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Identification of a perinuclear positioning element in human subtelomeres that requires A-type lamins and CTCF

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The localization of genes within the nuclear space is of paramount importance for proper genome functions. However, very little is known on the *cis*-acting elements determining subnuclear positioning of chromosome segments. We show here that the *D4Z4* human subtelomeric repeat localizes a telomere at the nuclear periphery. This perinuclear activity lies within an 80 bp sequence included within a region known to interact with CTCF and A-type Lamins. We further show that a reduced level of either CTCF or A-type Lamins suppresses the perinuclear activities of *D4Z4* and that an array of multimerized *D4Z4* sequence, which has lost its ability to bind CTCF and A-type Lamins, is not localized at the periphery. Overall, these findings reveal the existence of an 80 bp *D4Z4* sequence that is sufficient to position an adjacent telomere to the nuclear periphery in a CTCF and A-type lamins-dependent manner. Strikingly, this sequence includes a 30 bp GA-rich motif, which binds CTCF and is present at several locations in the human genome.

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Introduction

The spatial distribution of chromosomal domains within the nucleoplasm has an important function in the regulation of eukaryotic genomes (Csink and Henikoff, 1996; Maillet *et al*, 1996; Brown *et al*, 1997; Andrulis *et al*, 1998; Taddei *et al*, 2006; Nagai *et al*, 2008). Very little is known on the *cis*-acting elements governing the subnuclear localization of genes or groups of genes, especially in mammals. However, these elements are expected to contribute to accurate genetic and epigenetic regulations. As examples, the localization of chromosome regions at the periphery of the nucleus seems to have a central function in gene regulation and DNA repair (Therizols *et al*, 2006; Finlan *et al*, 2008; Kumaran and Spector, 2008; Reddy *et al*, 2008). Nevertheless, the precise regulation of genes located at the nuclear rim remains poorly understood as the expression of some genes appears unaffected by their proximity to the nuclear periphery (Finlan *et al*, 2008; Kumaran and Spector, 2008).

In metazoan, the lamina, which coats the inner surface of the nuclear envelope, has an important function in the perinuclear localization of chromosome segments. For instance, in mice, lamin B1 is involved in the perinuclear localization of chromosome 18 (Malhas *et al*, 2007) and recent large-scale mappings of chromosomal domains attached to B-type lamins performed in *Drosophila* and human cells, defined the Lamin-associated domains or LADs (Pickersgill *et al*, 2006; Guelen *et al*, 2008), which correlate with silenced regions. In human cells, some of the LADs are flanked by binding sites for CTCF, suggesting that the insulator protein allows a functional partitioning of the human genome by separating active and inactive domains.

The telomeres are not randomly localized within the nucleoplasm and can be found at the nuclear periphery (Gilson *et al*, 1993). This positioning varies greatly among organisms, cell types, cell cycle stages and individual telomeres. At the bouquet stage for instance, the clustering of all telomeres at the edge of the nucleus, is a nearly universal feature of meiosis (Scherthan, 2007). In budding yeast, the 32 telomeres gather into 4–6 *foci*, associated with the nuclear envelope (Gotta *et al*, 1996) that sequesters heterochromatic factors (Gotta *et al*, 1996; Maillet *et al*, 1996). The peripheral positioning of yeast telomere is mediated by at least two complexes bound to the telomeric chromatin, suggesting that, in this model organism, the telomere by itself behaves as a perinuclear positioning element (Taddei *et al*, 2005). Consistent with this hypothesis, *de novo* telomeres formed in yeast, devoid of natural subtelomeric repeats adopt a perinuclear localization (Tham *et al*, 2001). A perinuclear positioning of telomeres is also observed in *Plasmodium*, where it favours subtelomeric gene conversion (Freitas-Junior *et al*, 2000), whereas in plants, telomeres are observed either close to the nuclear periphery (Rawlins and Shaw,

1990) or around the nucleolus (Fransz *et al*, 2002). In mammalian nuclei, telomeres adopt different locations (Luderus *et al*, 1996). Although human telomeres are clustered at the nuclear periphery in sperm (Zalenskaya *et al*, 2000), most telomeres in lymphocyte nuclei are located in the interior of the nucleoplasm (Weierich *et al*, 2003). Thus, it seems that by default, human telomeres are localized internally in most cell types. However, some subtelomeric elements may antagonize this internal localization and target their proximal telomere to the nuclear envelope as suggested by the presence of LADs at different subtelomeres (Guelen *et al*, 2008). Such an example of localization at the periphery of the nucleus is the positioning of the 4q35 subtelomeric locus, involved in the facio-scapulo-humeral dystrophy (FSHD) (Masny *et al*, 2004; Tam *et al*, 2004).

The existence in metazoan of *cis*-acting DNA elements targeting chromosomal regions to the nuclear envelope remains elusive and a key question regarding the function of perinuclear localization is to know whether it is the cause or consequence of differential gene expression or genomic nature.

To identify and characterize such elements, we investigated the ability of *D4Z4*, a 3.3 kb macrosatellite repeat present at the 4q35 locus (Hewitt *et al*, 1994; Lyle *et al*, 1995), to direct the localization of an associated telomere towards nuclear periphery. *D4Z4* is repeated in tandem at several chromosomal loci including the short arm of acrocentric chromosomes, the pericentric region of chromosome 1q and the telomeric regions of the long arms of chromosomes 4 and 10 (Hewitt *et al*, 1994; Lyle *et al*, 1995). Normal 4q35 ends carry from 11 up to 150 copies of this element, whereas this number is reduced to 1–10 repeats in patients affected with the autosomal dominant FSHD (van Deutekom *et al*, 1993; Winokur *et al*, 1994). We report here that *D4Z4*, which we previously characterized as a CTCF- and A-type Lamins-dependent insulator (Ottaviani *et al*, 2009), behaves as a potent perinuclear *cis*-acting positioning element. Impressively, this property depends on CTCF and A-type Lamins binding and is lost upon multimerization of the repeats, uncovering a striking link between perinuclear localization, CTCF and A-type Lamins and transcriptional insulation.

