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Purification of Proteins Associated with Specific Genomic Loci

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SUMMARY

Eukaryotic DNA is bound and interpreted by numerous protein complexes in the context of chromatin. A description of the full set of proteins that regulate specific loci is critical to understanding regulation. Here, we describe a protocol called proteomics of isolated chromatin segments (PICh) that addresses this issue. PICh uses a specific nucleic acid probe to isolate genomic DNA with its associated proteins in sufficient quantity and purity to allow identification of the bound proteins. Purification of human telomeric chromatin using PICh identified the majority of known telomeric factors and uncovered a large number of novel associations. We compared proteins found at telomeres maintained by the alternative lengthening of telomeres (ALT) pathway to proteins bound at telomeres maintained by telomerase. We identified and validated several proteins, including orphan nuclear receptors, that specifically bind to ALT telomeres, establishing PICh as a useful tool for characterizing chromatin composition.

INTRODUCTION

Despite substantial characterization efforts, chromosomes remain poorly understood cellular organelles (Kornberg and Lorch, 2007), in part due to an inability to purify given chromatin segments in a manner that would allow the identification of bound factors. During the past 25 years, various chromatin isolation strategies have been pursued to establish locus-specific protein composition (Boffa et al., 1995; Ghirlando and Felsenfeld, 2008; Griesenbeck et al., 2003; Jasinskas and Hamkalo, 1999; Workman and Langmore, 1985; Zhang and Horz, 1982). While each achieved enrichment of the targeted region, none gave material of sufficient amount and purity to allow identification of bound factors.

Other methods have been developed that connect specific DNA sequences to the proteins that directly associate with them. These include yeast one-hybrid (Li and Herskowitz, 1993) and nucleic acid affinity capture (Kadonaga and Tjian, 1986), which identify sequence-specific DNA-binding proteins. These methods either use a “bait” DNA sequence outside of its endogenous context, or an in vitro capture approach. While these methods are useful, they do not provide a complete description of what is found at the loci in vivo. Chromatin immunoprecipitation (ChIP) is a powerful technology to assess whether a protein of interest is bound to a given genomic region. ChIP relies on the use of antibodies and thus is limited to analysis of the factors that are tested and does not establish a complete description of composition.

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SUPPLEMENTAL DATA

Supplemental Data include three figures and three tables and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(08)01520-1.

To gain insight into locus-specific composition, we sought to develop a strategy to purify an endogenous segment of chromatin in sufficient quantity and purity to identify the associated proteins. Such a technology would permit a detailed correlation between composition at a locus and phenotype, leading to a deeper understanding of chromosome biology. We wanted this method to be direct, relatively quantitative, and achievable without the requirement for genetic engineering. Since the DNA sequence provides a universal means of discriminating a specific chromatin locus from others, we used nucleic acid hybridization as the basis for purification. We demonstrate below that we are able to isolate specific formaldehyde-crosslinked chromatin regions and identify the proteins bound to those loci using mass spectrometric analysis (MS). We call this method *p*roteomics of *i*solated *ch*romatin segments (PICh) because it uses the DNA to retrieve the protein information; this is opposite to ChIP, which uses protein antigens to capture the associated DNA.

RESULTS

PICh Strategy

To determine whether a protein of interest is localized to a specific genomic region, a common approach is to combine immunostaining and DNA in situ hybridization (immuno-FISH) on fixed nuclei. We sought to develop a biochemical variation of this method to affinity purify the proteins from a genomic chromatin locus using nucleic acid hybridization and use MS to identify the associated factors (PICh, see Figure 1). Initial attempts to retrieve the target chromatin using conventional DNA capture/FISH probes and common FISH reagents suffered from very low yields and high levels of contamination from nonspecific proteins (data not shown).

To maximize the specific enrichment of telomere-associated proteins, optimization of the PICh procedure was necessary. The resulting protocol is outlined in Figure 1. In brief, cells were fixed, the chromatin was solubilized, a specific probe was hybridized to the chromatin, the hybridized chromatin was captured on magnetic beads, the hybrids were eluted, and the associated proteins were identified. Extensive crosslinking with formaldehyde was used to preserve protein-DNA and protein-protein interactions. Unlike strategies based upon antibody-antigen affinity, nucleic acid hybridization is insensitive to the presence of ionic detergents (Rose et al., 2002), which allows the use of these detergents throughout to limit contamination. To increase the stability of the probe-chromatin interactions, we used *l*ocked *n*ucleic *a*cid (LNA) containing oligonucleotides as probes because LNA residues have an altered backbone that favors base stacking thereby significantly increasing their melting temperature (Vester and Wengel, 2004). To minimize the steric hindrance (detrimental for yields) observed upon immobilization of chromatin (Griesenbeck et al., 2003; Sandaltzopoulos et al., 1994), we used a very long spacer (Morocho et al., 2005) between the immobilization tag and the LNA probe. Finally we limited the coelution of nonspecific factors by using desthiobiotin, a biotin analog with weaker affinity for avidin, permitting a competitive gentle elution using biotin (Hirsch et al., 2002).

Use of Telomeric Chromatin as a Proof-of-Principle

To establish this technology we chose to look at telomeres. Telomeres are an attractive target to test PICh because they are abundant. There are approximately 100 telomeres per cell, thus reducing the amount of starting material required to obtain sufficient purified material for analysis. Moreover, since telomeres are of significant biological interest they have been well characterized, allowing us to validate the technology. Telomeres must be maintained to support continued cell division, a characteristic feature of stem cells and cancer cells (Stewart and Weinberg, 2006). Maintenance is normally achieved by the activation of telomerase, a reverse-transcriptase able to compensate for sequence loss

inherent to linear DNA replication. In a subset of cancers (primarily those of mesenchymal origin) and in several in vitro immortalized cell lines, telomeres are maintained in the absence of telomerase by an *a*lternative *l*engthening of *t*elomeres pathway (ALT) that involves a poorly understood mechanism based on recombination (Cesare and Reddel, 2008). ALT telomeres exhibit heterogeneous length and can be found associated with PML nuclear bodies (PML-NBs) for unknown reasons (Molenaar et al., 2003). We used PICh to compare composition at ALT telomeres with composition at telomerase-maintained telomeres. These data were used to validate PICh by asking whether PICh could (1) identify the majority of previously characterized associations with these telomeres and (2) identify novel proteins whose association with telomeres could be verified using independent means, including fluorescence microscopy.

PICh Reveals Telomere Composition

We used PICh on three human cell lines with two distinct types of telomere: two *HeLa* clones that are telomerase positive and show different telomere length (*S3* and *1.2.11*) and the *WI38-VA13* ALT cell line (Figure 2A). We used a probe designed to hybridize with telomere sequences and, as a control, a probe with the same base composition but in a scrambled order (referred to as “scrambled” below). Among the hits not present in the scrambled purification, PICh identified 210 proteins associated with *HeLa 1.2.11* telomeres and 190 proteins associated with ALT telomeres of the *WI38-VA13* cells (see Tables S1 and S2). Consistent MS results were obtained in replicate purifications (86.3%, i.e., 26 out of 190 proteins were found in only one out of two ALT pulldowns, see Table S2). Ninety-eight proteins (about half) were found at both types of telomeres. These associations were specific to the telomere probe since these proteins were not retrieved when PICh was performed using the scrambled probe (see Table S3).

