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HP1 and BRCA1 proteins could enhance heterochromatin spread throughout the genome: The *Ctenomys* (Rodentia Ctenomydae) model

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ABSTRACT

The variation of heterochromatin amount and localization using the genus *Ctenomys* as a model is analyzed in this work. This genus exhibits a significant variation in heterochromatin localization that range from pericentric areas in few chromosomes to whole heterochromatic arms. Its karyotypic variation includes the occurrence of a large number of chromosomal rearrangements (2n=10 to 70; NF=16 to 80) during its evolution. The possible mechanism which generated heterochromatin variation was analyzed in the meiotic nuclei, mainly in the pachytene of *Ctenomys* species from Uruguay. In the pachytene stage, several bivalents merge in densely stained chromocenters that are positive for C-banding and DAPI staining. Additionally, "*in situ*" hybridization was used with the repetitive PvuII *Ctenomys* sequence (RPCS) as a probe which was also localized in the pachytene chromocenters. In order to understand the heterochromatin behaviour in the chromocenter, we applied immunolocalization for two proteins: HP1 a chromatin gene silencer which transforms euchromatin into heterochromatin and BRCA1 a double stranded break (DSB) DNA repair factor. Positive immune signals for both proteins in the pachytene chromocenters suggests that the mechanics of meiosis could break DNA in the chromocenters due to physical forces leading to metaphase I. Based on these data we propose that heterochromatin exchanges could imply double strand breaks produced by these forces that in the repairing process may conduce to the spreading of heterochromatin. This also can explain the colocalization of BRCA1 and HP1 signals.

KEYWORDS: HP1, BRCA1, heterochromatin, meiosis

INTRODUCTION

For the first time [1] described two types of chromatin in accordance with differential staining of nuclei: euchromatin and heterochromatin. He also hypothesized that ''euchromatin is genetic active and heterochromatin is genetic passive". In this way, heterochromatic chromosomes or zones of chromosomes with heterochromatin do not contain genes or at least active genes [2]. The possible function of heterochromatin was unknown until the discovery of position effect variegation (PEV) in *Drosophil*a [3]. James and Elgin described a protein associated to heterochromatin involved in gene silence and PEV called HP1 [4]. A striking characteristic of this protein is its high degree of evolutionary conservation [5] from *Drosophila* to man. This protein is involved in chromatin condensation and gene silencing [6] and gene large scale regulation by means of interaction with the methylated lysine 9 of histone H3 [7].

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Also was proposed as a chromosome rearrangement enhancer factor [8, 9]. Taking into account these previous data we used the meiosisof the genus *Ctenomys* from Uruguay as a model to analyze HP1 localization, due mainly to the variation in heterochromatin amount and location. In Uruguay three nominal species have been recognized: *Ctenomys pearsoni*, 2n=70 [10] in the Southwest; *Ctenomys torquatus*, 2n=44 [11, 12] located in the North; and *Ctenomys rionegrensis*, 2n=50 [13] situated in the West of the country. Species that inhabit the coast of the Rio de la Plata River constitute the so called "Ctenomys pearsoni complex" based on the similarities in cranial and penial morphology with respect to the *C. pearsoni* species. Chromosome differentiation also includes a high degree of variation in the amount and localization of heterochromatin. This variation ranges from C positive bands at the centromeric area of a few chromosomes (*C. pearsoni* complex) to karyotypes with totally heterochromatic short arms in their chromosomes as in *C. rionegrensis* [14, 15, 16, 17, 18]. In the heterochromatin is located the major satellite DNA of *Ctenomys* named RPCS (repetitive PuvII *Ctenomys* sequence) which is organized in long arrays of 348 bp-monomer units [19, 20, 14]. Several "*in situ*" hybridization studies developed in *Ctenomys* species from Uruguay and Argentina showed RPCS located in chromosomal regions positive for C-bands and DAPI [20, 14]. It is often assumed that repetitive DNA could be causally related with the occurrence of chromosomal rearrangements, [21, 22, 23] although the precise mechanisms related to these phenomena remain elusive. In order to gain understanding in heterochromatin variation we analyzed the pachytene of the meiosis in *Ctenomys* since in this stage, we observed the fusion of different bivalents establishing non homologous associations of chromosomes in chromocenters. We used immunolocalization with the conserved domain M31 of HP1 in pachytene cells to map this heterochromatin associated protein. We also map BRCA1 [24] an anti tumor protein directly implicated in DNA double strand break repair. Based on the results obtained we propose a model to explain the variation in amount and localization of heterochromatin throughout different karyotypes.