Results and discussion

D4Z4 behaves as a perinuclear positioning element

To evaluate the putative role of *D4Z4* in the subnuclear localization of telomeres, we artificially tagged different telomeres by transfecting human cells with DNA molecules carrying *D4Z4* sequences inserted between a seed of (TTAGGG)_n telomeric repeats and a *hygromycin-eGFP* cassette (Figure 1A and B). Telomere fragmentation is based on the non-targeted introduction of cloned telomeres into mammalian cells and *de novo* telomere formation after telomeric healing (Farr *et al*, 1992). Successful *de novo* formation of hygromycin-tagged telomeres was confirmed in individual clones by detection of a diffuse hybridization signal in Southern blot or in the population of transfected cells by fluorescence *in situ* hybridization (FISH) of metaphase spreads (data not shown and Figure 1A). In agreement with earlier data (Koering *et al*, 2002), the rate of *de novo* telomere formation in stably transfected C33A cells is very high reaching 85–94% of the hygromycin-resistant cells (Supplementary Table S1). Therefore, polyclonal populations of stably trans-

ected cells are representative of pools of independent clones of hygromycin-tagged telomeres and further experiments were performed on populations.

The tri-dimensional distribution of the FISH signal corresponding to the *hygromycin-eGFP* cassette was determined in different cell populations as a function of the nuclear volume after delineation of the nuclear periphery by simultaneous immunolocalization of B-type Lamins (Figure 1C; Supplementary Figure S1). Using this 3D-immuno-FISH method, we showed that *de novo* formed telomeres, devoid of natural subtelomeric sequences, have the tendency to localize in the innermost nuclear volume (T construct in Figure 1C–E). By contrast, the *eGFP-hygromycin* construct but devoid of telomere seed appears randomly distributed between the interior and the periphery of the nucleoplasm (Figure 1D and E). These results are in agreement with several reports suggesting that human telomeres have the intrinsic property to be anchored to internal sites (Luderus *et al*, 1996; Weierich *et al*, 2003; Tam *et al*, 2004).

As expected from earlier observations, the 4q35 locus is enriched in a peripheral zone of the nucleus (Figure 1C–E) (Masny *et al*, 2004; Tam *et al*, 2004). Strikingly, telomeres associated to a single copy of *D4Z4* have a more peripheral distribution (T1X construct) than *D4Z4*-less telomeres (T construct) (Figure 1C–E), suggesting that this element might be specialized in the subnuclear positioning of telomeres. The perinuclear localization of *D4Z4*-associated telomeres unlikely results from biases in telomere seeding or in chromosome choice as we observed a similar rate of telomere formation (Supplementary Table S1) and no preferential insertion between populations of cells transfected with seeding constructs with or without *D4Z4* (Supplementary Figure S2).

We recently showed that *D4Z4* act as a CTCF-dependent insulator protecting from position effect variegation and enhancer-promoter communication (Ottaviani *et al*, 2009). The protection against silencing is mediated in part by CTCF and A-type Lamins, which bind *in vivo* to *D4Z4* as shown by chromatin immunoprecipitation. Upon multimerization of *D4Z4*, both CTCF binding and insulator activity are lost suggesting that FSHD is associated with a gain of function of CTCF (Ottaviani *et al*, 2009). Interestingly, insulators are often anchored at fixed nuclear substructures including the nuclear periphery in eukaryotes (Gerasimova and Corces, 2001; Gaszner and Felsenfeld, 2006; Valenzuela and Kamakaka, 2006). Therefore, to test whether peripheral tethering of telomeres is a common property of CTCF-dependent vertebrate insulators, we compared *D4Z4* with the 5' *HS4* chicken β -globin CTCF-dependent insulator inserted near telomeres (Figure 1B). We showed that 5'*HS4*-tagged telomeres are localized in the innermost nuclear volume as observed for the T construct, suggesting that the peripheral positioning activity is an intrinsic property of *D4Z4* (Figure 1C–E).

We have previously identified a shorter fragment with anti-silencing activity within *D4Z4* and we mapped by deletion the region of *D4Z4* involved in this perinuclear positioning activity. We found three truncated forms of *D4Z4* that recapitulate both the insulator activity and the peripheral localization of telomeres of the full-length repeat (Figure 2, T1X Δ B1, T1X Δ B1-3, T1X Δ E), whereas the very proximal 382 bp at the 5' end of *D4Z4* (Figure 2, T1X Δ B2-3) harbours

