Am. J. Hum. Genet. 59:1091–1096, 1996

Analysis of Loss of Inactive X Chromosomes in Interphase Cells

J. Surrallés,[1] P. Jeppesen,[2] H. Morrison,[2] and A. T. Natarajan[1]

[1]MGC—Department of Radiation Genetics and Chemical Mutagenesis, Leiden University, Leiden; and [2]MRC—Human Genetics Unit, Western General Hospital, Edinburgh

Summary

We have developed a method that allows, for the first time, a specific analysis of the inactive X chromosome (Xi) in interphase cells. By combining immunolabeling of acetylated histone H4 with specific antisera and FISH with an X-chromosome centromere–specific DNA probe, micronucleated whole Xis in human female cells may be identified by their lack of histone H4 acetylation. As one example of the potential applications of this methodology in genetic studies in humans, an artifact-free X-chromosome aneuploidy detection in lymphocytes of women of different ages has been performed. Our results indicate that not only the Xi but also the active X chromosome is preferentially lost during aging, indicating that the high frequency of sex-chromosome aneuploidy in human females cannot be explained solely by a lack of negative selection of Xi aneuploid cells. Further applications of the proposed methodology in genetic studies are discussed.

Introduction

The X chromosome is unique among human chromosomes, both because of the phenomenon of X inactivation to compensate gene dosage in females (Lyon 1961) and because of the existence of many important X-linked diseases (Mandel et al. 1992). The inactivation status of all but one X chromosome in mammalian cells has attracted the attention of many geneticists, leading to the discovery of a number of special features of the inactive X chromosome (Xi), such as in DNA hypermethylation (Mohandas et al. 1981), underacetylation of histones (Jeppesen and Turner 1993; Turner 1993), and the requirement for the presence and transcription of the XIST gene (Brown et al. 1991, 1992; Penny et al. 1996). Nevertheless, the exact mechanism of X inactivation remains a mystery (Lyon 1996).

Aneuploidy is strongly correlated with spontaneous abortion, mental retardation, and carcinogenesis. Early studies suggested an association between aneuploidy and aging, mainly involving the sex chromosomes (Jacobs et al. 1961, 1963; Galloway and Buckton 1978; Nowinski et al. 1990). However, aneuploidy studies at metaphase are technically limited, owing to both chromosome loss during slide preparation and the difficulties of finding enough aneuploid metaphases to acquire statistically significant results. These factors are even more of a problem when the Xi is considered, since it lies peripheral in the nucleus and therefore is prone to artifactual loss during mitotic preparation. Until now, the analysis and visualization of the Xi has been limited to cells at metaphase, and this has required the use of either BrdUrd pulse treatments to identify the late-replicating Xi (Willard and Latt 1976; Abruzzo et al. 1985) or 5-azadeoxycytidine specifically to decondense the Xi (Haaf et al. 1993). An alternative is to use karyotypically abnormal cells with, for instance, a translocation involving only one X chromosome (Tucker et al. 1996). However, chemical approaches could modify actual in vivo aneuploidy levels, for instance, by causing mitotic delay to the already-late-replicating Xi. On the other hand, the effect of aging cannot be analyzed with karyotypically abnormal cells, where the influence of the translocation on the aneuploidy level is impossible to control. A previous study employed aged mentally retarded women to enhance the probability of finding X-chromosome aneuploid metaphases (Abruzzo et al. 1985). However, the possibility of including constitutional X-chromosome mosaicisms when retarded women are studied cannot be discounted.

The aim of the present study was to develop a method circumventing all the limitations of sex-chromosome aneuploidy studies at metaphase. Using rabbit antisera specific for acetylated isoforms of histone H4, one of us (P.J.) recently reported that the Xi in female mammals is distinguished at metaphase by a lack of histone H4 acetylation (Jeppesen and Turner 1993), seemingly reflecting the correlation between histone acetylation and gene transcription (David and Candido 1978; Chahal et al. 1980). In the present report, we combine immunola-

Received May 23, 1996; accepted for publication August 12, 1996.

Address for correspondence and reprints: Dr. Jordi Surrallés, MGC—Department of Radiation Genetics and Chemical Mutagenesis, Leiden University, Wassenaarseweg 72, 2333 AL-Leiden, The Netherlands. E-mail: surralles@rullf2.leidenuniv.nl

0002-9297/96/5905-0018$02.00

beling of acetylated histone H4 with specific antisera and FISH with an X-chromosome centromere–specific DNA probe. With this approach, whole Xis lost at anaphase of human lymphocytes were identified as micronuclei (MN) lacking histone H4 acetylation. Using this novel cytogenetic technique, we assessed and compared the levels of loss of the Xi and the active X chromosome (Xa) in healthy women of various ages. Further applications of the methodology introduced here are discussed.