Most of the proteins previously shown to bind telomeres, including low-abundance proteins such as Apollo (Lenain et al., 2006; van Overbeek and de Lange, 2006), were found in the telomere PICh (see Table 1). In total, 33 proteins previously shown to associate with telomeres were found (Table 1). We failed to identify four known or expected components of telomere chromatin: the Tankyrase 1 poly-ADP ribose polymerase (Smith et al., 1998); the Rad51D helicase (Tarsounas et al., 2004); the Werner Syndrome helicase WRN (Crabbe et al., 2004), although WRN interactors such as the WHIP ATPase and the TERA protein were found; and the SIRT6 deacetylase (Michishita et al., 2008). We also failed to identify the telomerase reverse transcriptase TERT and Dyskerin at *HeLa* telomeres. These proteins are unlikely to represent a constitutive component of telomere chromatin based upon previous analyses so would not be expected to be telomere bound in a significant percentage of cells, especially in these nonsynchronized populations. In any case, lack of detection might reflect low abundance at telomeres or might reflect inefficient crosslinking, although formaldehyde was used by others to demonstrate the presence of certain of these proteins at telomeres (Tankyrase 1, SIRT6, Rad51D, and WRN). We conclude that PICh is robust since a single experiment suffices to retrieve most of the known components of the targeted locus (about 85%). To further validate the technology, we asked whether other proteins present in these preparations are bona fide telomere-associated proteins.

Validation of Telomere Associations

To determine the utility of PICh, novel associations must be demonstrated to be bound to telomeres using independent methods. We present several lines of evidence that suggest that PICh is reliable.

First, we assembled a rank-ordered list of factors identified from the purified material based upon abundance, calculated using the ratio of peptide number to protein size. Among the top

25 scoring proteins from each of the two lists, 18 (*HeLa*) and 17 (*VA13*) are known or expected telomere-interacting proteins. We also detected maintenance type-specific associations (see Tables 1, S1, and S2 and references therein). For instance, protein components of the telomerase holoenzyme (GAR1, NHP2, and NAT10) were only found at *HeLa* telomeres. Their absence at ALT telomeres is consistent with the absence of hTR and hTERT expression in the *VA13* cell line (Bryan et al., 1997). Similarly, the PML protein, the 9-1-1 complex, the MMS21 complex, the BLM helicase, or Topoisomerase III alpha, proteins previously shown to be associated with ALT telomeres (Nabetani et al., 2004; Potts and Yu, 2007; Stavropoulos et al., 2002; Tsai et al., 2006), were only detected in the *VA13* PICh preparations, again confirming that maintenance-specific associations can be identified. Finally, OBFC1 (Ob-fold containing protein 1) is a putative human ortholog of yeast Stn1, a critical telomere-binding protein in this organism. OBFC1 is found at both types of telomeres by PICh, consistent with the anticipated role for this protein at human telomeres (Martin et al., 2007).

We next validated the association of a set of proteins from the list that had not previously been reported to interact with telomeres. As a stringent criterion for validity of the listed proteins, we analyzed two of the five lowest ranked proteins from the ALT list, the Fanconi Anemia Factor J (Fanc-J), a helicase ranking in the last position, and the transcriptional corepressor RIP140. We also analyzed the localization of NXP-2, a protein that ranked in the middle of the ALT list. Consistent with our results from PICh, all three proteins were found to associate with ALT telomeres by coimmunostaining (Figure 2B) but were not found to associate with *HeLa* telomeres (data not shown). While we found some cells harboring a pattern with RIP140 enriched at most telomeres (Figure 2B), this pattern was uncommon. Because this was seen in multiple cells, RIP140 is a valid association.

We then examined Homeobox containing protein 1 (HMBOX1), a putative transcription factor that had not been previously reported as binding to telomeres (Chen et al., 2006) but was identified by PICh in all cells tested (*HeLa S3, 1.2.11*, and *WI38-VA13*; see Tables S1 and S2 and data not shown). This protein is highly conserved in vertebrates but not in lower eukaryotes. Interestingly the two closest related sequences in *C. elegans* retrieved the F54A5.1 and CEH-37 proteins. The latter is known to be telomeric in this organism, which harbors a similar telomeric repeat sequence (TTAGGC) (Kim et al., 2003). Exogenously expressed HMBOX1-Flag colocalized with the telomere marker RAP1 in about 70% of *WI38-VA13* nuclei (Figure 2C). We could detect HMBOX1 at some telomeres in the *HeLa 1.2.11* cell line but in only ~10% of nuclei, with the majority of staining being nontelomeric. Thus we cannot rigorously exclude the possibility that it represents a false positive from the *HeLa* PICh. However, its MS identification at both types of telomeres and telomeric localization observed by immunostaining of a majority of *VA13* nuclei suggest that it is a true association in both cell lines.

Therefore, of the eight novel telomere-associated proteins identified by PICh that we tested (including four described below), seven showed clear association and one (HMBOX1) showed ambiguous but likely telomere association. This indicates that the false positive rate for telomere proteins identified by PICh is likely to be low.

Orphan Receptors at ALT Telomeres

Shelterin proteins and histones are expected to be the most abundant components at long telomeres because they play a structural role at that locus. In addition to these proteins in the ALT telomere preparations, we were surprised to identify orphan receptors. The two most abundant of these receptors were of the NR2 class: COUP-TF2 (#17) and TR4 (#18), which both had ~50% coverage by MS sequencing, thus ranking higher than two shelterin proteins, POT1 and TPP1 (see Table S2). Other NR2 proteins were also identified: COUP-TF1

(#118), EAR2 (#34), and TR2 (#99). These proteins belong to the nuclear hormone receptor superfamily and are well characterized transcription factors. It is unlikely that they originate from contaminating promoter elements adjacent to telomeres since these factors were not found at *HeLa* telomeres (short or long). We were intrigued that these factors were highly enriched at regions that have not been described as containing classical promoters.

The specificity for these associations was confirmed by ChIP using antibodies against COUP-TF2 and TR4 (Figure 3A) and by immunoblot analysis on PICh-purified telomeres (Figure 3B). Moreover these proteins were specifically enriched at telomeres compared to other nuclear regions, as shown by immuno-FISH and costaining experiments in the *WI38-VA13* (ALT) cell line (Figure 3D). COUP-TF1 was also found at ALT telomeres by costaining but at a lower frequency, consistent with it having significantly lower coverage in the MS (Figure 3D; Table S2). In contrast, none of these proteins could be detected at *HeLa* telomeres (see Table S1; Figures 3A and 3B). By immunoblot, we found that, while COUP-TF2 and TR4 are expressed at higher levels in the ALT cell line, COUP-TF1 is more abundant in *HeLa* cells (Figure 3C) and yet is not found at telomeres. The specific association of orphan receptors with ALT telomeres thus does not appear to reflect differences in expression levels in the cell lines tested but rather an association that is specific to ALT telomeres.