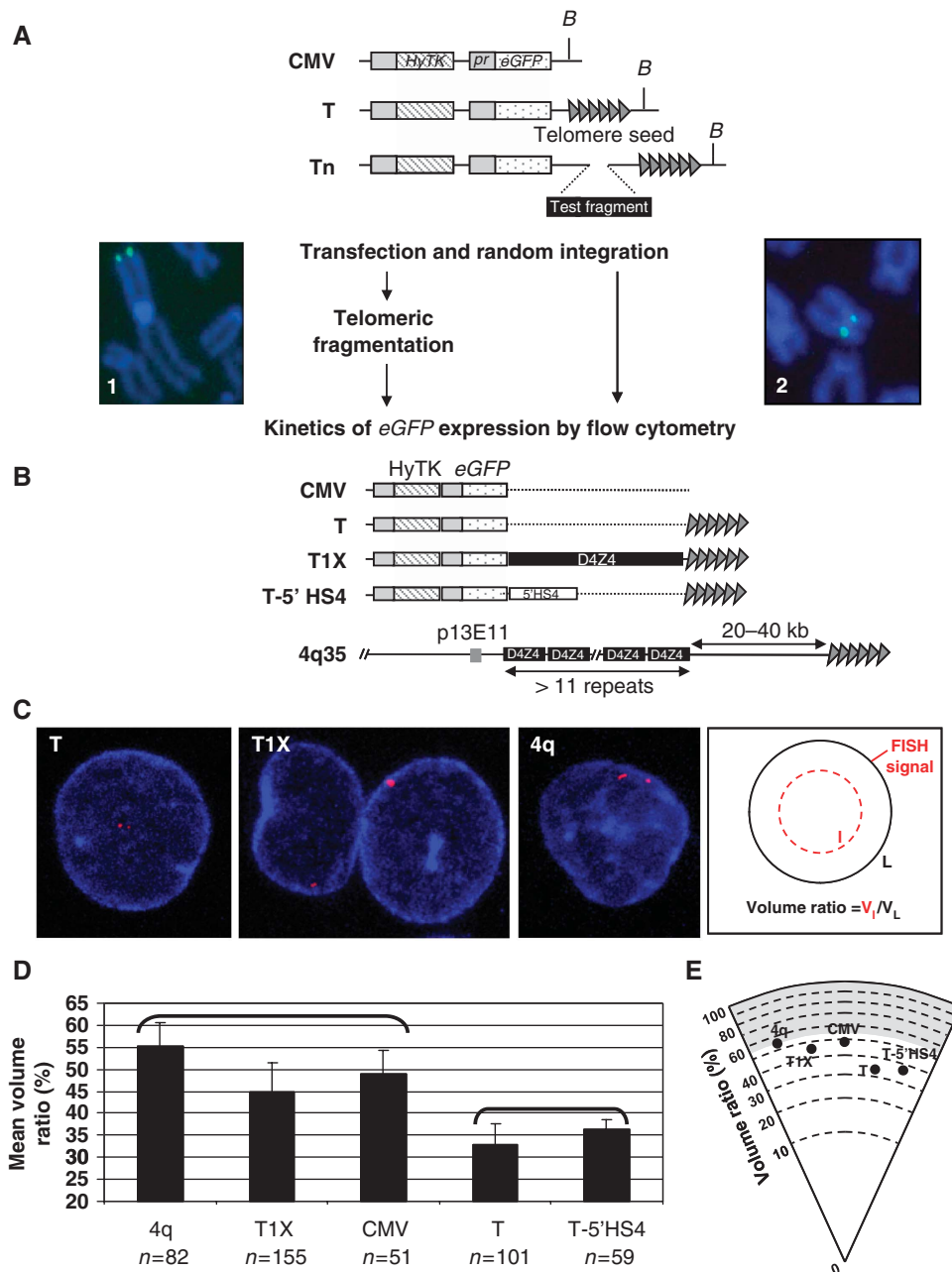


Figure 1 *D4Z4* relocates *eGFP*-tagged telomeres towards the nuclear periphery. **(A)** The constructs are derived from the pCMV vector, which carries a Hygromycin resistance gene fused to the herpes simplex virus type 1 thymidine kinase suicide gene (*HyTK*) and an *eGFP* reporter gene both driven by CMV promoters, and carry a telomere seed (depicted by arrows) that allows telomeric fragmentation. We inserted different sequences between the reporter and the telomere to investigate their respective effect on telomere positioning. Successful *de novo* formation of *eGFP*-tagged telomeres or internal integration of the CMV construct were confirmed by fluorescence *in situ* hybridization (FISH) on metaphase spreads (photographs 1, 2, respectively, Supplementary Table S1). M-FISH analysis performed on cells containing the pCMV Telo vector (T) or a vector with *D4Z4* (T1X) confirmed that constructs carrying the *D4Z4* sequence do not integrate preferentially at certain chromosomes (Supplementary Figure S2). **(B)** CMV: empty vector; T: empty telomeric vector; T1X: insertion of a 3.3 kb *D4Z4* element between *eGFP* and the telomere in T; T-5' HS4: insertion of the 1.2 kb chicken β -globin insulator (Chung *et al*, 1993). Schematic representation of the normal 4q35 allele. **(C)** Confocal section of T, T1X cells stained with an *eGFP* probe (single red dot) or endogenous 4q with a 4qtel probe staining both alleles (two red dots). Representation of the analysis of a nucleus after two-colour 3D-FISH (right panel). We considered the outer limit of the Lamin B signal (blue) as the edge of the nucleus (100%). Distribution of the FISH signals within the nuclear volume was calculated from the centre (0%) to the outer edge of the sphere after reduction of the outer signal (V_L = nuclear volume = 100%) until it overlaps with the FISH signal ($V_1 = x\%$ of V_L) (Supplementary Figure S1). Experiments were performed on three to four independent populations of cells (Supplementary Table S1). **(D)** Histogram displaying the mean positioning of natural and fragmented telomeres as their mean values of volume ratio (V_1/V_L) \pm s.d. shown by error bars (y-axis). Data sets were compared with the Kruskal–Wallis statistic test ($P < 2.4 \times 10^{-9}$, n = number of interphase nuclei). Brackets identify two groups where all conditions are significantly different from the other group, based on FDR determination. **(E)** Distribution of the FISH signal from the centre to the outer rim of the nucleus. Lamin B signal occupies the outermost 18% of the nuclear radius considered (grey shadow).

