Chromatin Organization and Remodeling of Interstitial Telomeric Sites During Meiosis in the Mongolian Gerbil (*Meriones unguiculatus*)

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ABSTRACT Telomeric DNA repeats are key features of chromosomes that allow the maintenance of integrity and stability in the telomeres. However, interstitial telomere sites (ITSs) can also be found along the chromosomes, especially near the centromere, where they may appear following chromosomal rearrangements like Robertsonian translocations. There is no defined role for ITSs, but they are linked to DNA damage-prone sites. We were interested in studying the structural organization of ITSs during meiosis, a kind of cell division in which programmed DNA damage events and noticeable chromatin reorganizations occur. Here we describe the presence of highly amplified ITSs in the pericentromeric region of Mongolian gerbil (*Meriones unguiculatus*) chromosomes. During meiosis, ITSs show a different chromatin conformation than DNA repeats at telomeres, appearing more extended and accumulating heterochromatin markers. Interestingly, ITSs also recruit the telomeric proteins RAP1 and TRF1, but in a stage-dependent manner, appearing mainly at late prophase I stages. We did not find a specific accumulation of DNA repair factors to the ITSs, such as γ H2AX or RAD51 at these stages, but we could detect the presence of MLH1, a marker for reciprocal recombination. However, contrary to previous reports, we did not find a specific accumulation of crossovers at ITSs. Intriguingly, some centromeric regions of metacentric chromosomes may bind the nuclear envelope through the association to SUN1 protein, a feature usually performed by telomeres. Therefore, ITSs present a particular and dynamic chromatin configuration in meiosis, which could be involved in maintaining their genetic stability, but they additionally retain some features of distal telomeres, provided by their capability to associate to telomere-binding proteins.

SINCE its identification in human telomeres (Moyzis *et al.* 1988; Meyne *et al.* 1989), the distribution of the repetitive (TTAGGG)_n sequence has been widely studied in a multitude of genomes. These long arrays of highly repeated DNA serve as binding sites for many proteins, mainly the shelterin and telosome complexes (Liu *et al.* 2004; de Lange 2005, 2009), preventing chromosome ends from activating DNA repair pathways through the formation of the T loop and thereby ensuring genome and chromosome stability (Blackburn 1994; de Lange 2005; Palm and de Lange 2008).

Interestingly, telomeric DNA repeats are not restricted only to chromosome ends, but have also been found interstitially along chromosomes (Meyne *et al.* 1990; Nanda and Schmid 1994; Abuín *et al.* 1996; Metcalfe *et al.* 1998). Two kinds of interstitial telomere sites (ITSs) have been defined: short ITSs, usually interspersed in the genome, and large ITSs, mainly located within or surrounding pericentromeric regions. ITSs have been proposed to originate following chromosomal rearrangements and DNA repair events (Azzalin *et al.* 1997, 2001; Lin and Yan 2008; Ruiz-Herrera *et al.* 2008). Large ITSs usually originate after the occurrence of chromosome rearrangements, especially Robertsonian (Rb) translocations, which result after loss of the chromosome end protection in telocentric chromosomes, allowing the fusion of two chromosomes and thus producing a neo-metacentric chromosome that may or may not retain telomeric sequences in the newly formed centromere (Garagna *et al.* 1995; Bouffler 1998). When the telomeric repeats are maintained, they result in ITSs (Hsu

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et al. 1975; Simons and Rumpler 1988; Meyne *et al.* 1990; Garagna *et al.* 1997; Hartmann and Scherthan 2004). Indeed, ITSs are mostly located at the pericentromeric regions of neometacentric chromosomes and, after the Rb fusion, they undergo amplification, allowing the stabilization of the neo-centromere through the formation of pericentromeric heterochromatin (Ruiz-Herrera *et al.* 2008; Rovatsos *et al.* 2011).

Although the role of ITSs in the genome is poorly understood, ITSs in somatic cells are break-prone, resembling fragile sites in the DNA (Slijepcevic *et al.* 1996; Bouffler 1998; Ruiz-Herrera *et al.* 2005). Thus, the ITSs could also correspond to sites of spontaneous and induced chromosome breakage, conferring fragility to the region where they are inserted. At the molecular level, it has been shown that some proteins of the shelterin complex, such as TRF1, TRF2, and RAP1, can also locate to ITSs (Zakian 1995; Mignon-Ravix *et al.* 2002; Krutilina *et al.* 2003; Simonet *et al.* 2011; Bosco and de Lange 2012), suggesting a role of these proteins in the organization and/or functioning of heterochromatic ITSs. In this sense, for example, the shelterin protein TRF1 is fundamental to preventing TTAGGG-repeat replication problems and protecting telomeres from breaking (Sfeir *et al.* 2009). Thus, it is possible that the presence of some components of the shelterin complex could be related to the maintenance of genome stability at ITSs (Slijepcevic 2006; Lin and Yan 2008; Misri *et al.* 2008).

While the organization and dynamics of ITSs have been widely studied in somatic cells, their molecular organization during meiosis is poorly understood. This becomes very relevant, considering that ITSs during meiosis could potentially organize differently from telomeres and somatic ITSs (Heng *et al.* 1996). In this sense, it is not known if the chromatin conformation of the ITSs, especially pericentromeric ITSs, may incorporate proteins characteristic of telomeres, such as members of the shelterin complex, or if they include proteins and/or chromatin modifications typical of the pericentromeric regions. Additionally, it has been reported that some ITSs may behave as hot spots for recombination during meiosis (Ashley and Ward 1993). Since recombination is in essence a DNA repair process, this feature might be related to the predisposition of ITSs to be sites of DNA breaks. Finally, while telomeres maintain a close association with the nuclear envelope during the first meiotic prophase (Scherthan 2007), provided by their association with the SUN1 and SUN2 proteins (Ding *et al.* 2007; Link *et al.* 2014), no data are available to date about the association of ITSs with the nuclear envelope and/or SUN proteins.

To address some of these issues, we have studied the chromatin organization and dynamics of ITSs in the Mongolian gerbil *Meriones unguiculatus* (Rodentia, Gerbillidae). The subfamily of gerbils has been demonstrated to present highly rearranged karyotypes, partially due to Rb translocations (Benazzou *et al.* 1982; Blackburn 1994; Dobigny *et al.* 2003), making this species an exceptional model for studying ITSs. We show the presence of highly amplified (TTAGGG)_n repeats in the centromeric region of all autosomes. These

regions are enriched in protein modifications characteristic of centromeric and pericentromeric heterochromatin regions, such as histone H3 trimethylated at lysine 9 (H3K9Me3). Moreover, we detected that the shelterin complex proteins TRF1 and RAP1 also associate with ITSs, but notably, their presence during meiosis seems to be stage-dependent. The location of these proteins was not correlated with the presence of DNA repair-related proteins in the ITSs during meiosis. Finally, we found that, although at a low frequency, some centromere regions may bind the nuclear envelope through the interaction with SUN1 protein, mimicking the role of telomeres during meiosis. We discuss and hypothesize possible roles of this specific and unique organization and behavior of ITSs during meiosis.