To determine if these associations are unique to the particular ALT cell line analyzed, we performed immuno-FISH experiments in two other ALT cell lines, *U2-OS* and *Sa-OS-2*. In contrast to the *WI38-VA13* cell line originating from SV40 immortalization in vitro, *U2-OS* and *Sa-OS-2* are human osteosarcoma tumor cell lines. Both COUP-TF2 and TR4 were detected at telomeres in *U2-OS* cells while only TR4 could be observed at *Sa-OS-2* telomeres (see Figure S1). We suggest that orphan receptor association with telomeres might be a common feature of ALT cells since this was detected in three distinct ALT cell lines.

Since a growing number of orphan receptors, including TR2 (Park et al., 2007), are shown to be targeted to the PML-NB or to discrete nuclear bodies (Chalkiadaki and Talianidis, 2005; Chen et al., 2004; Wu et al., 2002), their presence at ALT telomeres could be due to the nonspecific crosslinking of orphan receptors to telomeres localized at the PML-NB. We found, however, that orphan receptors could be found associated with telomeres in the absence of any PML signal (arrowheads in Figure S1), arguing against this possibility.

Furthermore, we examined colocalization of orphan receptors and telomeres using cells in metaphase. This experiment also addresses the possibility that orphan receptors associate with *e*xtra*c*hromosomal *t*elomere *r*epeats (ECTR). ALT cells are characterized by the presence of extrachromosomal telomere DNA repeats (ECTR) of unknown function (Tokutake et al., 1998). Despite the observation that the VA13 cells that we used have negligible amounts of ECTR (Ford et al., 2001; Tokutake et al., 1998), the recent proposal that ECTR localizes at PML bodies (Fasching et al., 2007) raised the formal possibility that orphan receptors are associated only with ECTR. If that were true, they would not be expected to be colocalized with telomeres at the ends of chromosomes. Immunostaining of metaphase chromosomes revealed prominent signals for orphan receptors colocalized with shelterin proteins at the tips of chromosomes (Figure 4). We observed a similar pattern of chromosomal tip localization for orphan receptors in the *U2-OS* cell line (not shown). We conclude that orphan receptors associate with telomeric DNA located at the tips of chromosomes, leading us to wonder what impact these nuclear receptors might have on ALT telomere maintenance.

Orphan Nuclear Receptor Proteins May Underlie Telomere PML-NB Colocalization

It has been proposed that a critical step in ALT maintenance occurs in the PML-NB because disruption of PML expression suppresses ALT (Jiang et al., 2007), and several proteins potentially involved in telomere recombination are localized at PML-NBs in ALT cells (Henson et al., 2002). We wondered if the binding of orphan receptors to variant sequences known to be at telomeres (see Discussion) might cause relocalization of telomeres to PML bodies and thus might impact telomere maintenance. According to the PICh data there are at least five different orphan receptors bound to telomeres. Knocking down the expression of all five of these proteins at the same time is technically challenging, so we examined whether knocking down the expression of the highest ranked associated protein (COUP-TF2) would produce an effect on the localization of telomeres to PML-NBs.

Using shRNA, we derived stable *WI38-VA13* cell lines in which *COUP-TF2* had been knocked down. In the absence of knockdown, an average of 80%–90% of telomeres (as identified by TRF2 immunostaining) are colocalized with the PML protein. In the clones in which COUP-TF2 levels were reduced (Figure S2A), telomeres were significantly delocalized from PML-NB (~60% colocalization, see Figure S2B). We conclude that *COUP-TF2* expression levels are related to telomere-PML-NB association frequency in the *WI38-VA13* cell line.

To address whether delocalization of telomeres from PML-NB impacts telomere maintenance, we measured telomere length in the orphan receptor-depleted cell lines. A subtle shortening of telomeres was observed in the two cell lines that also showed knockdown of *COUP-TF2*, as indicated by the smearing down of telomere restriction fragments from these cell lines (Figure S2C). Despite this phenotype, we did not notice an obvious proliferation defect. However, this analysis is complicated by the observation that *TR4* becomes upregulated in many of these stable cell lines (Figure S2A) and by the appearance of revertants in the cellular population (data not shown). Our results are consistent with a possible redundant function of orphan receptor action at telomeres. A full phenotypic effect (total disruption of PML-NB association and thus ALT maintenance) might require knocking down multiple orphan receptors found at *VA13* telomeres. Nonetheless, we conclude that there is a subtle but measurable impact on telomeres caused by knocking down COUP-TF2, offering further validation for a genuine association of this family of receptors with ALT telomeres.

Insights into Telomeric Chromatin Structure

In addition to providing information regarding the identity of proteins bound to a locus, PICh provides information on the relative levels of abundant proteins bound to a given sequence in distinct cell types. For example, in the process of developing PICh for use on telomeres, we noticed fewer histones in telomeric preparations from *HeLa S3* cells than in telomeric preparations from *HeLa 1.2.11* cells (Figure 2A). We pursued this finding because of its possible relationship to the silencing of subtelomeric regions, known as *t*elomere *p*osition *e*ffect (TPE), which increases with the length of telomeres (Baur et al., 2001). TPE is a classic example of gene silencing mediated by chromatin. Indeed histone modifications and chromatin structure have been suggested to play a major role in TPE in mammals (Blasco, 2007). In addition, we were interested in validating the efficacy of PICh in measuring protein level.

The telomeres from *HeLa 1.2.11* cells are longer than those in *S3* cells (20 kb on average versus <5 kb). Thus, the observation that the shorter telomeres have less histones might reflect a difference in nucleosome density or might simply reflect the fact that there is less total telomeric material in *S3* cells. When we loaded the same amount of shelterin proteins,

expected to represent an equal loading of telomeric DNA (Loayza and DeLange, 2003), we observed greater nucleosome signal at the longer telomeres (Figure 5A). ChIP with an anti-histone H3 antibody (Figure 5B) confirmed the lower nucleosomal density at *HeLa S3* telomeres (about 2.5-fold less nucleosomes/kb of telomeric DNA). Moreover, immunoblots performed on PICh purified telomere material, normalized for shelterin abundance, also confirmed the paucity of nucleosomes at the short *HeLa S3* telomeres (Figure S3A).