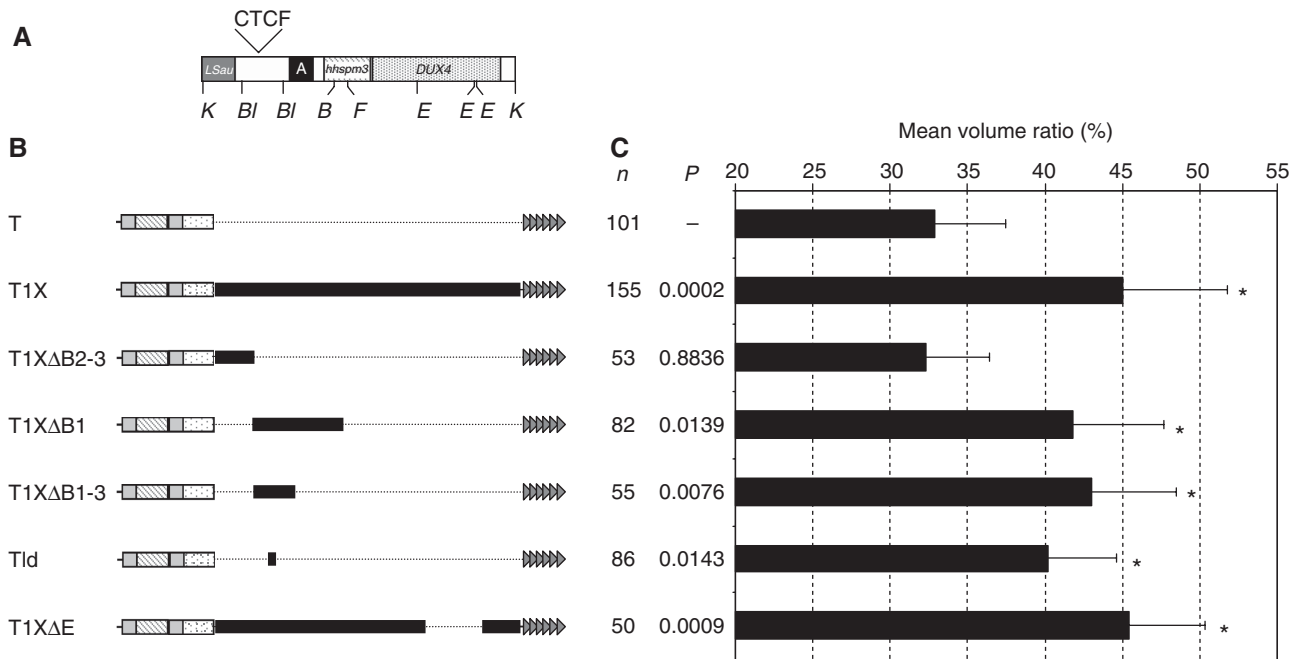


Figure 2 Identification of the tethering element within *D4Z4*. (A) Schematic representation of this element from position 1–3303 relative to the two flanking *KpnI* sites (K) (to scale), the different regions within *D4Z4* are shown. Each repeat contains two classes of repetitive DNA, *LSau*, a repetitive element associated with heterochromatin regions, a GC-rich low copy repeat, *hhspm3* displaying sperm-specific DNA hypomethylation and a region named region A. *DUX4* corresponds to an ORF with a double homeobox encoding the DUX4 protein putatively involved in the disease. The position of the CTCF site is shown (Ottaviani *et al*, 2009). The restriction sites used for the cloning of *D4Z4* subfragments are indicated (B: *Bam*HI; BI: *Bln*I; E: *Ehe*I) and the different constructs used are depicted (B). (C) Subnuclear positioning of *eGFP*-tagged telomeres in cells containing different fragments of *D4Z4*. The number of nuclei analysed per construct is indicated (*n*) together with the *P*-values determined with the Mann–Whitney tests using the T construct as the reference. Significant conditions after false discovery rate (FDR, $\alpha = 0.05$) correction for multiple comparisons are marked by asterisk. These results reveal that constructs containing the proximal insulator of *D4Z4* displace a telomere towards the nuclear periphery, whereas T1XΔ2–3 occupies the same positioning as the T constructs.

the same distribution than a *D4Z4*-less telomere. Impressively, a short sequence of 80 bp that harbours anti-silencing activity (Supplementary Figure S3) is sufficient to localize a telomere closer to the periphery (Figure 2, Tld). Therefore, we conclude that *D4Z4* has the ability to direct a subtelomeric region at or near the nuclear envelope and that this property is mediated, at least in part, by a segment present at the 5' end of the *D4Z4* repeat.

CTCF and A-Lamins contribute to the localization of *D4Z4* at the nuclear periphery

As the different fragments involved in the perinuclear positioning possess an anti-silencing activity mediated *in vivo* by CTCF and A-type Lamins (Ottaviani *et al*, 2009; Supplementary Figure S3), we wondered whether these proteins are involved in the positioning activity of *D4Z4* as well. When the expression of *CTCF* or *LMNA* is significantly reduced by RNA interference (Supplementary Figure S4), the distribution of *D4Z4*-tagged telomeres is more internal (Figure 3A and B) revealing an essential role for these proteins in the perinuclear distribution of *D4Z4*. Importantly, the effect of CTCF depletion is specific for *D4Z4* as our RNA interference conditions are (i) not accompanied by a modification of the localization of *D4Z4*-less telomeres (ii) nor by any global disturbance of the nuclear architecture as shown by the distribution of the 10q locus and X chromosome (Figure 3A, C and D) and (iii) the CTCF-dependent 5'*HS4* insulator of the chicken β -globin locus artificially integrated at chromosome ends (T-5' *HS4*) remains in the inner nuclear space (data not shown). We conclude

that *D4Z4* positions its associated seeded telomere to the nuclear periphery through an interaction with CTCF and A-type Lamins, whereas in the absence of CTCF, the wild-type 4q35 locus remains at the nuclear periphery (Figure 3A), suggesting additional pathways for the anchoring of this region.