Materials and Methods

Spermatocyte spreads

Testes of adult *M. unguiculatus* males were extracted and dissected in PBS [137 mM NaCl, 2.7 mM KCl, 10.1 mM Na₂HPO₄, 1.7 mM KH₂PO₄ (pH 7.4)] to obtain the seminiferous tubules. These were subsequently processed for surface-spreading technique as described by Peters *et al.* (1997) or squashing as described by Page *et al.* (1998). For spreading, seminiferous tubules were disaggregated with tweezers in PBS, and the cell suspension was centrifuged at 1200 × *g* for 8 min. Cells were then resuspended in PBS, centrifuged again, resuspended in 100 mM sucrose, and subsequently spread onto a slide. These preparations were fixed with 1% paraformaldehyde in distilled water containing 50 mM Na₂B₄O₇ and 0.15% Triton X-100. After air-drying, slides were washed with 0.04% Photo-Flo (Kodak) in distilled water and air-dried before being used for immunofluorescence. For squashing, tubules were fixed for 10 min in 2% formaldehyde in PBS containing 0.05% Triton X-100 (Sigma), and then small pieces were minced with tweezers, placed on a slide coated with 1 mg/ml poly-L-lysine (Sigma), and subsequently squashed. Slides were then frozen in liquid nitrogen and immediately immersed in PBS after removing the coverslip.

Colchicine treatment and bone marrow extraction

A stock solution of 0.1% colchicine (Sigma) in 0.9% ClNa was prepared. Gerbils were treated with 1.2 mL of this solution by intraperitoneal injection. Specimens were euthanatized 2 hr later, and femurs were removed for bone marrow extraction. The femurs were finely washed from inside out with a syringe containing a 0.75% KCl solution at 37°. Once the largest fragments had been decanted, the rest of the suspension was removed and incubated for 30 min at 37°. This suspension was centrifuged at 900 × *g* for 8 min, and the cells obtained were subsequently resuspended in 2 mL of a 3:1 ethanol–acetic acid mixture. Cells were then stored at 4° for 30 min. A drop of the cell suspension was left to fall on a slide, and the fixative was left to completely dry. Afterward, the slides were rinsed 2× for 5 min in PBS before the FISH procedure.

Immunofluorescence

Slides were incubated overnight at 4° with the following primary antibodies diluted in PBS: mouse monoclonal anti-SYCP3 (Ab-12452, Abcam, Cambridge, UK) at a 1:100 dilution; rabbit polyclonal anti-RAP1 (IMG-289 Imgenex, San Diego) at a 1:30 dilution; rabbit polyclonal serum raised against mouse TRF1 (TRF 12-S; Alpha Diagnostic International, San Antonio, TX) at a 1:100 dilution; rabbit polyclonal anti-H3K9Me3 (Abcam, 8898) diluted at 1:100; a human anti-centromere serum that recognizes centromeric proteins (ACA) (Antibodies Incorporated, 15-235) diluted at 1:100; rabbit anti-RAD51 (PC130, Calbiochem, Darmstadt, Germany) at 1:50 dilution; mouse monoclonal anti-MLH1 (551091, Pharmingen, San Diego) at 1:30 dilution; mouse monoclonal against histone variant H2AX phosphorylated at serine 139 (γ H2AX) (05-636, Millipore, Billerica, MA) at 1:1000 dilution; guinea pig against SUN-1 at 1:30 dilution. Slides were rinsed 3 $\times$ for 5 min each rinse in PBS and subsequently incubated with secondary antibodies in a moist chamber at room temperature for 1 hr: Alexa Fluor 350-conjugated goat anti-mouse IgG, Alexa Fluor 350-conjugated goat anti-rabbit IgG, Texas Red (TR)-conjugated goat anti-mouse IgG, TR-conjugated goat anti-rabbit IgG, fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG, FITC-conjugated goat anti-guinea pig, IgG and TR-conjugated goat anti-human IgG. For triple immunolocalization of proteins in which two of the primary antibodies were obtained from the same host species, we proceeded as described (Page *et al.* 2003). Secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA) and used at a 1:100 dilution. Slides were later stained with 10 μ g/mL of 4', 6-diamidino-2-phenylindole (DAPI) for 3 min and then mounted with Vectashield (Vector, Burlingame, CA) or rinsed in PBS 3 $\times$ for 5 min each rinse and processed following the FISH for telomeric DNA repeats protocol.

FISH for telomeric DNA repeats

After the last rinsing series in PBS, we followed the protocol described by Viera *et al.* (2003) with some variations. Briefly, slides were fixed in 4% formaldehyde in PBS for 10 min and dehydrated in an ascendant ethanol series (70, 90, and 100%) for 5 min each and allowed to air-dry. A hybridization mixture containing 70% deionized formamide (Sigma), 10 μ M FITC-labeled (C3TA2)₃ peptide-nucleic acid (PNA) probe (Applied Biosystems, Foster City, CA), and 2.1 mM MgCl₂ buffer (pH 7.0) in 8 mM Tris (pH 7.2) was added per slide, and DNA was denatured by heat for 3 min at 80°. Hybridization was performed for 2 hr at room temperature. Slides were then washed twice with 70% formamide in H₂O, containing 10 mM Tris (pH 7.2) and 10% BSA for 15 min, and three times with TBS [1 M Tris, 1.5 M NaCl (pH 7.5) containing 0.005% Tween-20] for 5 min. Slides were then dehydrated again with ethanol, air-dried, stained with 10 μ g/mL DAPI for 3 min and mounted with Vectashield (Vector, Burlingame, CA).

Image processing

Observations were made on an Olympus BX61 microscope equipped with a motorized Z axis. Images were captured with an Olympus DP72 digital camera using the Cell-F software (Soft Imaging System, Olympus, Hamburg, Germany) and processed by using public domain ImageJ (National Institutes of Health; <http://rsb.info.nih.gov/ij>) and Adobe Photoshop software.

Measurement of chromosome length and chiasma distribution

We measured the length of the synaptonemal complexes (SCs) in pachytene spermatocytes, as well as the length occupied by the ITSs over them, by using the “free hand” tool of ImageJ software. Both total and partial measurements were normalized against the length of the largest bivalent in each cell. Finally, the proportion of the SC occupied by the ITSs was calculated. Measurements were also used to discriminate between bivalents, taking into consideration their relative length and centromere position along the bivalents.