MATERIALS AND METHODS

Somatic chromosome preparations and banding procedures

Animals from Uruguay were captured alive using Oneida Victor traps N° 0 in the following locations: *C. pearsoni* (Autódromo Nacional), 2n=70 (eight individual), Canelones population (Jaureguiberry), 2n=58 (eight individuals), Solis population (Solis), 2n=70 (ten individuals), Chihuahua and Barra Portezuelo populations (Maldonado), 2n=66 (six individuals), José Ignacio population (Maldonado), 2n=64 (eight individuals), *C. rionegrensis* (Las Cañas, Rio Negro), 2n=50 (ten individuals), and *C. torquatus* (Tacuarembó), 2n=44 (ten individuals) and Villa Serrana (Lavalleja), 2n=44 (six individuals) (Fig. 1). Voucher specimens were deposited in the collection of the Sección Zoología Vertebrados, Facultad de Ciencias (Montevideo, Uruguay). To analyze karyotypes and somatic C bands, bone marrow cells were used after treatment with a hypotonic solution (KCl 0.075 M) during 20 min at RT. Chromosome preparations were obtained after 3 fixation rounds (10 min. each) of methanol-acetic acid 3:1 and stained with Giemsa (Merck, Darmstadt) 5% in distilled water for 5 min. To obtain C bands air dry slides were treated with HCl 0,2 N for 30 min. After a distilled water wash, slides were treated with a saturated barium hydroxide solution at 50°C during 6 min. and a 2x SSC solution for 20 min. at 60°C. Slides were stained with Giemsa (Merck, Darmstadt) 5% in distilled water for 8 minutes.

Meiotic prophase preparations

The meiotic prophase cells were obtained after treating minced testes with a hypotonic solution (KCl 0.075 M) during 20 min at RT. After three rounds of methanol-acetic acid fixation (10 minutes each) and cell centrifugation (650 g/5 minutes) after the last fixation step, cells were dispersed over clean slides and air dried.

Synthesis of fluorescent probes and *in situ* hybridization

The highly repetitive DNA from *Ctenomys* was labeled with Alexa 594 using nick translation (Molecular Probes, USA). The chromosome preparations were hybridized following [25]. Typically the hybridization mixture contained 10%

Fig. 1. Map from Uruguay indicating the sites where specimens were collected. *C. torquatus* ▲; *C. pearsoni* □; *C. rionegrensis* ●; Canelones ▲; Chihuahua/Barra de Portezuelo ■; José Ignacio △; Solís ○.

SDS 2x SSC, 50% dextran sulphate, 1 mg sheared salmon sperm DNA and 50-100 ng labeled probe. The chromosome preparations were denatured on a heat block for 8 min at 72-78°C (depending on age of preparation). Following overnight hybridization, preparations were washed and hybridization sites detected as appropriately; the most stringent washes were in 2x SSC with 40% formamide at 42°C. Slides were counterstained with DAPI (Sigma-Aldrich, St. Louis, USA) and photographed with a Nikon Microphot FX epi-fluorescence microscope with appropriately filters using a Nikon D70 digital camera.

Indirect immunofluorescence

Meiotic cells were fixed with 1% paraformaldehyde for 15 minutes, immersed in cold PBS (5°C) overnight and gently squashed between slide and cover slip, this was later removed after immersion in liquid nitrogen [26]. Heterochromatin protein was detected by incubating slides overnight (RT) with the M31 (Dako) primary antibody (1:200) and with the secondary antibody (Cy3-tagged) (1:400) for 90 min. washed in cold PBS for 5 min. BRCA1(Dako, USA) protein was detected after overnight incubation of the slides in primary antibody (1:200) and a 1:400 secondary Cy5 tagged antibody for 30 min and washed in PBS (Abcam, Cambridge USA). After immunostaining all the slides were mounted in antifade solution (p-Phenylenediamine (Sigma-Aldrich, St. Louis, USA) 0,2 % in non-fluorescent glycerol) with 2µg/ml of DAPI. A Nikon Microphot FX epi-fluorescence microscope with appropriate filters and a Nikon D70 digital camera were used for image capture.

