Maintenance of X- and Y-inactivation of the pseudoautosomal (PAR2) gene SPRY3 is independent from DNA methylation and associated to multiple layers of epigenetic modifications

M.L. De Bonis¹,†, A. Cerase¹,‡, M.R. Matarazzo¹,†, M. Ferraro¹, M. Strazzullo¹, R.S. Hansen², P. Chiurazzi³, G. Neri³ and M. D’Esposito¹,*

¹Institute of Genetics and Biophysics ‘A. Buzzati Traverso’ CNR, Naples Italy, ²Department of Genetics, University of Washington, Seattle, USA and ³Institute of Medical Genetics, Catholic University, Rome, Italy

Received November 14, 2005; Revised January 20, 2006; Accepted February 10, 2006

Maintenance of X-inactivation is achieved through a combination of different repressive mechanisms, thus perpetuating the silencing message through many cell generations. The second human X–Y pseudoautosomal region 2 (PAR2) is a useful model to explore the features and internal relationships of the epigenetic circuits involved in this phenomenon. Recently, we demonstrated that DNA methylation plays an essential role for the maintenance of X- and Y-inactivation of the PAR2 gene SYBL1; here we report that the silencing of the second repressed PAR2 gene, SPRY3, appears to be independent of DNA methylation. In contrast to SYBL1, the inactive X and Y alleles of SPRY3 are not reactivated in cells treated with a DNA methylation inhibitor and in cells from ICF (immunodeficiency, centromeric instability, facial anomalies) syndrome patients, which have mutations in the DNA methyltransferase gene DNMT3B. SPRY3 X- and Y-inactivation is associated with a differential enrichment of repressive histone modifications and the recruitment of Polycomb 2 group proteins compared to the active X allele. Another major factor in SPRY3 repression is late replication; the inactive X and Y alleles of SPRY3 have delayed replication relative to the active X allele, even in ICF syndrome cells where the closely linked SYBL1 gene is reactivated and advanced in replication. The relatively stable maintenance of SPRY3 silencing compared with SYBL1 suggests that genes without CpG islands may be less prone to reactivation than previously thought and that genes with CpG islands require promoter methylation as an additional layer of repression.

INTRODUCTION

X-chromosome inactivation (XCI) is the most studied long-range mechanism of epigenetic silencing, affecting most of the genes of a randomly selected X-chromosome in mammalian females (1). The establishment and maintenance of XCI depends on a number of molecular mechanisms, including XIST RNA coating, Polycomb 2 group protein recruitment, repressive histone modifications and DNA methylation (2).

Several genes escape XCI (3,4), many of which are located on two regions shared by X and Y chromosomes, the pseudoautosomal regions (PARs) (5,6). The PAR regions play a role in driving sex assortment in human male meiosis. Given the recombination between the X and Y in these regions, it was expected that their genes would escape XCI, being expressed also from the Y chromosome. Although true for the short-arm PAR (PAR1) genes, only the most telomeric of the long-arm PAR (PAR2) genes escape inactivation (IL9R and cXYorf1), whereas the centromeric ones (SYBL1 and SPRY3) are X and Y inactivated (7). Such different epigenetic behaviour correlates with the evolutionary history of the region: the inactivated genes arose from a more ancient X-chromosome acquisition than the escaping genes (8).
The unusual finding of Y-inactivation, however, has not been fully explored for mechanistic clues. We previously described a key role for DNA methylation in the allele-specific silencing of SYBL1 in XCI and Y-inactivation (9). We also found that repressive histone modifications and late replication play key roles in this repression (10,11). Although such epigenetic modifications are often inter-related, several studies suggest some independence between methylation, late replication and histone modification as repressive mechanisms (10,12,13).

Here we describe the novel finding of X-inactivation maintenance that is independent of DNA methylation. 

The unusual finding of Y-inactivation, however, has not been fully explored for mechanistic clues. We previously described a key role for DNA methylation in the allele-specific silencing of SYBL1 in XCI and Y-inactivation (9). We also found that repressive histone modifications and late replication play key roles in this repression (10,11). Although such epigenetic modifications are often inter-related, several studies suggest some independence between methylation, late replication and histone modification as repressive mechanisms (10,12,13).

Here we describe the novel finding of X-inactivation maintenance that is independent of DNA methylation. 