This depletion of nucleosomes might explain the previously observed smeary MNase digestion of short human telomeres, while longer telomeres show a more discrete pattern (Tommerup et al., 1994). These results suggest that, in the size range analyzed here, nucleosomal density increases with telomere length, consistent with the greater TPE observed with longer telomeres (Baur et al., 2001). This is also consistent with our failure to detect HP1 proteins at *HeLa S3* telomeres, while these factors were readily identified in the other cell line harboring longer telomeres. We hypothesize that a close relationship exists between histone density, heterochromatin protein associations, telomere length, and TPE. Importantly, these data indicate that nucleosomal chromatin is not refractory to PICh.

In addition to canonical histones H3, H2A, H2B, and H4, histone variants macroH2A and testis-specific H2B were identified by PICh in the cell lines that contain longer telomeres, *HeLa 1.2.11* and *WI38-VA13*. We confirmed the presence of macroH2A in these telomere preparations by western blot on PICh material (Figure S3B). We noticed an underrepresentation of the linker H1 (Figure 2A, compare the input with the purified telomere lanes), consistent with previous findings suggesting that macroH2A and linker H1 may not coexist at specific loci (Abbott et al., 2005). We confirmed the paucity of histone H1 at telomeres using ChIP to compare the relative abundance of H1 to that of H3 at telomeres or at Alu repeats (about 2.5- to 3-fold less, Figure 5C). The underrepresentation of H1 in the telomeric nucleosomal arrays might explain the ~40 bp shorter nucleosomal repeat length previously observed at telomeres (Lejnine et al., 1995). We conclude that for ubiquitous chromatin proteins expected at most loci such as histones, PICh reflects relative abundance of bound proteins and provides insights into chromatin structure.

DISCUSSION

PICh as a Tool for the Characterization of Chromatin Composition

The identification of the proteins that interact with genomic regions of interest is critical to the understanding of genome biology. These questions have primarily been studied using genetics, biochemical characterization of soluble complexes, structural studies, chromatin immunoprecipitation, and cell biology. These methods are powerful but do not provide information concerning the complete composition of a locus. By establishing the “chromatin formula” of factors bound at specific loci, PICh has the potential to advance the characterization of chromosomes by providing a method to examine the entire set of interacting proteins and how composition changes during regulation. We used PICh to study telomeres and identified most previously known factors and also new proteins that might be relevant to telomere biology. These studies validate the potential of PICh.

A major limitation for PICh is the need for a sufficient amount of purified protein for identification by MS, which can require starting with large amounts of material. Using conservative estimates, a standard MS requires about a picomole of a given protein. If the purification of a locus bound by a single protein is considered and if that locus is unique, MS necessitates at least half a picomole of diploid cells, amounting to several hundreds of liters of a standard mammalian cell line. To circumvent issues related to handling such large amounts of cells, we chose to target human telomeres, which represent a relatively abundant locus. Focusing on human telomeric chromatin decreases the need for starting material by

about 50-fold, as compared to single copy loci of similar individual size, since each chromosome has two telomeres. In terms of relative abundance, telomere sequences constitute 0.01% to ~0.07% of the genome in each cell. A technology that works on this type of target can immediately be applied to other repeat sequences or low-copy elements found in smaller genomes. For example, 0.01% corresponds to a 1.2 and 12 kb locus in the yeast and fly genomes, respectively, indicating that PICh as described here might be suitable for the purification of single copy segments in those organisms. Subsequent optimization might extend the use of PICh to smaller segments or low repeat/single copy elements in mammalian genomes.

PICh relies on nucleic acid hybridization to crosslinked chromatin and thus has the advantage that no genetic engineering is required. There are, however, potential limitations. Avoiding crosshybridization to other genomic regions is an issue that requires a careful capture probe design. Moreover, our failure to identify a few of the known telomere interactors suggests that either these factors are not abundant at telomeres or these factors fail to crosslink stably. We used formaldehyde, a 2 Å crosslinker, in this study; the use of other crosslinking compounds might help resolve such issues by capturing loosely bound, but biologically important, proteins.

Complexity of Proteins Found at Telomeres

The relatively high number of proteins found at each class of telomeres (~200) underscores the diversity of events occurring at this locus. While it is possible that telomeres are continually bound by a large number of proteins, this diversity might more likely be caused by each individual telomere harboring distinct composition at a given time. The cells used for these experiments were not synchronized, and it is therefore expected that PICh retrieved telomeres during various phases of the cell cycle: for instance, the bi-modal distribution of Fanc-J in ALT cells detected by immunflourescence (Figure 2B) might reflect this and might explain its weak MS detection in our samples. The results from PICh as performed here, therefore, give an average composition of this locus. The use of synchronized cultures and PICh might be informative in this regard.

It is inevitable that some proteins retrieved by PICh are bound throughout the genome and thus do not play a specific role at telomeres. For example, replication factors presumably fall into this category, although their enrichment at telomeres might also have a specific biological meaning (Gilson and Geli, 2007). On the other hand, PICh has the ability to identify factors that would be difficult to uncover using genetics because they either play vital roles elsewhere or are redundant (as might be the case for orphan receptors). It will be interesting to purify telomeres from more sources to correlate variations in the telomere formula with phenotypic changes. The purification of telomeres from other organisms will also be critical for a greater understanding of telomere biology since fundamental protection or maintenance mechanisms seem to be conserved.

Orphan Receptors at ALT Telomeres

Numerous proteins were seen that were specific to ALT telomeres, some of which were expected based upon previous literature (e.g., proteins involved in recombination) and some of which were unexpected (e.g., orphan receptors). We focused on the interaction between ALT telomeres and a subfamily of orphan nuclear receptors as characterizing this unexpected finding was important to validating the efficacy of PICh. The presence of orphan receptors at ALT telomeres might be viewed as a by-product of the ALT pathway (e.g., promoted by the deprotection of telomeres) or as one of the causes for ALT. We found that knocking down COUP-TF2, one of several orphan receptors, had a measurable impact on association of ALT telomeres with PML bodies and on overall telomere length (Figure S2).

While this is consistent with a potential effector role for orphan receptors in ALT maintenance, more work is needed to test this hypothesis.

Why might orphan receptors bind to telomeres? The proximal ~2 kb of human telomeres harbor variant sequences, interspersed with the normal TTAGGG repeats or other telomere variant motifs (Baird et al., 1995). One of these variant sequences, the “C-type” variant hexanucleotide repeat (TCAGGG), creates a high-affinity binding site for orphan receptors (Figure 6). It is possible that ALT telomeres are enriched in this motif. Alternatively, these sites could pre-exist in non ALT cells but be occluded by chromatin regulators (Benetti et al., 2007). The mechanisms that promote orphan receptor binding and function at ALT telomeres require further investigation.

While PICh has been proven effective for the characterization of the composition of telomeres, its applicability extends to other loci. We have used PICh to purify mouse pericentric heterochromatin and have obtained a distinct “protein formula” (data not shown). This approach can be used to characterize other nucleoprotein complexes, including nonchromatin targets. Although technically challenging, we hope to extend PICh to single copy elements. The ability to identify proteins bound to a given regulatory sequence based solely upon the identity of that DNA sequence will allow the unbiased discovery of regulatory interactions at key genomic loci.