RESULTS

Table I shows the characteristics of the karyotypes analyzed here. The location of heterochromatin in the species analyzed is showed in Fig. 2. Population Solís presented five pericentric C banded

chromosomes, four telocentric and one submetacentric (Fig. 2a). The *C. pearsoni* species present two telocentric and two metacentric chromosomes positive for C-bands (Fig. 2b). Population Canelones (Fig. 2c) showed two pairs of C band positive chromosomes (one telocentric and one submetacentric) while José Ignacio population has positive signals for six metacentric and four telocentric chromosomes (Fig. 2d). Chihuahua showed three pairs of telocentric and one pair of metacentric chromosome positives for C-banding (Fig. 2e) The *Ctenomys rionegrensis* species presented striking differences in C-banding patterns to those exhibited by the *Ctenomys pearsoni* populations, showing heterochromatic whole arms in all the chromosomes. Finally *C. torquatus* presented two pairs of metacentric chromosome with pericentric C bands.

Analysis of meiotic prophase

The highly repetitive DNA (RPCS) of *Ctenomys* was localized after "*in situ*" hybridization in three chromocenters in *C. rionegrensis* (Fig. 3a) and in only one in the pachytene cells of the *C. pearsoni* complex (Fig. 3b). The immunofluorescence with the M31 antibody (HP1) in pachytene cells of *C. rionegrensis* demonstrated strong positive signals coincident partially with the pattern described above (Fig. 3c). The BRCA1 signals in the same cells colocalize partially with the above mentioned (3d): After a detailed analysis of BRCA1 and M31 localization we found areas

Table I. Main values of the karyotypes analyzed here.

	Meta/subm.	Telocent.	2n	FN	C bands
C. pearsoni	6 +XY	28	70	80	2 pair
Pop.Chihuaua/BP	7 + XY	25	66	78	4 pair
Pop. Jose Ignacio	7 + XY	24	64	76	6 pair
C. torquatus	15 + XY	6	44	72	3 pair
Pop. Solis	6 + XY	28	70	80	6 pair
Pop. Canelones	11 + XY	17	58	78	2 pair
C. rionegrensis	10 + XY	14	50	68	7 pair

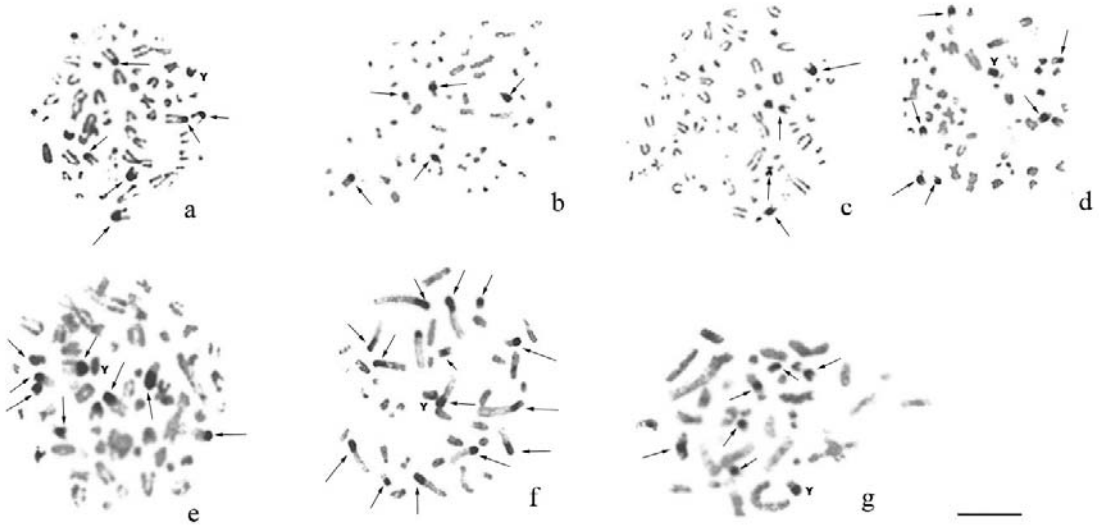

Fig. 2. C-banded chromosomes from: Solís (a), *C. pearsoni* (b), Canelones (c), José Ignacio (d), Chihuahua (e), *C. rionegrensis* (f) and *C. torquatus* (g). Arrow point C-band. Bar 10µm.