The multimerization of *D4Z4* suppresses peripheral localization

D4Z4 is repeated in tandem at several chromosomal loci including the 4q35 region linked to FSHD. Moreover, we demonstrated earlier that the binding of CTCF to *D4Z4* is lost on multimerization of the repeat (Ottaviani *et al*, 2009) and that the positioning activity of *D4Z4* is CTCF-dependent. Hence, we hypothesized that localization of a telomere-containing multiple *D4Z4* repeats would be more internal and explored whether the multimerization alters *D4Z4* perinuclear positioning activity. For this purpose, we transfected linearized DNA containing eight copies of the *D4Z4* sequence abutting a telomere seed (T8X construct). As observed with the other telomere seeding plasmids containing 0 or 1 *D4Z4* sequence, 94% of integrated T8X constructs form a *de novo* telomere (Supplementary Table S1) and the number of repeats remains stable after long-term culture (Supplementary Figure S5). As we hypothesized, T8X harbours the same subnuclear distribution than the *D4Z4*-less construct (T construct) and is more internal than telomeres carrying a single *D4Z4* sequence (T1X construct) (Figure 4A and B). Interestingly, this loss of peripheral localization on multimerization correlates with a decrease in CTCF (Ottaviani

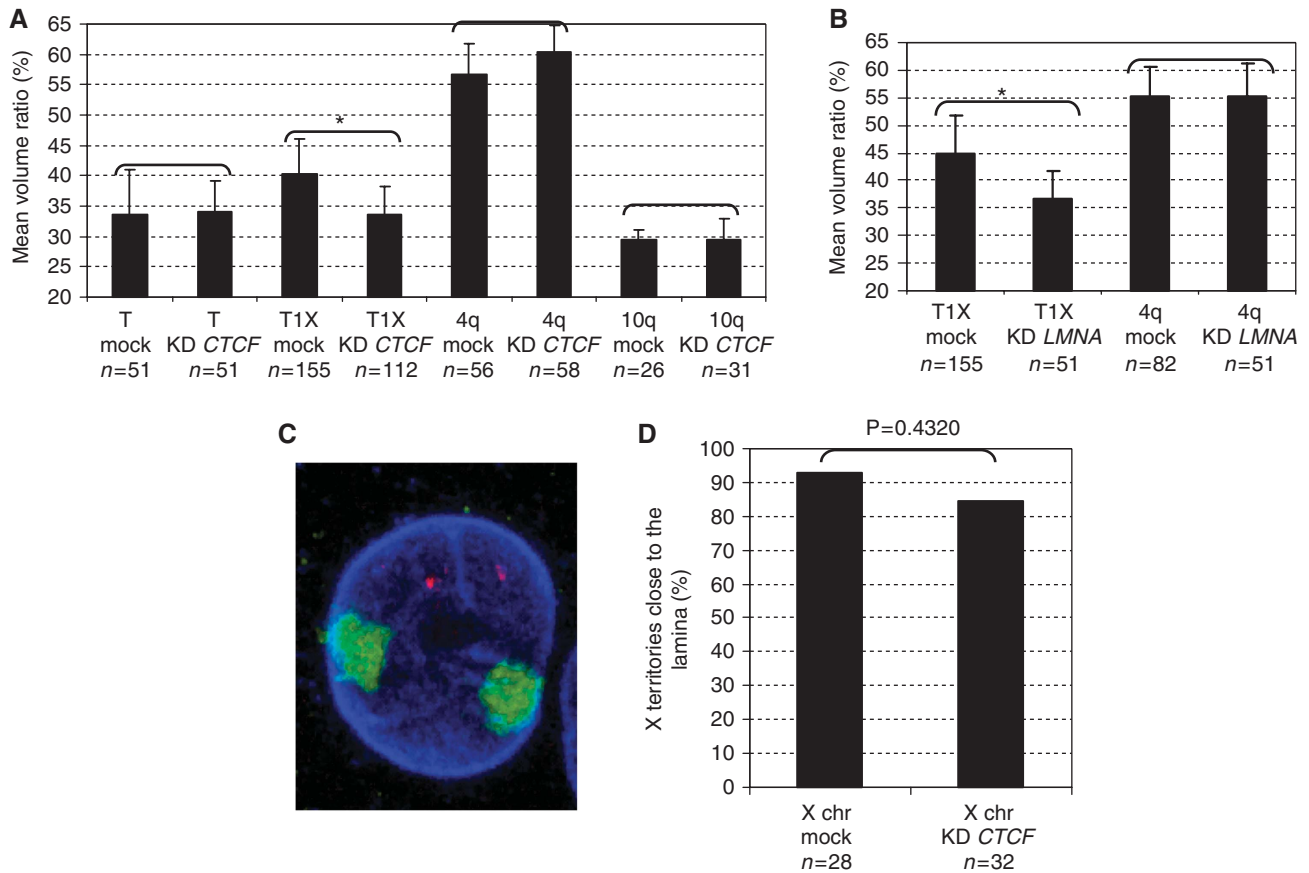


Figure 3 CTCF and A-type Lamins contribute to the positioning of *D4Z4*-tagged telomeres. **(A)** Cells containing the T1X or the T transgene transiently transfected with *CTCF* (KD *CTCF*) or negative (mock) siRNA were compared using the Mann–Whitney test. Depletion of CTFC correlates with the relocation of *D4Z4*-tagged telomere to the interior of the nucleus ($P=0.0198$) whereas the T construct ($P=0.597$) and the 4q ($P=0.536$) are not affected. **(B)** *LMNA* depletion significantly displaces the T1X construct ($P=0.0376$) but does not affect the distribution of the 4q telomere ($P=0.6973$) (Mann–Whitney). **(C)** To ascertain the global integrity of the nucleus architecture after the transient knock-down of *CTCF*, we analysed the distribution of the 4q or 10q telomeres (red signal) and the whole X chromosome (green signal) in T1X cells after transfection of *CTCF* siRNA by 3D-FISH as described earlier (the blue signal corresponds to Lamin B). We do not observe a redistribution of the FISH signal for the 4q and 10q probes (panel A) or X chromosome territories **(D)** in cells transfected with siRNA compared with the control, suggesting that the reduced *CTCF* expression does not globally perturb the distribution of these chromosomes inside the nuclear space as observed for the *D4Z4*-tagged telomeres. For 4q and 10q distributions, we compared mock and knock-down conditions with the Mann–Whitney statistical test. The numbers of X territories associated to the Lamin B signal in these conditions were compared with the Fisher exact test.