Material and Methods

Cell Culturing and Slide Preparation

Mononuclear white cells were obtained from heparinized blood of four women, ages 24, 27, 57, and 60 years, by use of Lymphoprep gradients. Cells were cultured at 37°C, 5% CO_2, in RPMI 1640 medium containing 10% FCS, 1 mM oxaloacetic acid, 0.4 mM pyruvic acid, 0.2 IU insulin/ml, 2 mM BL-glutamine, and 1% phytohemagglutinin. Forty-four hours after culture initiation, cells were incubated with 6 μg cytochalasin-B (Sigma)/ml to block cytokinesis and identify cycling first-division cells by their binucleated appearance. Twenty-eight hours later, cells were harvested by centrifugation at 1,000 rpm for 10 min and were resuspended in 2 ml of 75 mM KCl. After hypotonic swelling for 10 min at 37°C, the cell suspensions were ice-cooled. Cytospin preparations and subsequent immunolabeling were carried out essentially as described elsewhere (Jeppesen and Turner 1993), by use of 0.5×10^5 cells/slide, centrifugation for 10 min at 2,000 rpm, and an Ames Cyto-Tek centrifuge (Bayer Diagnostics).

Immunolabeling

After cytocentrifugation, slides were briefly dried and immersed in KCM (120 mM KCl, 20 mM NaCl, 10 mM Tris-HCl [pH 8.0], 0.5 mM EDTA, and 0.1% [v/v] Triton X-100) for ⩾10 min at room temperature (RT) to solubilize the cell membrane. Slides were then removed from KCM and dried around the sample area, onto which was gently pipetted 40 μl of a 1:100 dilution of a rabbit antiserum against histone H4 acetylated at Lys-12 (R5/12) in KCM containing 10% normal goat serum. The solution was covered by a 1-cm square of paraffin film, and the slides incubated at RT for 1–2 h in a humid chamber. After the first antibody incubation, the paraffin film was gently removed, and the slides were rinsed three times, 5 min each, in KCM. Primary antibody binding was detected with a secondary affinity-purified goat anti-rabbit IgG, conjugated to fluoroisothiocyanate (FITC), and diluted 1:20 in KCM and 10% normal goat serum. The second incubation was carried out for 30 min, as described above, and the slides were rinsed in a similar manner. Then the slides were post-fixed in a 10% solution of formaldehyde for 15 min at RT, were rinsed in water, air-dried, and aged for 2 d at RT before FISH analysis.

FISH

Slides were pretreated in the following way: 30 s in 0.1 M NaOH, 30 s in dH_2O, 30 s in 10 mM Tris pH 7.5, and 30 s in dH_2O (all washes at RT). The slides were then dehydrated in 70%, 90%, and 100% alcohol, 2 min each, and were vacuum dried. Slides were further immersed in a solution containing 50 ml H_2O, 43 μl 37% HCl, and 125 μl of 2% pepsin (Sigma) and were dehydrated as described above. Ten microliters hybridization mix was pipetted onto each slide. The hybridization mix was made of 50 μl deionized formamide, 10 μl of 20 × SSC, 500 μg salmon sperm DNA (10 μl; Sigma)/ml, 12 μl X-chromosome centromere–specific DNA probe, DXZ1, directly labeled with FluoroRed (rhodamine)-dUTP (Amersham), 10% dextran sulfate (10 μl; Pharmacia), and 8 μl dH_2O, to make a final volume of 100 μl. Slides were then covered with a coverslip, sealed with glue, denatured at 70°C for 5.5 min in a metal tray, and then were transferred to another metal tray at 45°C for overnight hybridization. The next day, the glue was removed, and the slides were washed four times, 3 min each, in 50% formamide and 2 × SSC at 45°C, followed by four washes, 3 min each, in 2 × SSC at 45°C. Slides were then immersed for 5 min in 4 × SSC and 0.1% Tween-20, were mounted in Vectashield antifading solution (Vector) containing 2 μg DAPI/ml, were sealed with glue, and were stored at 4°C until microscopy.