To analyze chiasmata distribution along chromosomes, we selected bivalent #10 as a representative example. This bivalent is easily recognizable since it is the largest of all telocentrics. Also, we took advantage of the fact that it never shows more than one chiasma. We measured the length of its long arm and divided it in 10 equally distant intervals (the ITSs being confined to the most proximal), in an approach similar to that followed by Libuda *et al.* (2013). Then, we measured the distance between the centromere and the MLH1 focus. As the length of the SC varies within the pachytene stage, we normalized both this distance and the total length of the bivalent to get a rigorous measurement of chiasmata distribution. We then assigned each MLH1 focus to the corresponding interval and calculated the frequencies over the total.

Results

Localization of interstitial telomeric DNA repeats in *M. unguiculatus* mitotic chromosomes

The karyotype of the Mongolian gerbil is composed of 44 chromosomes, with five telocentric pairs (Pakes 1969; Cohen 1970). We first studied the location of telomeric DNA repeats on mitotic chromosomes obtained from bone marrow cells. Telomeric (TTAGGG)_n repeats were detected as four dots per chromosome (one on each chromatid end) (Figure 1, A and B). In addition, autosomes showed an intense signal on an interstitial nontelomeric region that localizes in the primary constriction (Figure 1, A–D). No other telomeric signals were detected interstitially at the FISH resolution level. The centromere-located ITSs were observed in all autosomes and corresponded to larger TTAGGG blocks compared to the repeats located at the telomeres, suggesting a high level of amplification of these sequences. The extent of the FISH signal in the different pairs of autosomes was notably diverse, reflecting

Figure 1 FISH for telomeric DNA repeats (green) and counterstaining of the chromatin with DAPI (blue) on metaphase chromosomes from bone marrow spreads of *M. unguiculatus*. (A) Metaphase plate and (B) organized karyotype from this cell. The signal of the probe is notably present on the pericentromeric region of every autosome, including the telocentric ones (asterisks). (C) Telomeric signal in one selected telocentric chromosome. The presence of telomeric sequences is clearly evident on the centromeric region and more intense than in the telomeres. (D) A selected chromosome pair showing differences in the intensity of telomeric signal at the centromere. (E) Detail of the sex chromosomes. The X chromosome exhibits two discrete dots of the probe in the pericentromeric region, even smaller than those on the telomeres. No signal of ITS is detected on the Y chromosome.

a high variability in the size of ITS blocks from one chromosome pair to another (Figure 1B). This variability was also observed between homologous chromosomes, suggesting the existence of polymorphisms in the length of ITSs (Figure 1, C and D). Although telomeric signals were identified in the sex chromosomes, only two small dots were visible near the centromeric region of the X chromosome (Figure 1E), while no probe signal was detected interstitially on the Y chromosome (Figure 1E).

Structural organization of ITSs during the first meiotic division

We next studied the structural organization of the ITSs in meiotic chromosomes. During meiosis, the structure of chromosomes is greatly influenced by homologous synapsis and the assembly of a meiotic specific structure, the synaptonemal complex, which is formed by two axial elements and lateral elements (LEs), one per homolog, and a central element that holds LEs together. Homologous chromosome synapsis is initiated during zygotene, completed during pachytene, and vanishes during diplotene at the end of the first meiotic prophase. We used an antibody against SYCP3 protein, the main component of the SC LEs, to localize meiotic chromosomes during prophase I. Identification of the different stages of the first meiotic prophase was performed on the basis of homologous synapsis and the morphology of the sex chromosomes, as we previously reported (de la Fuente *et al.* 2007).

Using immunofluorescence to detect SYCP3 and centromeric proteins and FISH for telomeric (TTAGGG)_n repeats, we observed that in chromosome spreads the telomeric

signals localized as discrete and quite compacted foci at both chromosome ends (Figure 2). During the zygotene, the telomeric DNA signals were detected as single dots at the ends of either unsynapsed or synapsed LEs (Figure 2, A' and A''). Thus, each dot in unsynapsed LEs corresponded to telomeres of both sister chromatids; conversely, foci in synapsed LEs corresponded to four signals from the telomeres of each chromatid from the two homologous chromosomes (Figure 2, A' and A''). In pachytene bivalents, which are fully synapsed, a single focus was located at each SC end that corresponded to the telomeric DNA of both homologs (Figure 2, B–C''). During diplotene, the autosomal LEs desynapse, and in this species they also tend to fragment (Figure 2, D–D''). This feature makes it appear that some telomeric signals are separated from LEs. In addition to telomeric signals, ITSs were also detected in each bivalent during prophase I. The pericentromeric location of the ITSs was corroborated by the co-immunolocalization of centromeric proteins. No additional ITSs were observed outside the centromeric regions. Therefore, all the ITSs features described from here onward should be understood as involving only this kind of interstitial telomere.

During meiosis, the ITSs appeared as flame-like signals that localized to the SC and expanded from it (Figure 2). This lengthy appearance of ITSs was already observed at zygotene (Figure 2A) and maintained during pachytene (Figure 2, B and C) and diplotene (Figure 2D). The ITSs began to achieve a higher compaction at the end of the diplotene, similar to the repeats present at telomeres, and seemed to be completely condensed at metaphase I and anaphase I (Supporting Information, Figure S1).

Figure 2 Double immunolocalization of centromeric proteins (green) and SYCP3 (red) and FISH for telomeric DNA repeats (blue) on spread spermatocytes. (A) Late zygotene. The telomeric DNA signal is detected over the pericentromeric region of every bivalent, coinciding with the signal of the ACA serum. Note the late-synapsing bivalent (asterisk) enlarged in A' and A''. Sex chromosomes (XY) lack interstitial the PNA signal, which is observed only on their telomeres. (B and C) Pachytene. Differences between the signals of the ACA serum and the telomeric repeats are evident at the centromeres. They are indicated by arrows in the enlarged bivalents (asterisk) shown in B' and B'' and C' and C''. The ITS signal occupies a larger domain than the distal ones (arrowheads). (D) Early diplotene. The same features are observed at the ITSs, while distal signals tend to fragment and partially disorganize.

Interestingly, the ITS signal of the X chromosome was usually weak or even undetectable at the centromere (Figure 2). This could be a result of the condensation status of the chromatin during the first meiotic prophase, since its ITS is again visible on the X chromosome during metaphase I (Figure S1). We also observed that the axial elements of sex chromosomes sometimes exhibited a distal connection of telomeric DNA during pachytene (Figure S2). This connection is transient, may involve two or more chromosomal ends, and may be either intra- or interchromosomal. They were not detected by late diplotene (not shown) and were completely absent at metaphase I (Figure S1).