EXPERIMENTAL PROCEDURES

Telomeric Chromatin Isolation by PICh

Chromatin Sample Preparation—From 20 l *HeLa S3* cell equivalent (~10^6 cells/ml): cells were crosslinked in PBS–3% formaldehyde for 30 min at room temperature (RT) and washed four times in PBS. Cells were then equilibrated in sucrose buffer and dounced 20× with a tight pestle. Cells were equilibrated in glycerol buffer and pellet was collected following centrifugation at 3200 g for 10 min at 4°C. The pellet was resuspended into the same volume of glycerol buffer. The pellet was frozen into liquid nitrogen and stored at −80°C or used immediately for the next step.

The following volumes and numbers are given for one purification (that is ~3×10^9 cell equivalent): the material was centrifuged at 2000 g for 2 min at RT and the pellet was resuspended into the same pellet volume of 1× PBS—0.5%Triton X-100 and 90 μl of RNaseA (QIAGEN 100 mg/ml) were added. The mixture was incubated for 60 min at RT with shaking then at 4°C for 12–16 hr and was washed six times in PBS. The material was equilibrated in LBJD solution and the pellet was resuspended into 55% pellet volume of LBJD solution. Samples were sonicated (Micro-tip, Misonix 3000) using the following parameters: Power setting 7 (36–45 Watts), 15 s constant pulse, and 45 s pause for a 7 min total process time. Sample was collected by centrifugation at 16000 g for 15 min at RT. Chromatin sample was then applied to Sephacryl S-400-HR spin columns and incubated at 58°C for 5 min.

LBJDLS pre-equilibrated streptavidin beads were added (Pierce Ultralink streptavidin, 0.5 ml) and the sample was incubated for 2 hr at RT. Beads were discarded and supernatant was saved.

Chromatin Capture—Samples were centrifuged 15 min at 16,000 g at RT and 1/100th final volume of 20% SDS was added together with the LNA probe (1 μM final concentration).

Hybridization was conducted as follows (25°C for 3 min, 70°C for 6 min, 38°C for 60 min, 60°C for 2 min, 38°C for 60 min, 60°C for 2 min, 38°C for 120 min, 25°C final temperature). The sample was centrifuged at 16000 g for 15 min and the supernatant was diluted twice with milliQ water and the LBJD pre-equilibrated MyONE C1 beads solution was added (typically 0.6–1 ml). The sample was incubated for 12 hr at RT before increasing the volume to 10 ml with LBJD. Beads were washed seven times with 10 ml with LBJD at RT and two times with 1 ml with LBJD at 42°C. Beads were resuspended into 1 ml of elution buffer.

The sample was incubated for 1 hr at RT with shaking and temperature was raised to 65°C for 10 min. The eluate was precipitated using tri-chloroacetic acid (18% final) and the pellet was resuspended into 50 μl of crosslinking reversal solution.

The sample was incubated at 99°C for 25 min. The proteins were separated using a 12% Bis-Tris acrylamide pre-cast gel (Invitrogen) or stored at −80°C.

The proteins were revealed using the SilverQuest staining kit (Invitrogen) or Colloidal Blue (Invitrogen) according to manufacturer's instructions.

Colloidal blue-stained gels were used for subsequent MS analysis. Laneswere sliced in regions according to banding pattern and submitted to MS analysis.

Buffers Composition for PICh

PBS: 8 mM Na_2HPO_4; 2 mM KH_2PO_4; 137 mM NaCl; 2.7 mM KCl. Sucrose buffer: 0.3 M Sucrose; 10 mM HEPES-NaOH, pH 7.9; 1% Triton X-100; 3 mM $CaCl_2$; 2 mM MgOAc. Glycerol buffer: 25% glycerol; 10 mM HEPES-NaOH, pH 7.9; 0.1 mM EDTA; 0.1 mM EGTA; 5 mM MgOAc. LBJD: 10 mM HEPES-NaOH, pH 7.9; 100 mM NaCl; 2 mM EDTA, pH 8; 1 mM EGTA, pH 8; 0.2% SDS; 0.1% Sarkosyl, protease inhibitors. LBJDLS: 10 mM HEPES-NaOH, pH 7.9; 30 mM NaCl; 2 mM EDTA, pH 8; 1 mM EGTA, pH 8; 0.2% SDS; 0.1% Sarkosyl, protease inhibitors. Elution buffer: 12.5 mM Biotin (Invitrogen cat#B20656), 7.5 mM HEPES-NaOH, pH 7.9; 75 mM NaCl, 1.5 mM EDTA, pH 8; 0.75 mM EGTA, pH 8; 0.15% SDS, 0.075% Sarkosyl. Crosslinking reversal solution: 250 mM Tris, pH 8.8, 2% SDS, 0.5 M 2-mercaptoethanol. Capture probes are as follows:

Telomere: Desthiobiotin-108Carbons-5′TtAgGgTtAgGgTtAgGgTtAgGgt-3′

Scramble: Desthiobiotin-108Carbons- 5′GaTgTgTgGaTgTggAtGtGgAtg Tgg-3′

where capitalized letters are LNA residues and small letters are DNA residues. Synthesis was performed by Fidelity Systems.

Chromatin Immunoprecipitations

ChIP assays have been performed essentially as described (Lee et al., 2006). Antibodies used for ChIP were anti-H3 (abcam 1791), anti-H1 (clone AE-4, Santa Cruz), anti-TR4 (Perseus Proteomics), and anti-COUP-TF2 (abcam ab50487). Immunoprecipitated DNA was spoted on a slot-blot apparatus and probed with a telomere-specific probe or an Alu-specific probe. Error bars represent standard deviation (SD) of enrichments values obtained from independent experiments.

Cell lines, Culture Conditions, shRNA Treatment, and Plasmid Constructs

HeLa S3 cells were grown in Joklik's modified MEM with 5% Calf Serum by the National Cell Culture Center. *HeLa 1.2.11*, *WI38-VA13*, *U2-OS*, and *Sa-OS2* were cultured in DMEM, 10% Calf Serum.

The HMBOX1 clone was obtained from the Invitrogen Human Ultimate ORF collection and cloned in-frame with Flag in a pEGFP vector (Clontech) lacking the GFP sequence. Flag sequence was introduced by PCR using the following primer pair: 5′HMBOX1: AAGCTTCACCATGCTTAGTTCCTTTCCAGTGG and 3′HMBOX1-FLAG: GCGGCCGCTCACTACTTGTCGTCATCGTCCTTGTAGTC AGCAGCAGCGTCATCATCCAGGGCCTCTGTCTTGATTTC. The PCR product was ligated into a pGEM-T easy vector and sequenced. The gene was then inserted into the pEGFP vector at HindIII-NotI sites.

shRNA constructs were obtained from Sigma (Mission shRNA collection). Of the five sequences tested by transient transfection assays, only the following was efficiently knocking down COUP-TF2 expression (COUP-TF2.1): CCGGG CCGTATATGGCAATTCAATACTCGAGTATTGAATTGCCATATACGGCTTTTT. Control shRNA is the scramble sequence available from Sigma.