Microscopy

Images were captured with a Zeiss Axioplan fluorescence microscope equipped with a triple-bandpass filter set (Omega) to allow FITC, rhodamine, and DAPI images to be seen by selection of the appropriate excitation filter. Images were collected with a cooled CCD camera (Photometrics) and were processed with IP-Lab software in a Power PC Apple Computer. Slide scanning was performed with a Zeiss Axioscope fluorescence microscope also equipped with a triple-bandpass filter and three different single filters to allow rhodamine, FITC, and DAPI to be seen separately. A total of 3,000–4,000 cells/donor were analyzed for MN; once an micronucleus was localized with the DAPI filter, the presence of X-chromosome centromere–specific signal as well as antiacetylated histone H4 signal, was checked with specific filters. Only binucleated cells with a correct distribution and number of X-chromosome centromere–specific signals as well as clearly visible acetylated histone H4 immunolabeling in both nuclei were analyzed.

Results and Discussion

In order to assess accurately X-chromosome loss in normal individuals of different ages and to distinguish the Xa from the Xi in female interphase cells, we developed a new method based on a novel cytogenetic technique. We have previously reported that the acetylated form of histone H4 is a useful cytogenetic marker of gene expression (Jeppesen and Turner 1993). By including the histone deacetylase inhibitor sodium butyrate (Sealy and Chalkley 1978) to reduce deacetylation during preparation, even weak sites of acetylation, such as that seen in the pseudoautosomal active region of the Xi, can be detected (Jeppesen and Turner 1993). By inhibiting deacetylation for longer periods during the cell cycle, it also was inferred that histone H4 in the Xi remains underacetylated throughout interphase. We now have obtained further evidence for this deduction, by extending the previous approach to study X-chromosome aneuploidy during interphase in human lymphocytes. A previous study has demonstrated that chromosomes that fail to attach to the spindle at metaphase lag at anaphase and are eliminated in MN and that micronucleation thus is a major cause of hypodiploidy (Ford et al. 1988). That study was conducted in cytokinesis-blocked cells obtained with cytochalasin-B, a drug that specifically inhibits cytokinesis but that does not affect chromosome segregation.

In the present study, randomly micronucleated X chromosomes in cytokinesis-blocked binucleated cells were identified by FISH (Pinkel et al. 1988) using an alphoid X-chromosome centromere–specific DNA probe. Simultaneously, the histone H4 acetylation status of MN was determined by immunofluorescence with antiacetylated histone H4 antiserum. MN labeled by the X-specific alphoid probe showed two distinct patterns of antiacetylated histone H4 immunofluorescence: those that were positively labeled and those that were unlabeled, which we interpret as MN harboring Xas and Xis, respectively (fig. 1). Our results, summarized in table 1, show that, of a total of 353 MN analyzed, 86 (24.4%) harbored X chromosomes (X+), in accordance with previous studies reporting an overrepresentation of the X chromosome in MN of women (Catalán et al. 1995, and references therein). Eighty-one percent of MN lacking histone H4 acetylation (H−) were found to carry an X-chromosome centromere–specific FISH signal, unequivocally indicating that they harbored an X chromosome. MN lacking both histone H4 acetylation and the X-chromosome centromere–specific signal (H−X−) were presumed to be derived from acentric fragments of the Xi. This assumption was verified by relocating the H−X− MN after FISH painting with a whole X chromosome–specific DNA probe (data not shown). The low frequency of H−X− MN found (3.1% of total MN) further supports our interpretation of the data. Table 1 and figure 2 show that approximately half of the X+ MN were unlabeled by antiacetylated histone H4 antiserum, from which it seems reasonable to infer that unlabeled MN contain the Xi, since the Xi constitutes half of the total X-chromosome complement. If a proportion of all MN remain unlabeled for some possibly artifactual reason other than that they are derived from the Xi, we should expect as many as half of the non-X–derived MN to be H−, which is clearly not the case (96% of X− MN are indeed H+) . Since this study focuses on the loss of whole X chromosomes, H−X− MN have not been included in the numerical analysis.

Interestingly, our data show that not only Xis but also Xas are commonly lost in human lymphocytes of karyotypically and mentally normal women and that this effect is strongly increased with age. Thus, when young (24–27 years old) and older (57–60 years old) women are compared, the frequencies of MN harboring the Xa and the Xi are increased 2.5-fold ($P = .007$) and 3.4-fold ($P = .0002$), respectively (table 1 and fig. 2).