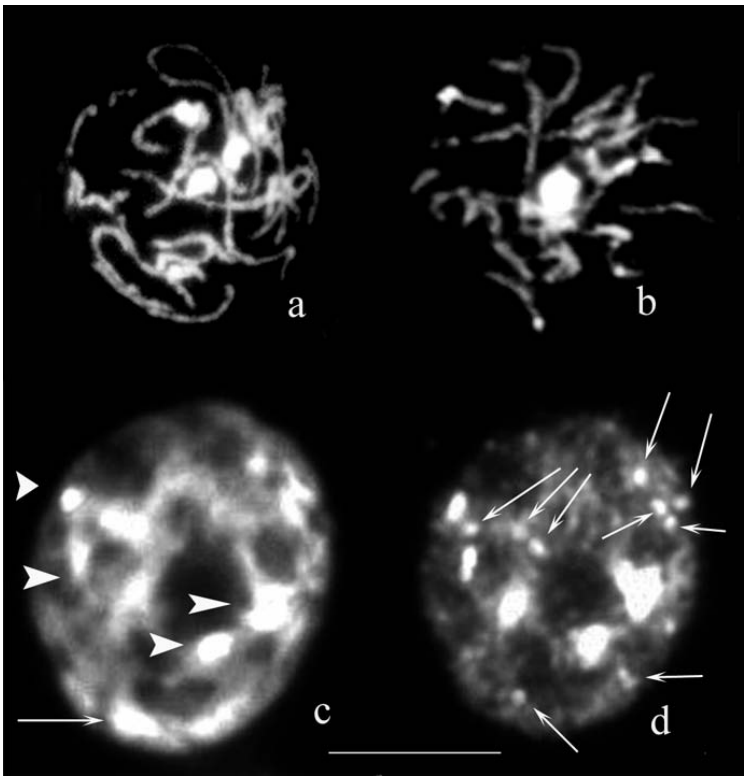

Fig. 3. *In situ* hybridization with the RPCS probe in: (a) *C. rionegrensis,* (b) Barra de Portezuelo (*C. pearsoni* complex). Inmunostaining with M31 antibody in: (c) *C. rionegrensis* pachytene and d) BRCA1 inmunostaining in the same cell; areas with non- co-localizing signals marked with arrows and arrow tip for co-localizing signals.
Bar 10µm.

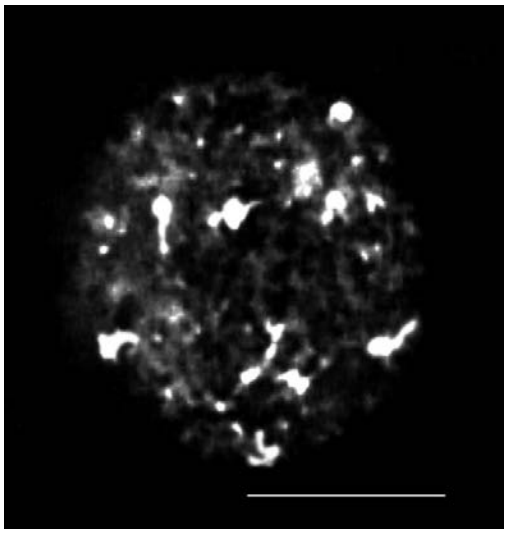

Fig. 4. Diffuse stage of *C. rionegrensis* after HP1 immunostaining showing the trace of pachytene chromocenter.

where both signals do not colocalize. The BRCA1 signals are often observed out of the chromocenters as indicated in Fig. 3d and by arrows, the other signals colocalize with M31 as those marked with arrow heads (Fig. 3c). In the diffuse stage in *C. rionegrensis* after HP1 localization no chromocenters were observed since they seem to be collapsed in a more open nuclei structure except in those places where heterochromatin maintain their condensed structure (Fig. 4).

DISCUSSION

Meiotic prophase analyses

It is important to remark the effect of heterochromatin as a recombination suppressor during meiosis, suggesting a regulatory function of the HP1 protein at a large scale [27, 28]. To analyze heterochromatin dynamics, we compared