Heterochromatin modifications associated to ITSs

Using FISH, we observed that ITS signals composed a wider area than the centromere, which was detected by anticentromere antibodies (Figure 2). This indicates that ITSs are present not only in the centromere region marked by centromeric proteins, but also in the surrounding pericentromeric region. To test if the ITSs could be a part of pericentromeric heterochromatin, we colocalized (TTAGGG)_n repeats using FISH along with a heterochromatin marker such as H3K9Me3. We observed that ITSs were indeed included in a heterochromatic domain, but, interestingly, not all the pericentromeric heterochromatin contained ITSs (Figure 3). This suggests that, as in

voles, telomeric repeats in the Mongolian gerbil are not the only origin of the pericentromeric heterochromatin (Rovatsos *et al.* 2011). Additionally, H3K9Me3 was also detected over a whole bivalent that presented delayed synapsis (de la Fuente *et al.* 2007) and over a large interstitial segment of another bivalent (Figure 3A). These bivalents retained their histone signal during the entire first meiotic prophase (Figure 3, A–D). As regards sex chromosomes, H3K9Me3 was present in both the X and Y up to early pachytene (Figure 3, A and B), but began to disappear at late pachytene and remained only at the centromeric region of the X chromosome from this stage onward (Figure 3, C and D).

Association of telomeric proteins to ITSs

It has been observed that in somatic cells, interstitial telomeric sites are inherently common fragile sites that require TRF1 for their stability (Sfeir and de Lange 2012). To test whether ITSs could also associate shelterin complex proteins during meiosis, we performed immuno-FISH analysis detecting (TTAGGG)_n repeats along with RAP1 or TRF1 in spermatocyte spreads. As expected, we observed that RAP1 (Figure 4) and TRF1 (Figure S3) always colocalized with the telomeric signals at chromosome ends. The signal at the telomeres was very similar in both cases, showing a compacted organization of the chromatin. We also found that both proteins appear associated with

Figure 3 FISH for telomeric DNA repeats (green) and double immunolocalization of SYCP3 (red) and H3K9Me3 (blue) proteins on spread spermatocytes. (A) Late zygotene. The signal of the anti-H3K9Me3 antibody reveals the presence of this protein in the pericentromeric region of the autosomes and also on two highly heterochromatic bivalents (H and h). Additionally, sex chromosomes show a notable signal of this histone. (B) Early pachytene. H3K9Me3 is clearly visible on the pericentromeric region corresponding to the spread chromatin loops, as occurs with the nontelomeric DNA repeats. A selected bivalent (asterisk) is enlarged in B'–B'''. (C) Late pachytene. The histone signal is maintained on the autosomes, whereas it is restricted to the centromeric region of the X (arrow). (C'–C'') Selected bivalent from C. (D) Early diplotene. The signal of the probe and H3K9Me3 are still detectable on the pericentromeric region of the autosomes.

ITSs, but in a stage-dependent manner. That is, RAP1 and TRF1 signals were very faint or even undetectable at the ITSs during zygotene and early pachytene (Figure 4A); however, both proteins were greatly enriched at ITSs at mid-late pachytene (Figure 4B; Figure S3). This expression pattern persisted during early diplotene, but RAP1 and TRF1 signals were considerably reduced from ITSs at late diplotene (Figure 5; Figure S3). Sex chromosomes exhibited neither RAP1 nor TRF1 at their centromere region at any stage.

The signals observed for the telomeric DNA and the shelterin complex proteins were mostly coincident at the ITSs. Only a few differences could be observed; *i.e.*, telomeric DNA detection yielded a more dotted pattern than the labeling of telomeric proteins. These differences can be explained by the different nature of FISH and immunodetection protocols, but we cannot dismiss that the telomeric repeats are separated by nontelomeric sequences, yielding a scattered pattern.

To determine if the particular expression pattern that TRF1 and RAP1 exhibited during meiosis was related to changes in the organization of heterochromatin, we per-

formed a double immunolocalization of H3K9Me3 and RAP1 (Figure 5). As described above, H3K9Me3 was enriched in the pericentromeric heterochromatin from early stages of prophase I, when RAP1 localized principally at the ends of the chromosomes (Figure 5, A–A'''). RAP1 signal was increased at the ITSs at mid-late pachytene (Figure 5, B–B'''). No changes in the organization of pericentromeric chromatin were observed on the basis of H3K9Me3 labeling at this stage, but this event is contemporaneous with the disappearance of the H3K9Me3 mark from the sex chromosomes. The signals of RAP1 and H3K9Me3 presented a similar pattern. However, RAP1 signal did not cover the whole heterochromatic region, paralleling the result obtained with the telomeric DNA probe (Figure 3) and suggesting that heterochromatin covers a wider area than ITSs. This distribution of RAP1 and H3K9Me3 was maintained during early diplotene (Figure 5C), but RAP1 labeling tended to decrease throughout diplotene (Figure 5D) and was not detectable in later stages (not shown). This event is concurrent with the progressive disappearance of H3K9Me3 from the pericentromeric region of autosomes (Figure 5D),

Figure 4 Double immunolocalization of SYCP3 (red) and RAP1 (blue) proteins combined with FISH for (TTAGGG) n repeats (green) on spread spermatocytes. (A–A''') Early pachytene. RAP1 is clearly seen at chromosome telomeres but is not detectable in the pericentromeric region of the bivalents. (B–B''') Late pachytene. RAP1 is detected on the telomeric DNA-containing chromatin loops of the pericentromeric region. Bivalents marked with an asterisk in both A and B are enlarged in the right column.

indicating changes in the organization of heterochromatin at the end of the first meiotic prophase. H3K9Me3 was no longer detectable at the centromeres of autosomes during metaphase I (data not shown).