Although, in three of the four donors studied, the frequency of MN harboring Xis slightly exceeded the frequency of those containing Xas, one (JF; table 1) showed the reverse result. It is possible that statistical variation in sampling is obscuring a small excess, when compared with Xas, of Xis lost in the cell population, which would be evident in a larger sample. Within the limits of experimental error, however, our results show no significant differences between the rates of Xa loss and Xi loss, in contrast to previous findings (Abruzzo et al. 1985; Tucker et al. 1996), which have suggested a preferential involvement of the Xi.

If there is, at anaphase, an increased tendency to lose the Xi compared with the Xa, then our data show that it is very small, much less than has been suggested by the study by Tucker et al. (1996). However, the Xa elements in that study were part of a balanced autosomal translocation, and the effects of the autosomal partners on the loss of the Xa components cannot be predicted. Abbruzzo et al. (1985) found a preferential participation of the Xi in X-chromosome aneuploidies in the lymphocytes of aging retarded women. That study, by its very nature, could not exclude the possibility that both X chromosomes are equally at increased risk of nondisjunction or anaphase lagging but that subsequent selection removed cell lineages harboring Xa aneuploidies, which, on the basis of our data, we would suggest is the case. The present study cannot be influenced by selection, since, because of the use of cytochalasin-B, it scores X chromosomes lost immediately after the anaphase at which the loss occurred. Our data clearly (*a*) show that sex-chromosome aneuploidies in women cannot be ex-

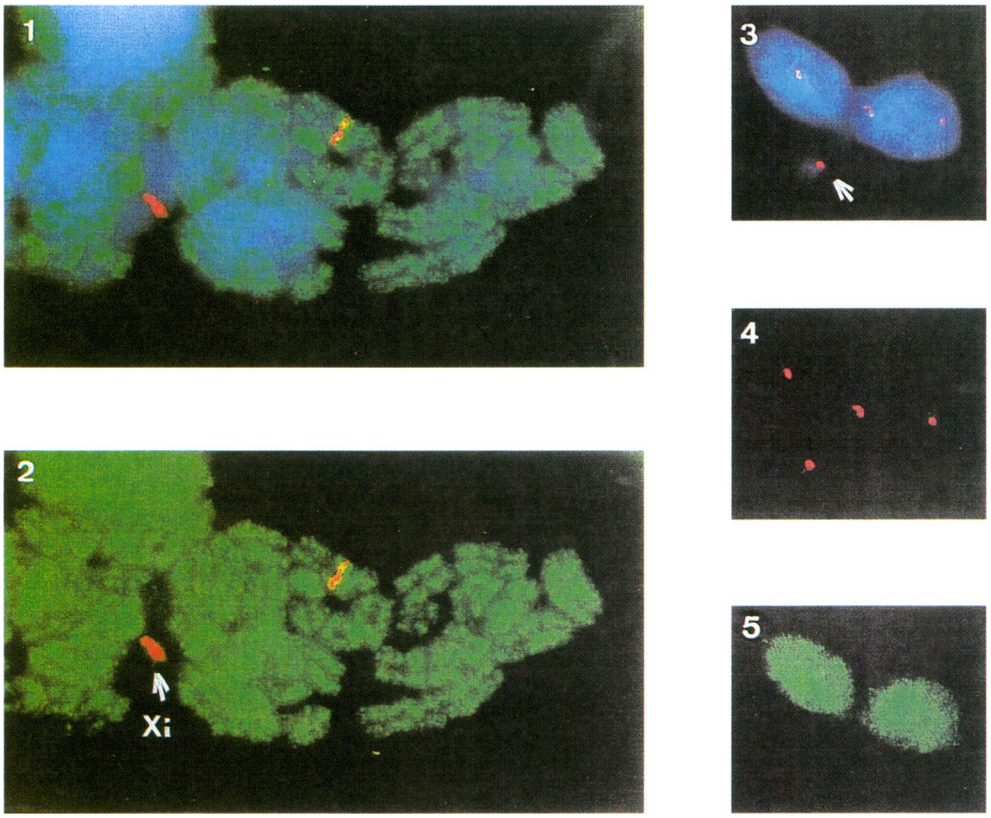

Figure 1 Fluorescence microscopic visualization of the Xi in metaphase and interphase. *1*, Non–acid-fixed metaphase showing triple-color DAPI (*blue*), FITC (*green*), and rhodamine (*red*), corresponding to DNA counterstaining, acetylated histone H4 immunolabeling, and X-chromosome centromere–specific FISH, respectively. *2*, Same metaphase as in *1*, with FITC and rhodamine signal, clearly showing the underacetylated Xi (*arrow*). *3*, Interphase 72 h–cultured, cytokinesis-blocked human lymphocyte with an micronucleus harboring an Xi (*arrow*). *4*, Same cell as in *3*, under rhodamine excitation, showing the presence of an X chromosome within the micronucleus. *5*, Same cell again, with an FITC excitation, demonstrating underacetylation of histone H4 within the interphase micronucleus.