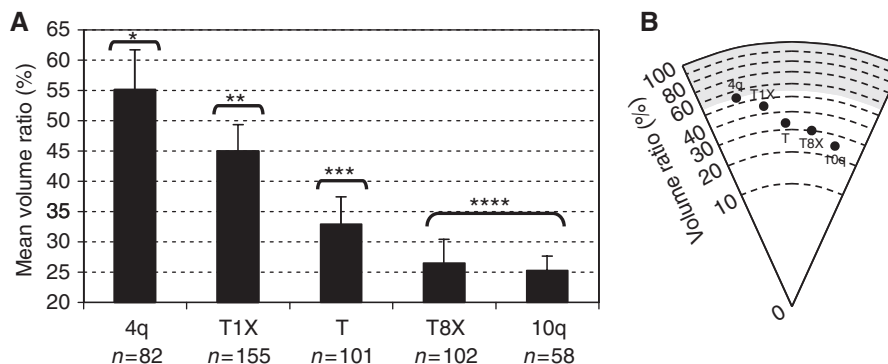


Figure 4 Loss of CTFC binding upon *D4Z4* multimerization abrogates peripheral tethering. **(A)** The position of the FISH signal was scored as described. The mean values \pm s.d. (y-axis) are presented for different populations compared with the Kruskal–Wallis statistic test ($P < 2.2 \times 10^{-16}$, n = number of interphase nuclei). Multiple pairwise comparisons corrected for 5% FDR define four independent groups (identified by *, **, ***, **** above the brackets). **(B)** Mean positioning of the FISH signal for constructs with different number of *D4Z4* from the centre (0%) to the outer (100%) edge of the nucleus.

et al, 2009) and A-type Lamins (Supplementary Figure S6) binding confirming the importance of CTFC for the peripheral positioning activity of *D4Z4*.

The fact that the wild-type 4q telomere is not delocalized on *CTCF* knock-down in our cells together with the particular behaviour of the 4q35 locus, which remains at the nuclear

periphery even in FSHD patients with only one copy of *D4Z4* (Masny *et al*, 2004; Tam *et al*, 2004), suggests the existence of at least two Lamins-dependent pathways for the perinuclear targeting of the 4q35 region. The first one is CTCF-independent and likely acts through a sequence located upstream of the *D4Z4* array on the 4q35 region, as suggested earlier (Masny *et al*, 2004) (Figure 5A). According to our results, the second pathway might be linked to a gain of function of CTCF in patients carrying a single or a weakly oligomerized *D4Z4* array (Ottaviani *et al*, 2009). In this configuration, a region located upstream of *D4Z4* (Masny *et al*, 2004; Guelen *et al*, 2008; Petrov *et al*, 2008) might cooperate with the remaining *D4Z4* repeats bound to CTCF to tighten the bonds between the corresponding region and the nucleus rim (Figure 5B). Consistent with the existence of two positioning elements at the 4q35 end, the proximal 4q35 FISH probe (D4S139) that hybridizes 215kb upstream of the region containing the repeats is more peripheral than the *D4Z4* array in normal cells (Masny *et al*, 2004) where CTCF is absent (Ottaviani *et al*, 2009). The putative existence of another region upstream of *D4Z4* mediating perinuclear positioning and the inability of long *D4Z4* arrays to bind CTCF and A-type Lamins in normal cells might also explain why the *D4Z4* repeats alone do not tether the 10q26 telomere

to the rim of the nucleus (Masny *et al*, 2004) and why *LMNA* knock-down does not affect the 4q35 positioning.

Both loci are 98% homologous for the regions extending 40 kb proximal to *D4Z4* and at least 10 kb distal to the repeat at 4q35 but does not contain the element located upstream the region of homology between the two chromosomes speculated earlier (Tam *et al*, 2004; Guelen *et al*, 2008). Consistent with this hypothesis, we observed a stronger degree of internal location (25.29%) than the *de novo* formed telomeres (Figure 4A) for the 10q terminus that does not possess the proximal positioning element postulated for 4q35 (see above). A second explanation might be linked to the *D4Z4* sequence itself. Indeed, *D4Z4* from 4q show a 98% homology to the one found on 10q but the major differences between the two types of repeat are found in the vicinity of the CTCF site that we identified earlier (Ottaviani *et al*, 2009) and consists in the insertion of 7–8 bases in the *D4Z4* from 10q (Supplementary Figure S7). CTCF and A-type Lamins, together with yet unidentified factors might thus be present only on the type 4 *D4Z4*. However, one cannot exclude at this step that the level of *LMNA* KD might not be sufficient as we and others were only able to see the repositioning of the 4q35 locus can only be observed in cells with homozygous invalidation of the *LMNA* gene (Masny *et al*, 2004) (Ottaviani *et al*, unpublished observations).