DNA damage-response proteins do not specifically associate with ITSs

ITSs are sites susceptible to DNA break and damage repair (Bouffler 1998). On the other hand, the particular structure of the telomeric sequences is highly protected by shelterin complex proteins, preventing the activation of DNA damage repair signaling in the telomeres (Sfeir and de Lange 2012). It is then possible that TRF1 and RAP1 could protect ITSs from DNA damage. To test if ITSs are affected by DNA damage in late stages of prophase I, we studied the progression of DNA repair during the first meiotic prophase. During the leptotene stage, massive DNA damage is endogenously induced by SPO11 protein in meiotic cells. Thereafter, a mechanism of homologous repair is set up, and the DNA is thoroughly repaired during subsequent stages of first meiotic prophase (Keeney and Neale 2006). In mammals, the phosphorylated form of histone H2AX

(γ H2AX) is one of the characteristic responses to this damage, and thus it is used as a common marker of DNA damage during meiosis (Mahadevaiah *et al.* 2001). Additionally, γ H2AX is accumulated on sex chromosomes from pachytene up to metaphase I, where it functions in the meiotic silencing of sex chromosomes (Mahadevaiah *et al.* 2001; Turner *et al.* 2004; Page *et al.* 2012). We followed the location of γ H2AX during pachytene in combination with labeling of the centromeres to ascertain if the centromeres were specifically marked for DNA damage and repair during these stages (Figure 6, A–D). We found that during early pachytene γ H2AX was abundantly distributed on the sex chromosomes, which showed a fuzzy appearance (Figure 6A). In addition, some small signals were detected over the autosomes. However, while some marks were located close to the centromeric regions, most of these marks localized at other interstitial regions (Figure 6, A–C''). In mid-pachytene, γ H2AX on the sex chromosomes becomes more compacted and regular, while the number of autosomal signals tended to decrease and then completely disappeared during late pachytene (Figure 6D). Therefore, the presence of telomeric proteins at the ITSs during pachytene does not seem to correlate to the accumulation of endogenously produced DNA damage at these regions.

We also analyzed the location of the recombinase RAD51 (Figure 6, E–H). This protein acts in the repair of DNA double-strand breaks (DSBs) during early stages of meiotic prophase and may remain associated with unrepaired regions after γ H2AX labeling has disappeared (Plug *et al.* 1998). As previously reported (de la Fuente *et al.* 2007), RAD51 is distributed along autosomes and sex chromosomes during zygotene in the Mongolian gerbil (Figure 6E). We did not observe any accumulation of RAD51 at the pericentromeric regions, indicating that ITSs may not be a preferential location for the action of DNA repair processes. Only in some instances were RAD51 foci located near the centromeres, but in those instances they seemed to be excluded from the heterochromatin (Figure 6, F–G''). In pachytene, while some unresolved DNA repair sites are still marked by RAD51 along chromosomes, ITSs appear devoid of it (Figure 6H). No RAD51 foci were detected in late pachytene or diplotene when RAP1 and TRF1 accumulate on ITSs (not shown). Therefore, the pattern of localization of both γ H2AX and RAD51 indicates that ITSs are not specific sites for the accumulation or persistence of unrepaired DNA during pachytene.

Chiasmata quantification and localization

In the Armenian hamster, it has been described that ITSs are hot-spot sites for recombination (Ashley and Ward 1993). To test if ITSs exhibit a similar behavior in the Mongolian gerbil, we localized and quantified the distribution of MLH1, a protein that is restricted to mid- and late pachytene and associates with chiasma sites (Plug *et al.* 1998), along with the localization of RAP1 during prophase I (Figure 6, I–J''). We identified one or two MLH1 foci per bivalent and a mean of 22.02 (SD $\pm$ 1.51) signals per spermatocyte (n = 83). We assumed that for ITSs (or any other region) to be considered

Figure 5 Triple immunolabeling of spread spermatocytes with antibodies against RAP1 (green), SYCP3 (red), and H3K9Me3 (blue). Bivalents indicated with an asterisk are enlarged in the details. (A–A''') Early pachytene. RAP1 is visible only on the telomeres of both the autosomal bivalents and sex chromosomes, but is not detected on the pericentromeric region at this stage, as detailed in a selected bivalent (A'–A'''). (B–B''') Late pachytene. Each bivalent exhibits RAP1 on the pericentromeric region at the end of this stage, maintaining the signal of H3K9Me3. (C–C''') Early diplotene. No obvious differences are visible from late pachytene, excepting a slightly higher condensation of the chromatin, as shown by the signals of H3K9Me3 and RAP1 proteins. Both proteins maintain their pattern during the beginning of this stage. (D–D''') Late diplotene. RAP1 has detached from the pericentromeric region of the autosomes and is not detected in some telomeres. H3K9Me3 has begun to dissociate from a few bivalents.

a hot spot, they should accumulate more MLH1 foci than expected from the proportion of the SC length occupied by these regions. We observed that 1.95% of MLH1 signals partially colocalized with RAP1 (Figure 6, J–J'). We then measured the total length of the SCs and the region occupied by ITSs in 16 cells. We found that the ITSs represent 7.69% ($SD \pm 0.67$) of the total length of meiotic chromosomes in the Mongolian gerbil. Therefore, since only 1.95% of chiasmata occur in these regions, ITSs may in fact be regions of low recombination rate in this species. To further support this conclusion, we analyzed the distribution of chiasmata along one of the bivalents in 115 spermatocytes. We observed that, in the telocentric bivalent #10, chiasmata very rarely localize to the close proximity of the centromere (where ITSs are found) (Figure 6K). By contrast, their distribution peaks at the first third of the arm length and decreases from there to the distal segment of the chromosome. These results strongly

suggest that the presence of ITSs is not correlated to an increase of crossovers in the Mongolian gerbil.

Association of centromeric regions with the nuclear envelope

The capability of ITSs to bind RAP1 and TRF1 raises the question as to whether ITSs also can interact with other telomere-associated components. One of the main features of meiotic chromosomes is the attachment of telomeres to the nuclear envelope, which is provided by their binding to the inner nuclear membrane protein SUN1. The absence of this protein leads to defects in chromosome synapsis, recombination, and meiotic progression in mammals (Ding *et al.* 2007; Link *et al.* 2014). We investigated the location of SUN1 protein using an antibody against mouse SUN1 (Adelfalk *et al.* 2009). We found that SUN1 localized at the telomeres of meiotic bivalents in the Mongolian gerbil (Figure 7A)

Figure 6 (A–D) Triple immunolabeling of spread spermatocytes with antibodies against centromeric proteins (blue), γ H2AX (green), and SYCP3 (red). (A) γ H2AX appears greatly concentrated over the sex chromosomes (XY). Labeling is also observed over some autosomes, where γ H2AX mostly appears as small foci located over the synaptonemal complex. Two bivalents (asterisks) are enlarged in B–B'' and C–C''. While most γ H2AX foci lie outside the centromeric regions (B–B''), in some rare occasions γ H2AX signal may overlap with the centromeric signal (C–C''). (D) Late pachytene. γ H2AX appears only on the sex chromosomes while autosomal foci are no longer detected. (E–H) Triple immunolabeling of spread spermatocytes with antibodies against H3K9Me3 (blue), RAD51 (green), and SYCP3 (red). (E) Zygotene. Some of the autosomal bivalents are still undergoing synapsis while others have completed their synapsis. In both cases, abundant RAD51 foci appear scattered along SCs. The pericentromeric regions, labeled with H3K9Me3 (which also labels the sex chromosomes and two autosomal bivalents), are mostly devoid of RAD51 foci (see enlarged detail of asterisk-marked bivalent in F–F''). On some occasions, RAD51 foci appear close to the pericentromeric area but usually with no overlapping (see detailed bivalent in G–G''). (H) Pachytene. Most RAD51 foci have disappeared. Some signal is still present in some autosomes and mainly on the sex chromosomes (XY). No overlap of RAD51 and pericentromeric regions was observed. (I–J'') Triple immunolabeling of spread spermatocytes with antibodies against RAP1 (blue), MLH1 (green), and SYCP3 (red). (I) Pachytene spermatocyte in which the distribution of MLH1 foci, corresponding to the location of chiasmata, can be appreciated. Most MLH1 foci appear located outside the pericentromeric regions. However, in some cases there is overlap with the RAP1 signal (asterisk). The overlapping can be appreciated in the enlarged detail shown (J–J''). (K) Analysis of chiasmata distribution along bivalent #10. Top left