plained adequately by just a lack of negative selection of Xi aneuploid cells and (*b*) indicate a constitutive defect in the machinery involved in mitotic segregation of X chromosomes.

This new method combining immunocytogenetics and in situ hybridization circumvents possible artifacts, since it is performed in intact, non–acid fixed, karyotypically normal, interphase cells spun directly onto slides, and

Table 1

Simultaneous Immunolabeling of Acetylated Histone H4 and X-Chromosome Centromere–Specific FISH in MN of 72 h–cultured Cytokinesis-Blocked Human Lymphocytes from Four Women

Donor (Age [in Years])	No. of Cells Analyzed	No. of MN Found	Acetylated Histone H4 and X-Specific Signals in MN[a]			
			H+X−	H−X−	H+X+	H−X+
JF (24)	3,532	69	58	1	6	4
SB (27)	4,093	115	97	3	7	8
ML (57)	3,491	81	47	2	14	18
SM (60)	2,984	88	54	5	13	16

[a] H+/H− = positive/negative signal after immunolabeling with antibodies against acetylated form of histone H4 (R5/12); and X+/− = positive/negative signal after in situ hybridization with X-chromosome centromere–specific DNA probe.

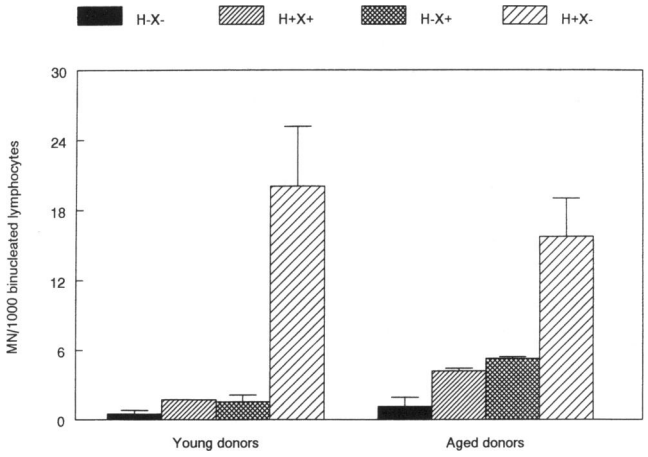

Figure 2 Frequency of MN (designated by "MN") with different content in 72 h–cultured binucleated lymphocytes from young (24–27 years old) and older (57–60 years old) women. MN are classified according to the presence or absence (designated, respectively, by "H+" and "H−") of immunolabeling signal for acetylated histone H4, as well as for positive or negative signal (designated, respectively, by "X+" and "X−") after X-chromosome centromere–specific FISH. Four types of micronucleus content can be distinguished: fragments from the Xi (H−X−); whole Xas (H+X+); whole Xis (H−X+); and whole autosomal chromosomes or fragments of active chromosomal material (i.e., autosomes or Xas). The SD is shown by vertical bars.

since the discrimination of the Xi is done in situ, not requiring the addition of any chemical to the culture. Furthermore, the analysis of the Xi can be performed in all kinds of mammalian cells, including uncultured cells, as long as pancentromeric or X-chromosome centromere–specific DNA probes are available.

The method that we describe also allows genetic analysis of the interphase Xi. In this respect, we provide the first direct evidence that histone H4 in the Xi remains underacetylated not only in metaphase, as has been shown elsewhere (Jeppesen and Turner 1993), but throughout the cell cycle, which is a necessary condition for histone acetylation to play a role in X inactivation. The identification of *cis*-acting factors such as XIST RNA (Brown et al. 1992), colocalizing with the Xi and possibly accounting for the inactive status of one X chromosome in interphase, will be more meaningful if it is restricted to isolated Xi, by following the methodology introduced here. Other potential applications of in situ hybridization combined with acetylated histone H4 immunolabeling to identify both X chromosomes are genetic and biochemical studies of active and inactive chromatin, studies on the behavior of sex chromosomes in interphase, analysis of the position effect of translocations involving inactive chromatin and the implications in cancer, and Xi breakage and the role of chromatin structure and transcription in DNA repair.