Microscopy

Cells were grown in two chambers slides (Lab-TekII, Nunc). Cells were washed in PBS and fixed in PBS containing 1% Triton X-100 and 3.7% formaldehyde for 5 min. After two washes in PBS, cells were blocked in PBS-10% milk-3% BSA containing 0.2% Tween and 0.2% NP-40 for 1 hr at RT. Incubation with primary antibody was in blocking solution for 2 hr at RT or overnight at 4°C. Two 15 min washes were performed after antibody incubation using PBS solutions containing 0.2% Tween, 0.2% NP-40 and 300, then 400 mM NaCl. Secondary antibodies (conjugated with Cy2, Cy3, or Cy5) were from Jackson laboratories. Slides were counterstained with DAPI and mounted in Mowiol. Antibodies used in this study are RAP1 1:40 (Abcam, ab4181), TRF2 1:40 (Santa Cruz H300 or Abcam ab13579), PML 1:100 (Santa Cruz, PGM3 or H238), COUP-TF2 1:100 (Abcam ab50487), TR4 and COUP-TF1 1:100 (Perseus Proteomics), Flag 1:1000 (Sigma, M2), Fanc-J 1:100(Sigma), and RIP140 1:100 (Abcam ab 42126). A standard FISH procedure was used whenever mentioned after post-fixation of the antibody staining, using the capture probe at 1 μM in a standard hybridization buffer. An anti-biotin-FITC (Vector-slab) was used to detect probe binding. Metaphase chromosome spreads and staining were performed as described (Sullivan and Schwartz, 1995). Stainings were analyzed using a Zeiss epifluorescence microscope and a Hammamatsu CCD camera. Images were obtained using the Openlab software, and the Photoshop CS software was used to color panels and prepare the figures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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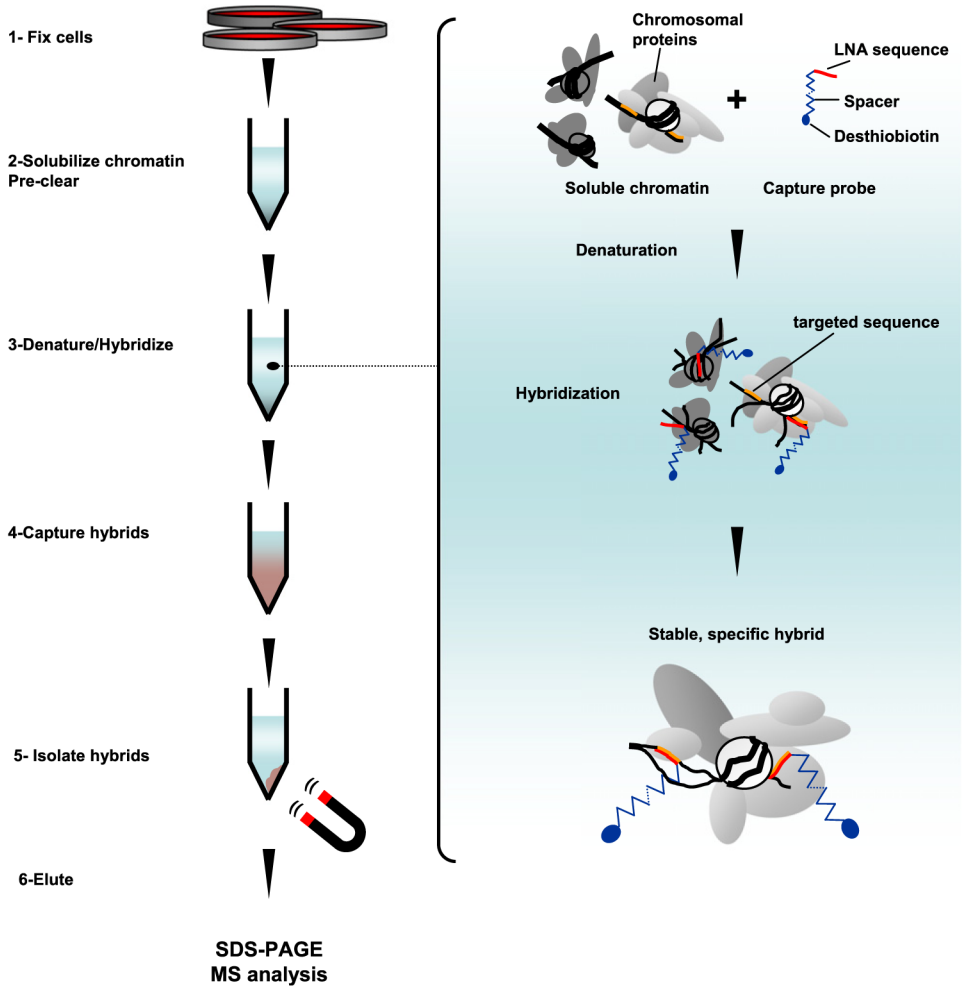