its localization in the meiotic pachytene of the *C. rionegrensis* (higher heterochromatin amount: 25%) and the *C. pearsoni* complex (lower heterochromatin amount: from 5% to 11, 6%) [29]. The RPCS "*in situ*" hybridization and the immunostaining using the HP1 antibody showed colocalization and a direct association between the number of the chromocenters in the pachytene stage and the heterochromatin amount, observed in metaphases [29]. In *C. rionegrensis* pachytene, chromosomes bivalent merge to shape three chromocenters in a non-homologous chromosome association [18]. While in the *C. pearsoni* complex only one chromocenter was observed, where non-homologous association occurs. This kind of non-homologous association was also observed in insects [30], plants [24, 31] and mammals [32, 33, 34]. We suggested [18, 29] the conversion of euchromatin into heterochromatin, as one of the possible mechanisms involved in chromosome evolution mainly because of the DNA amount preservation [16, 35] through the *Ctenomys* species. Our model is based on the mechanics of meiosis that could enhance the exchange of heterochromatin between the non-homologous bivalents attached to the chromocenters. Heterochromatin exchanges might occur between the end of the pachytene stage, through the diffuse stage and the beginning of mitosis I in meiotic cycle, where physical stress could generate double strand breaks [36, 37, 38, 29]. These double strand breaks (DSB) might be repaired [39] by BRCA1 and the localization of positive BRCA1 signals in the late pachytene and at the beginning of the diffuse state (previous to metaphase I), strongly supports this fact. BRCA1 is involved in different functions as nuclear process of transcription, chromatin remodelling and repair mechanisms of double strand breaks (DSB) [40]. Recently [41] reported that, in *Drosophila*, double strand breaks in heterochromatin, induced by radiation, are repaired by homologous recombination (HR) which is different from what occurred in euchromatin. The main difference is that heterochromatic DNA repair is situated outside of the bulk of chromocenters in order to avoid error prone repair. The results showed here strongly suggest the occurrence of some BRCA1 foci which are located outside of the bulk of heterochromatin chromocenters that do not colocalize with M31 fluorescence mass. This could suggest the occurrence in *Ctenomys*, of a heterochromatin repairing process for DSB, in the way described by [41]. The whole model that states that heterochromatin could enhance chromosome variation should include: a) non-homologous heterochromatin merging in pachytene, to shape chromocenters to which HP1 protein are associated; b) reparation by BRCA1 of DSB in heterochromatin DNA, when bivalents begin to break the chromocenter structure before the diffuse stage. The separation of bivalents could include physical forces producing stress enough to produce minimal differential heterochromatin interchange between non homologous bivalents [42]. This process could produce the horizontal movement of heterochromatin throughout the genome (repeated DNA and HP1 chromatin modifying factor), increasing copy number change and possibly chromosome changes. Possibly also the amount of heterochromatin could be increased in new places of the chromosomes or the karyotype due to the action of HP1. Furthermore the chromosome rearrangement landmarks, as fragile sites are located in heterochromatic as well as in euchromatic regions of the chromosomes [43]. In this paper we analyzed the location of heterochromatin (M31) and the repair factor BRCA1 in meiotic chromocenters. These areas showed to disaggregate at the end of pachytene through the diffuse stage where M31 presented abundant signals. These changes introduce the physical properties of chromatin and DNA as an important factor leaving clear evidence about the plasticity of chromatin, even in the more condensed state [44, 45, 46].

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REFERENCES

1. Heitz, E. 1928, I. Jahrb. Wiss. Botanik, 69, 762-818.
2. Heitz, E. 1930, Ber. Bot. Ges., 47, 274-284.
3. Muller, H. J. 1930, J. Genet., 22, 299-234.
4. James, T. C. and Elgin, S. C. 1986, Mol. Cell Biol., 6, 3862-72.