Acknowledgments

We thank Dr. Bryan Turner for a gift of antiacetylated histone H4 rabbit antiserum. J.S.'s contribution to this research at Leiden University and visit to the MRC at Edinburgh were made possible by a long-term postdoctoral fellowship awarded by the Commission of the European Union, Environmental Programme, contract EV5V-CT-94-5240.

References

Abruzzo MA, Mayer M, Jacobs PA (1985) Aging and aneuploidy: evidence for a preferential involvement of the inactive X chromosome. Cytogenet Cell Genet 39:275–278

Brown CJ, Ballavio A, Rupert JL, Lafrenière RG, Grompe M, Tonlorenzi R, Willard HF (1991) A gene from the region of the human X inactivation centre is expressed exclusively from the inactive X chromosome. Nature 349:38–44

Brown CJ, Hendrich BB, Rupert JL, Lafrenière RG, Xing Y, Lawrence J, Willard HF (1992) The human XIST gene: analysis of a 17 Kb inactive X-specific RNA that contains conserved repeats and is highly localized within the nucleus. Cell 71:527–542

Catalán J, Autio K, Wessman M, Lindholm C, Knuutila S, Sorsa M, Norppa H (1995) Age-associated micronuclei containing centromeres and the X chromosome in lymphocytes of women. Cytogenet Cell Genet 68:11–16

Chahal SS, Matthews HR, Bradbury EM (1980) Acetylation of histone H4 and its role in chromatin structure and function. Nature 287:76–79

David JR, Candido EP (1978) Acetylated histone H4 is preferentially associated with template-active chromatin. Proc Natl Acad Sci USA 75:3574–3577

Ford JH, Schultz CJ, Correll AT (1988) Chromosome elimination in micronuclei: a common cause of hypoploidy. Am J Hum Genet 43:733–740

Galloway SM, Buckton KE (1978) Aneuploidy and ageing: chromosome studies on a random sample of the population using G-banding. Cytogenet Cell Genet 20:78–95

Haaf T, Werner P, Schmid M (1993) 5-Azadeoxycytidine distinguishes between active and inactive X chromosome condensation. Cytogenet Cell Genet 63:160–168

Jacobs PA, Brunton M, Court Brown WM, Doll R (1963) Change in human chromosome count with age: evidence for a sex difference. Nature 197:1080–1081

Jacobs PA, Court Brown WM, Doll R (1961) Distribution of human chromosome counts in relation to age. Nature 191: 1178–1180

Jeppesen P, Turner BM (1993) The inactive X chromosome in female mammals is distinguished by a lack of histone H4 acetylation, a cytogenetic marker for gene expression. Cell 74:281–289

Lyon MF (1961) Gene action in the X chromosome of the mouse (Mus musculus). Nature 190:372–373

——— (1996) Pinpointing the centre. Nature 379:116–117

Mandel J-L, Monaco AP, Nelson DL, Schlessinger D, Willard HF (1992) Genome analysis and the human X chromosome. Science 258:103–109

Mohandas T, Sparkes RS, Shapiro LJ (1981) Reactivation of an inactive human X chromosome: evidence for X inactivation by DNA methylation. Science 211:393–396

Nowinski GP, Van Dyke DL, Tilley BC, Jacobsen G, Babu VR, Worsham MJ, Wilson GN, et al (1990) The frequency of aneuploidy in cultured lymphocytes is correlated with age and gender but not with reproductive history. Am J Hum Genet 46:1101–1111

Penny GD, Kay GF, Sheardown SA, Rastan S, Brockdorff N (1996) Requirement for Xist in X chromosome inactivation. Nature 379:131–137

Pinkel D, Straume T, Gray JW (1988) Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. Proc Natl Acad Sci USA 83:2934–2938

Sealy L, Chalkley R (1978) The effect of sodium butyrate on histone modification. Cell 14:115–121

Tucker JD, Nath J, Hando JC (1996) Activation status of the X chromosome in human micronucleated lymphocytes. Hum Genet 97:471–475

Turner BM (1993) Decoding the nucleosome. Cell 75:5–8

Willard HF, Latt SA (1976) Analysis of deoxyribonucleic acid replication in human X chromosomes by fluorescence microscopy. Am J Hum Genet 28:213–227