Figure 1.
Outline of the PICh Protocol

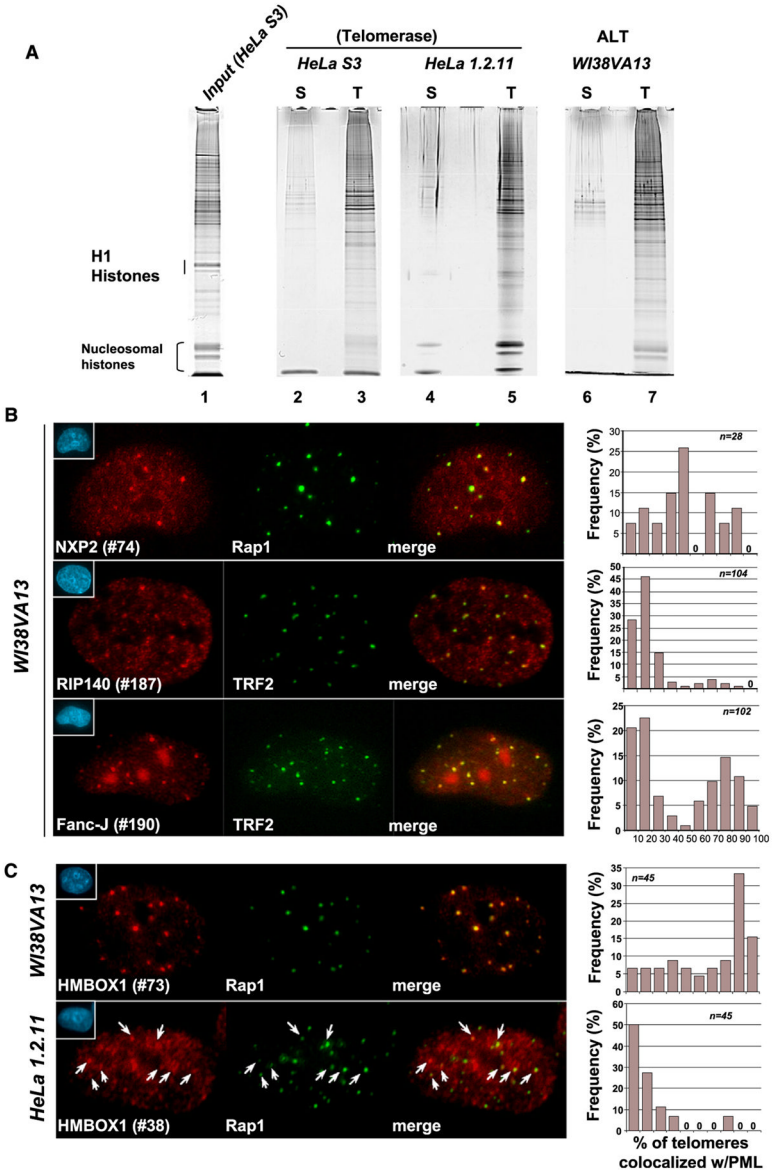

Figure 2. Purification of Telomeric Chromatin from Transformed Human Cell Lines

(A) Silver staining of material obtained from PICh purified telomere chromatin. Purifications from the two HeLa clones (*S3* and *1.2.11*) and the *WI38-VA13* ALT cell line are shown. T: PICh performed with the telomere-specific probe. S: PICh performed with the "scrambled" probe. Input represents 0.001% of the starting material (3×10^4 cell equivalent), and purifications were from 5×10^8 cell equivalents/lane.

(B) Validation of selected PICh associations to ALT telomeres by immunostaining. Protein names are followed by their ranking order in the ALT list. NXP2 was an HA-tagged construct transiently transfected (experiment performed 48 hr post-transfection). Endogenous RIP140 and Fanc-J are detected using specific antibodies.

(C) Coimmunostaining with the Flag-tagged HMBOX1 and RAP1 in the *WI38-VA13* and *HeLa 1.2.11* cell lines (72 hr post-transfection).

(B and C) Quantification on the right is expressed as the fraction of nuclei showing the extent of telomere colocalization with the tested proteins in the analyzed population.

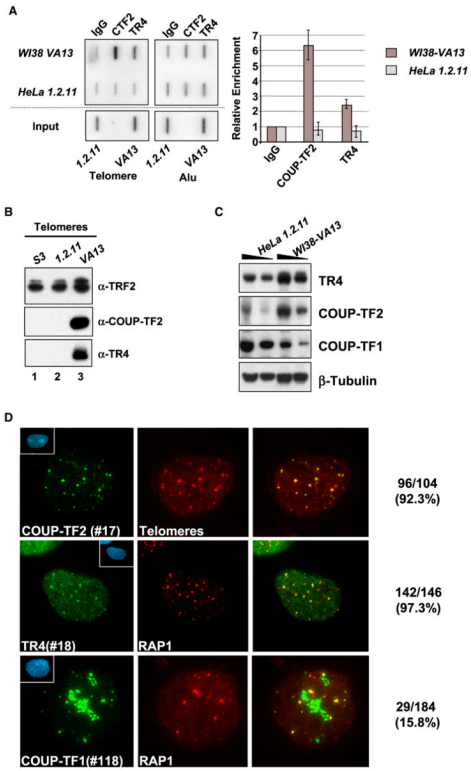

Figure 3. Analysis of COUP-TF2, COUP-TF1, and TR4 Association with WI38-VA13 ALT Telomeres

(A) CHIP with anti-COUP-TF2 and anti-TR4 antibodies. Immunoprecipitated DNA (from *VA13* or *HeLa 1.2.11*) was probed on a slot-blot using a telomere-specific probe (Telomere) or an Alu probe (Alu). Inputs represent 0.002% of starting material. Twenty percent of the IP was loaded. The right panel shows quantification performed from two independent experiments (error bars represent SD from enrichment values). Immunoblots for COUP-TF2 and TR4 on PICh-purified telomeres (6% loaded) are shown.

(B) Western blot analysis for COUP-TF2 and TR4 levels in material purified from the indicated cell lines; TRF2 is a loading control for telomere material.

(C) Immunoblots showing COUP-TF2, COUP-TF1, and TR4 protein levels in *HeLa 1.2.11* or *VA13* cell lysates. β-tubulin is probed to control for loading.

(D) Immuno-FISH (COUP-TF2 and telomere probe) and coimmunostaining of RAP1 and TR4 or COUP-TF1 in *VA13* nuclei. Frequency of colocalization is shown on the right.

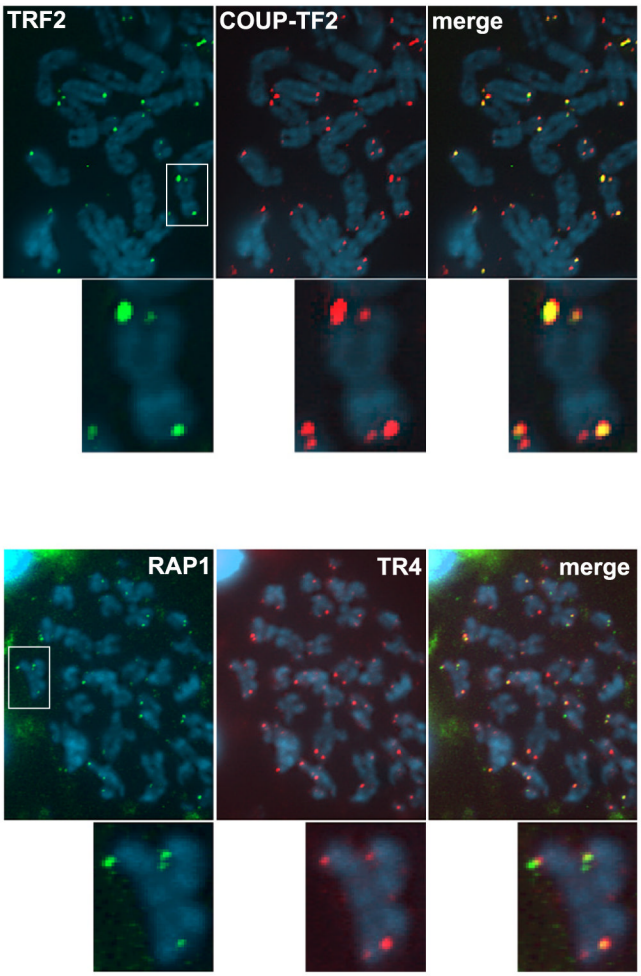

Figure 4. Coimmunostaining of Orphan Receptors with Shelterin Proteins on VA13 Metaphase Spreads

Top panels: COUP-TF2 and TRF2. Bottom panels: TR4 and RAP1.