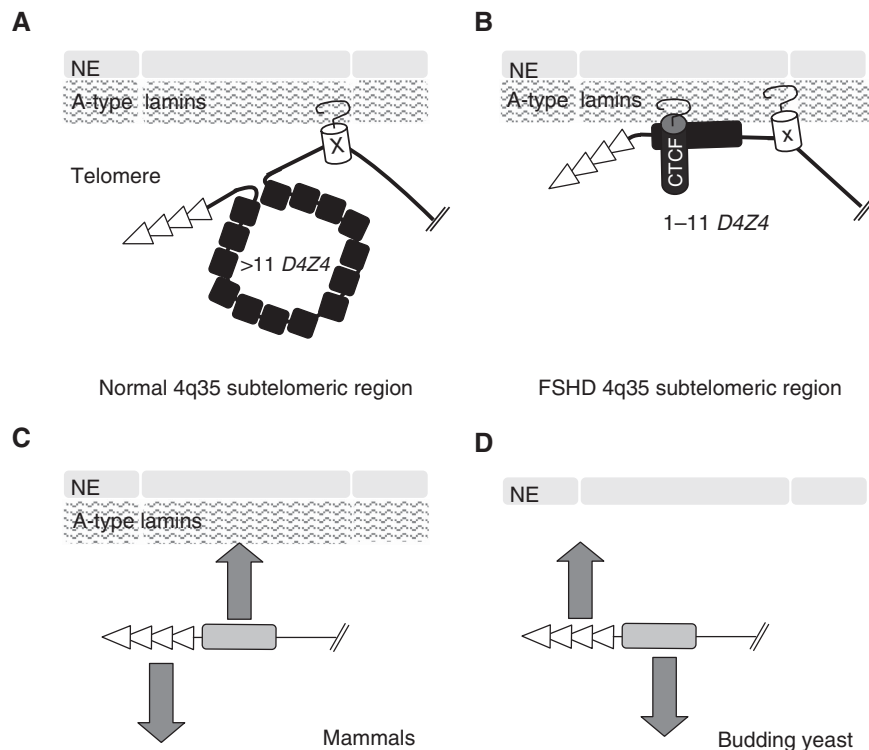


Figure 5 Model for the tethering of the 4q35 locus at the nuclear periphery. **(A)** In normal cells, *D4Z4* repeats are methylated (van Overveld *et al*, 2005) and not bound by CTCF (Ottaviani *et al*, 2009). On the basis of our data and those from Masny *et al* (2004); Tam *et al* (2004); Guelen *et al* (2008), we propose that this subtelomeric region is attached to the periphery through a Lamin-A-dependent tethering site localized outside the *D4Z4* array, likely on the centromere side. **(B)** A model for the tethering of 4q35 at the nuclear periphery in FSHD cells. The contraction of *D4Z4* allows the binding of CTCF (Ottaviani *et al*, 2009) and changes the functional organization of the 4q35 region. The *D4Z4* repeats become specifically attached to the periphery (our work). We propose that this higher-order switch modifies the expression of FSHD-associated genes probably by preventing their repression (Gabellini *et al*, 2002). The involvement of CTCF opens new strategies for the identification of these genes. **(C)** In human cells, most of the telomeres are localized within the nucleoplasm, probably anchored at defined subnuclear sites. These sites might serve to nucleate heterochromatin at telomeres. The peripheral localization might be caused by specific subtelomeric elements. **(D)** In baker's yeast, the nuclear periphery acts as a subnuclear compartment where telomeres are clustered to recruit and bind key heterochromatic factors. The telomere-NE association is antagonized by subtelomeric insulator elements called STAR, linking subnuclear localization and transcriptional insulation (Magdinier *et al*, 2008). Similarities with yeast suggest that *D4Z4* could represent a human equivalent of *S. cerevisiae* STAR elements.

Consistent with a key role for the 4q-specific repeat, it is interesting to note that in the Caucasian population at least 20% of individuals carry 10-type 3.3 kb repeats on chromosome 4 and *vice versa* (van Deutekom *et al*, 1996; van Overveld *et al*, 2000). However, the disease is always associated with a low number of four-type repeats (<11) on the 4q locus only, regardless of the size of the 10q-4q-hybrid array.

In summary, our data together with Masny *et al* (2004) and Guelen *et al* (2008), suggest that a normal 4q35 locus is attached to the nuclear rim through a *D4Z4*-independent anchor, whereas a pathogenic 4q35 locus is also tethered through a second anchor depending on *D4Z4*. A corollary of this hypothesis is that the peripheral environment of the pathogenic 4q35 allele might be different for the wild-type allele, leading to a differential expression of genes causing the disease. In agreement with this model, the FSHD displays some features of muscular laminopathies (Bakay *et al*, 2006). Moreover, we showed that A-type Lamins contribute to the regulation of *D4Z4* activity (Ottaviani *et al*, 2009) but also to *D4Z4*-associated telomere positioning, suggesting that the Lamins-dependence of *D4Z4* might contribute to the epigenetic regulation of the FSHD-associated gene(s) and put A-type Lamins as key regulators of *D4Z4* function (Figure 5B).

***D4Z4* as a prototype of CTCF/Lamins-dependant perinuclear anchoring elements**

As the perinuclear positioning *cis*-acting element of *D4Z4* also behaves as a transcriptional insulator (Ottaviani *et al*, 2009), we propose that this element defines a particular class of CTCF-dependent insulator that acts in cooperation with A-type Lamins to position certain chromosome regions at the nuclear periphery. The *in vivo* binding of Lamins A/C to *D4Z4* suggests that the nuclear lamina directly recruits the repeat (Ottaviani *et al*, 2009). In this case, CTCF and A-type Lamins could be part of the same complex positioning short *D4Z4* arrays to the nuclear lamina. Consistent with the hypothesis of a functional Lamins/CTCF interaction, these proteins were previously copurified in soluble extracts from HeLa cells overexpressing CTCF (Yusufzai *et al*, 2004) and A-type Lamins depletion reduces *D4Z4* anti-silencing activity (Ottaviani *et al*, 2009).

Alternatively, CTCF might counteract the internal anchoring pathway acting on telomeres, for instance through its insulator activity, thereby allowing its associated telomere to be displaced towards the periphery. Interestingly, a recent report revealed that A- and B-type Lamins form separate meshworks with a preferential association of B-type Lamins with silenced regions and A-type Lamins with euchromatin (Shimi *et al*, 2008), suggesting that CTCF-Lamins A/C interactions might control a subset of chromosomal domains regulated at the nuclear rim.

Our findings, together with the colocalization between some CTCF insulators and the nucleolus (Yusufzai *et al*, 2004) and recent data showing that Cohesins (Parelho *et al*, 2008; Wendt *et al*, 2008) or Emerin and B-type Lamins (Guelen *et al*, 2008) can colocalize with CTCF throughout the genome, suggests that CTCF acts as a global regulator of nuclear architecture by interacting with different key components of human chromosomes. In agreement with the possible existence of different classes of CTCF-dependent insulators, we have been unable to detect a significant

association of *D4Z4*-tagged telomeres and the nucleolus (data not shown). Strikingly, we found that a 30 bp GA-rich motif, that is included into the 80 bp perinuclear positioning sequence and binds CTCF *in vitro* (Ottaviani *et al*, 2009) is present in different unrelated regions of the human genome (Supplementary Figure S8; Supplementary Table S2). Therefore, this motif might be the signature of a subset of CTCF-binding sites able to function together with A-type Lamins to position various chromosomal segment to the nuclear envelope.

