8