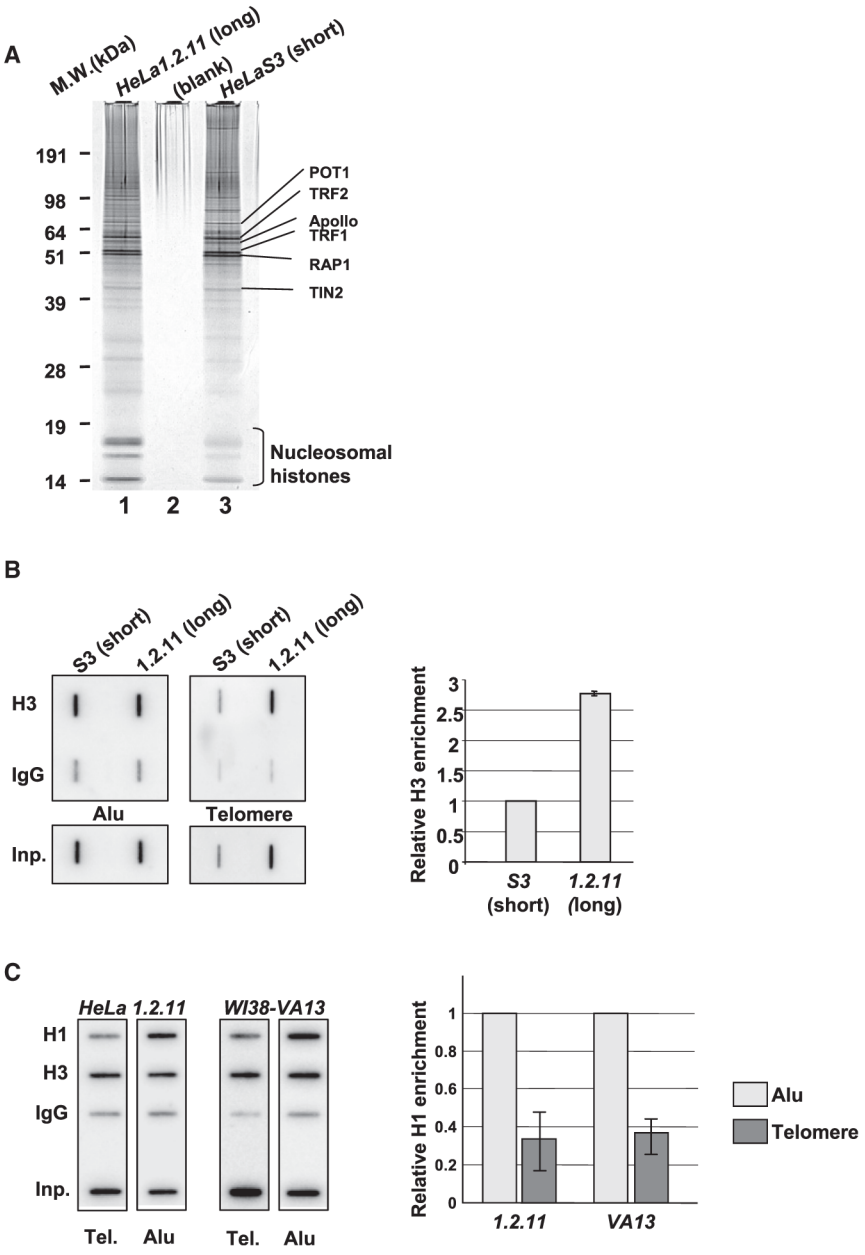

Figure 5. Nucleosomal Density and Composition at Telomeres

(A) similar amounts of shelterins from *HeLa 1.2.11* or *S3* were loaded to compare nucleosomal histone to shelterin signals.

(B) CHIP with an anti-H3 antibody. The immunoprecipitated DNA (from HeLa *S3* or *1.2.11*) was probed on a slot-blot using a telomere-specific probe (Telomere) or an Alu probe (Alu); the panel on the right shows quantitation from two independent experiments. Inputs represent 0.002% of starting material. Twenty percent of the IP was loaded. Right panel shows quantifications normalized to input telomere DNA and H3 IP at Alu repeats (error bars represent SD from enrichment values).

(C) CHIP with anti-H1 and anti-H3 antibodies. Immunoprecipitated DNA (from *VA13* or *HeLa 1.2.11*) was probed on a slot-blot using a telomere-specific probe (Telomere) or an Alu probe (Alu); the panel on the right shows quantification from two independent

experiments (error bars represent SD from enrichment values). Inputs represent 0.002% of starting material. Twenty percent of the IP was loaded. H1 versus H3 enrichments at telomeres are normalized using the signals obtained for these proteins with the Alu repeats.

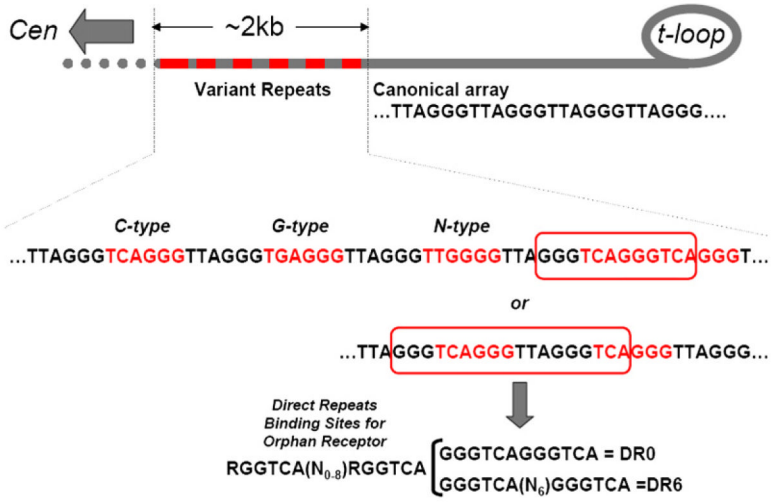

Figure 6. Possible Scenario for Orphan Receptor Binding to ALT Telomeres

R: purine (A or G), DR: direct repeat. A combination of telomere variant repeat may constitute binding sites for orphan receptors, which upon association to telomeres target the locus to PML-NB.

Table 1

Incidence of Known Telomere Proteins Retrieved by PICh

WI38-VA13 (ALT)	HeLa 1.2.11	Both	Not Found
PML	Gar1	TRF1	SMC5 (ALT)
Rad9	NHP2	TRF2	SIRT6
Rad1	NAT10	RAP1	Tankyrase 1
Hus1		POT1	WRN
MMS21		TIN2	Rad51D
SMC6		TPP1	TERT
TOPO 3α		OBFC1	DKC
BLM		Apollo	
		MRE11	
		Rad50	
		NBS1	
		DNA-PKc	
		Ku70	
		Ku86	
		Fen-1	
		RPA	
		CDK1	
		HP1α	
		HP1β	
		HP1γ	
		PARP1	
		SP100 (ALT)	