as a single or double spot at the end of the SC. Sex chromosomes also showed a SUN1 signal at each telomere. On the contrary, most centromeric regions were devoid of SUN1 protein. However, we observed that a small but significant fraction of bivalents were able to bind SUN1 at the centromeric regions. This SUN1 signal presented very interesting features: (1) it appeared as a small dot that colocalized with centromere proteins; (2) the presence of SUN1 was associated with a stretching of the SC and the centromere; and (3) SUN1 was present only in metacentric and submetacentric chromosomes (Figure 7, B–B’). The SUN1–centromere association was especially notable in one of the individuals analyzed (46.8% of pachytene spermatocytes, $n = 62$), which allowed us to ascertain additional features about this association. We observed that the association was usually shown by only 1 or 2 bivalents per spermatocyte, although up to 4 bivalents could present it (mean = 1.4). This number was independent of whether spermatocytes were in early or late pachytene. Additionally, we identified the bivalents by size and centromere position and found that at least 13 of the 16 metacentric bivalents were able to show this signal. To determine if this feature could represent an actual association of the centromeres with the nuclear envelope, we determined the location of SUN1 in squashed spermatocytes, which retain their three-dimensional configuration. We could confirm that the bivalents showing a SUN1 signal at their centromeric region presented a third point of attachment to the nuclear envelope, in addition to the two telomeric ones (Figure 7C and File S1). A further inspection into the location of MLH1 and γ H2AX revealed that the presence of SUN1 does not correspond to unrepaired DNA or crossing-over positions (not shown).

Discussion

Origin of ITSs in *M. unguiculatus*

The appearance of telomere sequences in inner positions of the chromosomes has been explained as a result of a complex karyotype evolution process, with chromosomal rearrangements such as Rb translocations and amplification of telomeric DNA repeats being the most plausible (Meyne *et al.* 1990; Azzalin *et al.* 2001; Viera *et al.* 2004; Rovatsos *et al.* 2011). The sequences found in the metacentric/submetacentric chromosomes of *M. unguiculatus* shown here would have probably arisen from Rb translocation events. Subsequently, amplification events could have increased the original amount of telomeric repeats located around the centromere. Some authors have suggested that this amplification may arise from unequal crossing over and posterior stabilization (Ruiz-Herrera *et al.* 2008), which may provide an explanation for the centromeric

role of such newly formed sequences. Our results on the location of MLH1 showed that, although at a low rate, recombination is possible within ITSs, giving support to the model of amplification by unequal crossover. Likewise, the notable presence of histone modifications related to heterochromatin, such as H3K9Me3, strongly supports the capability of ITSs to adopt a heterochromatic configuration. Heterochromatinization of ITSs has been previously observed in other species (Garagna *et al.* 1997) and seems to be a common process concomitant with the amplification of telomeric DNA repeats.

Among the most striking aspects of the ITSs observed in *M. unguiculatus* is the variability in the length of the repeats in the chromosomes, a feature that is shared by other species of mammals, such as many members of the Arvicolidae family (Rovatsos *et al.* 2011). In our case, telocentrics seem to present greater ITSs than metacentrics. It is then possible that these differences originated from Rb fissions produced after chromosome fusions and subsequent amplification of the ITSs. In support of this model, it should be recalled that ITSs are sensitive sites for chromatin breaks and DNA repair (Bouffler 1998), favoring then the breakage of the ancient chromosome within these regions and the subsequent DNA repair in a process known as “telomere healing” (Matsumoto *et al.* 1987). After chromosome fission, it is also possible that amplification and extension processes could have occurred, increasing the differences in ITS length within the different chromosomes, as previously proposed (Ruiz-Herrera *et al.* 2008).

Sex chromosomes seem to present a different situation in relation to ITSs, since only a small signal is present in the X, while the Y chromosome does not show any ITS. Rb fusions involving autosomes in *M. unguiculatus* had been previously suggested to represent a relatively recent event in evolution (Gamperl and Vistorin 1984). In this context, the appearance and amplification of ITSs currently found in autosomes could correspond to a stratum in the karyotype evolution in *M. unguiculatus* that did not involve the sex chromosomes, as it has been proposed for the shrew *Sorex araneus* (Zhdanova *et al.* 2005).

Structural organization of ITS chromatin during meiosis

Our results show that nontelomeric DNA repeats present an organization during meiosis that is notably different from the organization observed at telomeres (Figure 8). In this sense, ITSs exhibit three exceptional features during meiosis. First, the chromatin comprising the ITSs appears to be more extended than telomeric chromatin. Although we have not characterized the length of the telomeric sequences at ITSs, we favor the interpretation that these repeats present a more open conformation because they form and/or appear in a region of longer DNA loops (Figure 8). Therefore, it is possible

image shows an enlargement of bivalent #10 where the MLH1 focus is visible. A schematic representation of the bivalent is depicted on the top right. Below the image, intervals show the different locations considered for chiasmata. The chart shows the distribution of chiasmata in bivalent #10 analyzed in 115 spermatocytes. The frequency of chiasma occurrence within each interval of the bivalent is represented. Crossing over is rarely found near the proximal region (intervals 1 and 2) while the highest incidence takes place within the middle segment of the bivalent (intervals 4–6).