Modulation of telomere perinuclear anchoring: a conserved feature of subtelomeric insulators

This work identifies, for the first time, a human subtelomeric element that positions a telomere at the nuclear periphery. Hence, it emerges that a basic principle governing telomere subnuclear organization in human cells is the ability of specific subtelomeric sequences to counteract the tendency of human telomeres to remain in the innermost nuclear space (Figure 5C). These findings corroborate the notion that the localization of a telomere within the nucleoplasm is determined, at least in part, by the nature of its subtelomeric region. Moreover, our work suggests that A-type lamins play a role in the function of telomeres by contributing to their subnuclear distribution. In agreement with this view, Gonzalez-Suarez *et al* (2009) recently showed that A-type lamins play a general role in telomere function and localization in the mouse. However, if the main pool of nuclear lamins is found at the nuclear periphery, soluble lamins and lamin speckles have also been observed in the inner nuclear space (Kumaran *et al*, 2002) and the integration of A-type lamins at the nuclear periphery depends on the phase of the cell cycle (Muralikrishna *et al*, 2001). Therefore, together with the components of the lamina, internal lamins might also contribute to the subnuclear positioning of a subset of telomeres (this work and Gonzalez-Suarez *et al*, 2009).

It is worth noting that the opposite happens in budding yeast, that is, specific subtelomeric sequences antagonize the tendency of telomeres to be at the periphery (Berthiau *et al*, 2006; Hediger *et al*, 2006) (Figure 5D). Strikingly, in both organisms, the subtelomeric sequences modulating telomere positioning are coupled to transcriptional insulation (Fourel *et al*, 1999; Hediger *et al*, 2006; Ottaviani *et al*, 2009). Therefore, subnuclear positioning and insulation might have been conserved in subtelomeric regions during evolution to fulfill critical functions for telomere maintenance, transcriptional programming and chromosome organization.

Materials and methods

Cell culture

The human epithelial cervix carcinoma C33-A cell line was maintained in Dulbecco's Modified Eagle's Medium with L-alanyl-L-glutamine (GlutaMAXTM), D-glucose and sodium pyruvate (Invitrogen). Media were supplemented with 10% FBS (Invitrogen) and 1% Penicillin-Streptomycin (10 000 units/ml; Invitrogen) and grown at 37°C, 5% CO₂, in a humidified atmosphere.

Constructs, transfections and telomeric fragmentation procedure

The constructs containing or not a telomeric seed are described in Ottaviani *et al* (2009). Linearization downstream of the 1.2 kb (TTAGGG)_n seed at the *Bst*XI site (B) allows the non-targeted

introduction of cloned telomeres into mammalian cells. Details are available on request. The conditions of transfection of the linearized vectors with a modified calcium phosphate method (Koering *et al*, 2002) were optimized for each construct to obtain a single integration per cell. Three days after transfection, Hygromycin B is added to the medium. The percentage of eGFP-positive cells and the average level of eGFP is monitored by flow cytometry (FACS) (data not shown). Successful *de novo* formation of eGFP-tagged telomeres and single integration were confirmed in the polyclonal population of transfected cells and in a set of clones by FISH on metaphase spreads (Figure 1A, photographs 1, 2; Supplementary Table S1) and by detection of a diffuse hybridization signal in Southern blot (data not shown).

RNA interference

We used pre-annealed small interfering RNAs (siRNAs): siGENOME SMARTpool reagent, human *CTCF* (M-020165-01); human *LMNA* (NM_170707), Silencer Negative Control #1 siRNA (Ambion). Transfections were performed with DharmaFECT 1 (Dharmacon) with 200 pmoles siRNA for 2×10^5 cells.

3D immuno-FISH

To detect our constructs, the pCMV vector was used as a probe and labelled with the DIG-Nick Translation Kit (Roche Diagnostics). TelVysion 4q and 10q probes (Vysis) and Star FISH X-painting (Cambio) were used for the detection of natural chromosomes. All probes were denatured at $80 \pm 1^\circ\text{C}$ for 5 min before hybridization. Conditions for slides preparation, hybridization and immunodetection are available on request. For detection, we used mouse anti-DIG antibodies (Roche Diagnostics), rabbit anti-Lamin A/C antibodies (H110, Santa-Cruz) and goat anti-Lamin B antibodies (M-20, Santa-Cruz), all diluted 1/50, followed by incubation with secondary donkey antibodies coupled with different ALEXA fluorochromes, diluted 1/300 (Molecular Probes). Nuclei were counterstained with DAPI (Sigma) diluted to 1 $\mu\text{g}/\text{ml}$ in PBS and mounted in Vectashield (Vector Laboratories).

Images were acquired with the confocal scanning laser system, LSM510, from Zeiss (Germany). A $63 \times$ Plan-APOCHROMAT, oil immersion, NA 1.40 objective (Zeiss) was used to record optical sections at intervals of 0.48 μm . The pinhole was set the closest to 1

Airy with optical slices in all wavelengths with identical thickness (0.8 μm). Images were averaged four times to improve the signal to noise ratio. Generated .ism files had a voxel size of $0.1 \mu\text{m} \times 0.1 \mu\text{m} \times 0.48 \mu\text{m}$ and were processed through the Imaris software (Bitplane AG). After 3D analysis, data sets were presented as the distribution of FISH signals between three concentric zones of equal volume or as the mean ratio between two volumes. *P*-values were obtained by performing non-parametric statistical tests (Statistics were done with R. <http://www.R-project.org>). After Kruskal-Wallis tests, subsequent pairwise comparisons were performed using the Mann-Whitney test and the significance threshold ($\alpha = 0.05$) was corrected for multiple comparisons using the Simes method for false discovery rate (FDR) determination. Regular Mann-Whitney test was used to compare knock-down with control conditions.

Supplementary data

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

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Conflict of interest

The authors declare that they have no conflict of interest.

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