5. Wreggett, K. A., Hill, F., James, P. S., Hutchings, A., Butcher, G. W. and Singh, P. B. 1994, Cytogenet. Cell Genet., 66, 99-103.
6. Weiler, K. S. and Wakimoto, B. T. 1995, Annu. Rev. Genet., 29, 577-605.
7. Singh, P. B. and Georgatos, S. D. 2003, Journal of Structural Biology, 140, 10-16.
8. Kellum, R. 2003, Curr. Top. Microbiol. Immunol., 274, 53-77.
9. Wang, G., Chow, M. A., Horsley, C. M., Brown, N. R., Cowell, I. G. and Singh, P. B. 2000, Mol. Cell Biol., 20, 6970-6983.
10. Lessa, E. P. and Langguth, A. 1983, Resúmenes y Comunicaciones de las Jornadas de Ciencias Naturales de Montevideo, 86-88.
11. Kiblisky, P., Brum-Zorrilla, N., Pérez, G. and Sáez, F. A. 1977, Mendeliana, 2, 85-93.
12. Freitas, T. R. O. and Lessa, E. P. 1984, J. Mammal., 65, 637-642.
13. Reig, O. A., Bush, C., Ortells, M. O. and Contreras, J. R. 1990, Evolution of Subterranean Mammals at the Organismal and Molecular Levels, Edited by Nevo, E., Reig, O. A. and Alan, R. Liss, pp. 71-96.
14. Freitas, T. R. O. 1994, Cytogenetics and Cell Genetics, 67, 193-198.
15. Rossi, M., Redi, C., Viale, G., Massarini, A. and Capanna, E. 1995, Cytogenet. Cell Genet., 69, 179-184.
16. Novello, A., Cortinas, M. N., Suárez, M. and Musto, H. 1996, Chromosome Research, 4, 335-339.
17. Villar, S., Martinez, W., Folle, G. and Novello, A. 2005, Mamm. Biol., 70, 255-260.
18. Novello, A. and Villar, S. 2006, Genetica, 127, 303-309.
19. Novello, A., Brum-Zorrilla, N., Musto, H., Bruzzoni, H. and Stoll, M. 1988, Montevideo, Uruguay: V Reunión Iberoamericana de Conservación y Zoología de Vertebrados Res., p 56.
20. Rossi, M., Reig, O. and Zorzopulos, J. 1990. Mol. Biol. Evol., 7, 340-350.
21. Bradley, R. and Wichman, H. 1994, Chromosome Res., 2, 354-360.
22. Garagna, S., Zuccotti, M., Redi, C. and Capanna, E. 1997, Nature, 390, 241-2.
23. Slamovits, C. H., Cook, J. A., Lessa, E. P. and Rossi, M. S. 2001, Mol. Biol. Evol., 18, 1708-1719.
24. Less-Miller, S. and Meek, K. 2003, Biochimie., 85, 1161-1173.
25. Schwarzacher, T. and Heslop-Harrison, P. 2000, Practical "*in situ*" Hybridization, 60-65, BIOS Scientific Publishers UK.
26. Novello, A., Kralewski, M. and Benavente, R. 1996, Chromosome Res., 4, 622-623.
27. Singh, P. B. and Georgatos, S. D. 2003, J. Struc. Biol., 10-16
28. Xu, Y. and Brendan, D. Price. 2011, Cell Cycle, 10(2), 261-267.
29. Novello, A., Villar, S. and Urioste, J. 2010, Cytogenet. Genome Res., 128, 152-161.
30. Chubykin, V. 2009, Russian Journal of Genetics, 45, 1027-1039.
31. Naranjo, T. and Corredor, E. 2008, Cytogenet. Genome Res., 120, 320-330.
32. Oud, J. L. and Reutlinger, A. H. 1981, Chromosoma, 83, 395-407.
33. Switonsky, M. and Gustavsson, I. 1991, Cytogenet. Cell Genet., 57, 1-8.
34. Kauppi, A., Jeffreys, J. and Keeney, S. 2004, Nature Reviews Genetics, 5, 413-424.
35. Ruedas, L. A., Cook, J. A. and Yates, J. W. 1992, Genome, 36, 449-458.
36. Cui, Y. and Bustamante, C. 2000, Proc. Natl. Acad. Sci. USA, 97, 127-132.
37. Bennink, M. L., Sanford, L., Leno, G. H., Zlatanova, J., de Grooth, B. and Greve, J. 2001, Nature (Structural Biology), 8, 606-610.
38. Meena, S., Leyma, P. and Nickoloff, J. 2008, Cell Research, 18, 134-147.
39. White, D., Rafalska-Metcalf, I. U., Ivanov, A. V., Corsinotti, A., Peng, H., Sheng-Chung Lee, Trono, D., Janicki, S. M. and Rauscher, F. 2011, Mol. Cancer Res., 1-14.
40. Stanta, L. and Parvin, J. 2003, Curr. Op. Cell Biol., 15, 345-350.
41. Chiolo, I., Minoda, A., Colmenares, S. U., Polyzos, A., Costes, S. V. and Karpen, G. H. 2011, Cell, 144, 732-744.
42. Nicklas, R. B. 1983, J. Cell Biol., 97, 542-548.
43. Eissenberg, J. C. and Reuter, G. 2009, Int. Rev. Cell Mol. Biol., 273 , 1-47.
44. Marko, J. F. 2008, Chromosome Research, 16, 469-497.
45. Mazumder, A. Roopa, A., Basu, A., Mahadevan, L. and Shivashankar, G. V. 2008, Biophysical Journal, 95, 3028-3035.
46. Ortiz, V. and de Pablo, J. J. 2011, Phys. Rew. Letters (PRL), 106, 238107.