Figure 7 Triple immunolabeling of spermatocytes with antibodies against centromeric proteins (blue), SUN1 (green), and SYCP3 (red). (A) Pachytene spread spermatocyte in which SUN1 can be clearly seen at the ends of bivalents. Most centromeric regions do not show any SUN1 signal, but one bivalent shows a small signal at the centromere (arrow). (B–B'') An enlarged bivalent showing a centromeric SUN1 signal (arrow). The synaptonemal complex appears stretched at this region (arrowhead). (C) Pachytene squashed spermatocyte. Five different focal planes were superimposed to give rise to a single image. The three-dimensional organization of the nucleus was preserved, and the attachment point of bivalents at the nuclear periphery can be observed. A small metacentric chromosome, similar to that shown in B–B'', shows a three-point attachment configuration: two at telomeres (arrowheads) and one at the centromere (arrow), which presents a SUN1 signal. Note the presence of many centromeric signals, corresponding to metacentric chromosomes, located in the nuclear interior. For a complete reconstruction of the nucleus, see [File S1](#).

that the chromatin organization of the telomeric repeats depends on their genomic context. Indeed, this dependence was already observed when telomeric repeats were ectopically inserted interstitially into chromosomes (Heng *et al.* 1996). In this context, it seems reasonable that protein factors that associate with DNA, including histone variants and/or modifications, may play a main role in this chromatin organization. Since ITSs present a chromatin condensation status more similar to the rest of the chromosome, it seems plausible that telomeric regions could bear specific factors that determine the formation of shorter chromatin loops. The intimate association of telomeres to the nuclear envelope during the first meiotic prophase may represent a structural constraint that favors short chromatin loops. Furthermore, a specific association of telomeres with components of the condensin complex has been reported in mouse meiosis (Viera *et al.* 2007), indicating that specific condensation factors could be acting at chromosome ends.

Second, the epigenetic modifications of the chromatin differ between ITSs and the telomeric sequences located at chromosome ends. During meiosis, ITSs are characterized by a massive presence of histone modifications such as H3K9Me3, a typical marker of heterochromatin, while the telomeric repeats at telomeres are devoid of this protein. This feature again suggests that the conformation of the chromatin greatly depends on its position within the chromosome and not only on the nature of the DNA sequence.

Third, despite the different chromatin organization of ITSs and telomeres, the ITSs are still able to associate specific

telomeric proteins. However, in contrast with telomeres, which exhibit constant levels of the shelterin complex proteins throughout meiosis, the incorporation of TRF1 and RAP1 onto the ITSs seems to be stage-dependent, appearing mainly during mid-pachytene and disappearing during diplotene. This intriguing result indicates that the chromatin dynamics of ITSs is different from that of DNA telomeric repeats at the telomeres. Furthermore, it raises the possibility that these proteins may play a role in the regulation and/or function of ITSs in meiosis.

The particular dynamics of the shelterin complex proteins in the ITSs suggests a role in chromatin stabilization and protection during meiosis

Both TRF1 and RAP1 proteins are essential in organizing and maintaining the T-loop conformation of the telomeres and also in protecting chromosome ends from DNA damage repair proteins (Zhong *et al.* 1992; Li *et al.* 2000; Scherthan *et al.* 2000). In addition, these proteins could be important for the proper attachment of chromosomes into the nuclear envelope (Scherthan *et al.* 2000; Scherthan 2007). Nevertheless, many other roles have been described for these proteins. In somatic cells, TRF1 is essential for preserving the integrity of fragile sites in the genome and telomeres, and it protects them from DNA breakage and repair (Krutilina *et al.* 2001, 2003; Bosco and de Lange 2012). Indeed, low levels of TRF1 are associated with activation of the ATR kinase and breakage of fragile sites of the genome and of telomeres (Bosco and de Lange 2012). On the other hand, it has been shown that RAP1 can

Figure 8 Schematic representation illustrating the different patterns of chromatin configuration of distally vs. interstitially located telomeric DNA repeats during prophase I. A submetacentric autosomal bivalent from early pachytene (A–C) to late diplotene (J–L) is depicted, in which a synaptonemal complex (double red and yellow lines) and RAP1-TRF1 complexes (purple spheres) are represented. Chromatin loops of each homolog are colored in light and dark gray, respectively, while those loops containing telomeric (TTAGGG)_n repeats are depicted in green. Also, chromatin loops containing both telomeric DNA repeats and H3K9Me3 histone are represented in blue. Note that RAP1-TRF1 complexes are present only in the pericentromeric region during late pachytene–early diplotene (D, E, G, and H). By contrast, RAP1-TRF1 complexes are always detected in the telomeric loops as a constitutive factor (C, F, I, and L). It is worth noting the different level of condensation of the chromatin during diplotene (G–L), in which the loops and the protein complexes appear more compacted. The same situation takes place in telocentric chromosomes.

bind not only to telomeres, but also to nontelomeric regions, having essential telomeric and extratelomeric roles. Although mammalian RAP1 is dispensable for telomere capping, it is fundamental to prevent telomere recombination and fragility (Martinez *et al.* 2010). Moreover, in somatic cells, >70% of RAP1-binding sites are found at intragenic positions or at the vicinity of gene-coding chromatin, indicating that RAP1 is an important factor for telomere integrity and for transcriptional gene regulation in mammals (Martinez *et al.* 2010).

It is then possible that the particular dynamics that RAP1 and TRF1 exhibit during prophase I could be related to protection and integrity maintenance of the ITSs, especially from pachytene up to diplotene, when chromatin in autosomes becomes decondensed and transcriptionally active (Page *et al.* 2012). This becomes particularly important since meiosis is a critical process for genome stability. The induction of hundreds of DNA double-strand breaks at the beginning of meiosis by SPO11 represents a great challenge to maintaining genome integrity in these cells (Lichten 2001). Soon after the induction of DSBs, the homologous repair pathway involving RAD51 is triggered, and by mid-pachytene most of the DNA damage has been properly repaired. In this scenario, it is then possible that ITSs could be especially sensitive to the induction of DSBs and that a great number of DNA breaks or repair proteins could be associated with ITSs during leptotene and up to mid-pachytene. Nevertheless, our results suggest that γ H2AX and RAD51 have no specific accumulation at the ITSs, suggesting that ITSs behave similarly to the rest of the ge-

nome in terms of occurrence of SPO11-dependent DNA DSBs and in relation to efficiency of DNA damage repair by the early homologous-repair mechanisms. During these early stages, this feature does not seem to depend on the presence of RAP1 or TRF1 at the ITSs, since these proteins appear mainly at mid-late pachytene when the bulk of DNA damage has already been repaired.

Nevertheless, RAP1 or TRF1 may have a function in protecting ITSs from DNA repair in later meiotic stages. There is increasing evidence that additional DNA repair events can be triggered during late stages of prophase I, either under normal conditions or after artificial induction of DNA damage (Goedecke *et al.* 1999; Ahmed *et al.* 2007). Interestingly, the mechanisms of DNA repair acting at these later stages would be preferentially nonhomologous pathways, mainly nonhomologous end joining (NHEJ) (Ahmed *et al.* 2010). Furthermore, some telomere-associated proteins like Ku-70 have been reported to be important for the proper repair of DNA by the NHEJ pathway (Ahmed *et al.* 2013). Thus, the presence of RAP1 and TRF1 during late pachytene and diplotene could be attributed to a role in the regulation and/or protection of ITSs once these DNA repair mechanisms are triggered.

A role of ITSs during meiosis?

Even though the origin and dynamics of ITSs have been widely discussed (for a review, see Lin and Yan 2008), their role remains unknown. Moreover, the possible functions that ITSs could have during meiosis have barely been discussed.

Telomeres are well known to play a main role in tethering chromosome ends to the nuclear envelope during meiosis (Scherthan *et al.* 1996). This is an important step before the formation of the “bouquet” arrangement during leptotene–zygotene and the initiation of synapsis between homologs. We did not expect a similar role of ITSs in meiosis of the Mongolian gerbil. Our previous results (de la Fuente *et al.* 2007) and those presented here indicate that centromeric regions of metacentric chromosomes are for the most part not attached to the nuclear envelope during zygotene or pachytene. Likewise, although synapsis has been previously observed to start either from the ends or from the inner positions of the chromosomes in *M. unguiculatus*, we did not find evidence of a centromeric initiation of synapsis in metacentric chromosomes (de la Fuente *et al.* 2007). However, the amazing finding that some centromere regions can bind SUN1 and attach to the nuclear envelope suggests that ITSs can retain their capability to perform some telomere-associated functions. Such ability is remarkable, considering that the ITSs are embedded in a very different chromatin landscape. On these grounds, the study of ITSs could serve as a model for the understanding of the requirements for nuclear envelope attachment. So far, our results have shown that the association of ITSs to the nuclear envelope may precede the incorporation of RAP1 and TRF1, which occurs mainly at mid-pachytene. Further analyses would be needed to characterize the involvement of additional proteins, such as TERB1, which has been proposed to bridge between telomeric repeats and SUN1 (Shibuya *et al.* 2014).

Although the attachment of telomeres to the nuclear envelope is crucial for the outcome of meiosis (Ding *et al.* 2007; Link *et al.* 2014), we do not think that ITSs attachments could be essential during Mongolian gerbil meiosis, since they are found in only a fraction of cells and they seem not to be related to DNA damage or meiotic recombination. Instead, we propose this could be a remnant of a previous evolutionary state of metacentric chromosomes. The three point attachment figures observed in squashed spermatocytes resemble those displayed by trivalents in Rb heterozygote mice (Berrios *et al.* 2014). Considering that metacentric chromosomes in *M. unguiculatus* were most likely originated by Rb translocations (Benazzou *et al.* 1982; Dobigny *et al.* 2003), it emerges that this capability may be reminiscent of a (recent?) heterozygote state of these chromosomes. Obviously, it would be relevant to explore if this behavior could also be found in other species, such as voles, which present similar features in relation to ITSs (Rovatsos *et al.* 2011).

On the other hand, it has been proposed that ITSs may represent hot spots for recombination in some mammalian species (Ashley and Ward 1993; Ashley 1994). However, our results showed that MLH1 is not preferentially localized at ITSs, indicating that ITSs do not necessarily behave as hot spots, at least after endogenous DNA-damage conditions. Indeed, the chiasma frequency reported here indicates that ITSs in *M. unguiculatus* act as cold spots for recombination. Similar results have been reported in the short ITSs present in

chicken chromosomes (Galkina *et al.* 2005), indicating that high recombination rates could not be an intrinsic feature of ITSs during meiosis. Additionally, since ITSs in the Mongolian gerbil are located flanking the centromeres and within the surrounding heterochromatin, it is possible that centromeres and/or the heterochromatic conformation of ITSs could mediate the decreased frequency of meiotic recombination, an effect that has been repeatedly observed in many other species (John and King 1985; Anderson *et al.* 1999; Zickler and Kleckner 1999; Mehrotra *et al.* 2008). This could explain the different behavior in comparison to the Armenian hamster ITS, which is also located near the centromere although it is not known whether it is included in a heterochromatic domain.

Finally, although the frequency of MLH1 foci may be lower at ITSs than in the rest of the chromosome, their presence is still notable. In mouse chromosomes, MLH1 recombination nodules seem to be excluded from the pericentromeric heterochromatin (Anderson *et al.* 1999; Froenicke *et al.* 2002). The fact that in the Mongolian gerbil some homologous recombination activity is observed within the centromeric region may indicate the ITSs can influence the usually inactive state of heterochromatin in relation to recombination. This, as mentioned above, may partially explain the tendency of ITSs to expand within the pericentromeric region.

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GENETICS

Supporting Information

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Chromatin Organization and Remodeling of Interstitial Telomeric Sites During Meiosis in the Mongolian Gerbil (*Meriones unguiculatus*)

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Figure S1 FISH for telomeric DNA repeats (green) and counterstaining of the chromatin with DAPI (blue) on squashed spermatocytes during first meiotic division. **(A)**. Metaphase-I. All the bivalents appear alienated at the metaphase-I plate. The telomeric signal of the ITs appears completely compacted at this stage. The differences in size with the distal telomeric signals are due to the great amplification present at telomeres and revealed also in somatic cells. Sex chromosomes (XY) enlarged in **(B)** show a distal connection of chromatin, but the telomeric signals do not contact between both chromosomes. **(C)**. Anaphase-I. All chromosomes have segregated and two chromosome poles are clearly discernible. No connection between telomeres of chromosomes of different poles is observed. This also applies for the sex chromosomes (enlarged in **D**).

Figure S2 (A-D) Details of sex chromosomes in different spermatocytes during pachytene labeled with anti-SYCP3 (red) and FISH for telomeric DNA repeats (green) and their corresponding schematic representation (A'-D'). Distal connections are observed in different configurations, involving one or both chromosomes.

Figure S3 Double immunolocalization of TRF1 (green) and SYCP3 (red) proteins in spread *M. unguiculatus* spermatocytes. Arrowheads point to the bivalents depicted in the right column. (A) Early pachytene. As expected, the telomeric protein is detected as one dot at every tip of the bivalents and sex chromosomes. (B) Late pachytene. TRF1 accumulates noticeably on interstitial segments of the autosomes that coincide with the centromeres until the beginning of diplotene (C). (D) Late diplotene. No interstitial TRF1 signals are detected.

File S1
Supplementary movie 1

(A) 3D reconstruction of the squashed pachytene spermatocyte immunolabeled with antibodies against centromeric proteins (blue), SUN1 (green) and SYCP3 (red) shown in Figure 7C. The whole nucleus is reconstructed in the main figure. (B) The bottom insert shows the specific region of the nucleus where a metacentric bivalent presents a three-point attachment configuration (arrow). File S1 is available for download as an .avi file at

<http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.114.166421/-/DC1>.