SPRY3, the proximal PAR2-inactivated gene that lacks a canonical CpG island, is not reactivated at both Xi and Y alleles, following treatment with DNA methylation inhibitors, nor in cells from human syndromes affecting DNA methylation. As in the case of SYBL1, a clear difference in histone methylation is evident between the promoters of active and repressed alleles; in addition, H3K27 histone methyltransferase E(Z)H2 together with the PRC2 Polycomb complex binds the inactive alleles. In contrast to SYBL1, however, late replication and repression of the Y allele was maintained in ICF (immunodeficiency, centromeric instability, facial anomalies) cells where a relatively sharp early–late replication border was seen in the 99 kb region between the escaping and repressed genes. We suggest that genes containing CpG island promoters may be more prone to reactivation than those without, explaining the need for XCI and other silencing systems, such as genomic imprinting, to adopt promoter methylation as an additional layer of silencing.

RESULTS

DNA methylation does not affect the allele-specific expression of the human PAR2 gene SPRY3

SPRY3 is localized at the centromeric end of PAR2, about 99 kb upstream from the gene encoding the synaptobrevin like protein 1, SYBL1 (14). Similar to SYBL1, inactive X (Xi) and Y chromosome alleles of SPRY3 are inactivated, in spite of their identical sequences owing to the relative high frequency of recombination. We began a systematic analysis of the elements controlling SPRY3 transcription in order to gain insights into its allele-specific silencing.

The gene is composed of two exons, with the initial codon residing in exon 2 (Fig. 1). Genomic sequencing and expressed sequence tag analysis identified a long 30- untranslated region, and 5'-RACE experiments positioned the start site 434 bases upstream of translation initiation. Using a promoter scanning strategy, the 1 kb region (Fig. 1) spanning the transcription start site has been found by in vitro assay using a luciferase reporter to contain the strongest promoter activity. An internal, minimal promoter region from nucleotides -180 to +21, however, is capable of sustaining transcription (De Bonis, unpublished data).

Given the sequence identity between active and inactive alleles, we sought to determine the role of epigenetic mechanisms, in primis DNA methylation, in the maintenance of allele-specific silencing at SPRY3 locus. To determine whether there is a CpG island associated with the gene, we scanned a 20 kb region centered on the promoter region for the presence of CpG clusters (using the EMBOSS CpG software at: http://www.ebi.ac.uk/emboss/cpgplot/). No canonical CpG islands were found, even using low stringency parameters. To determine the role of DNA methylation in SPRY3 regulation, if any, we examined the methylation status of all the clusters surrounding the promoter and first exon by digestion of genomic DNA from somatic cell hybrids with two methylation-sensitive enzymes (HpaII and BssHII), followed by PCR analysis: no signs of differential methylation were seen between expressed and silent alleles (data not shown). In contrast, the adjacent gene, SYBL1, which is subject to allele-specific DNA methylation (11), did show allele-specific amplification of repressed alleles.
following methylation sensitive genomic restriction (data not shown).

A more thorough analysis was performed by examining the effect on \textit{SPRY3} allelic expression of genomic DNA hypomethylation induced either by 5aCdr treatment or by using the genetic model of cell lines from patients affected by ICF syndrome, which are deficient in the DNMT3B cytosine methyltransferase (15).

Somatic cell hybrids retaining the human Xi or the Y chromosomes as a sole human counterpart were treated with 5aCdr and examined at various times after treatment for reactivation of \textit{SPRY3} and \textit{SYBL1} by RT–PCR, using the escaping gene \textit{MIC2} as an internal control (Fig. 2). As previously shown (9), \textit{SYBL1} reactivation on both the Xi and Y chromosomes is readily detected under these conditions; however, \textit{SPRY3} reactivation could not be detected at any time point. In addition, treatment of a male lymphoblastoid cell line with 5aCdr resulted in a partial reactivation of \textit{SYBL1}, but no sign of \textit{SPRY3} reactivation was detected (data not shown).

ICF syndrome is an autosomal recessive human disease caused by germline mutations affecting \textit{DNMT3B} gene (16–18). Patients show hypomethylation at heterochromatic loci, including the inactive X-chromosome (19). Among many genes on the X-chromosome showing hypomethylation of their associated CpG island, but retaining their monoallelic status of expression, \textit{SYBL1} shows hypomethylation of its CpG island and advanced replication time, resulting in biallelic expression (10). Therefore, we checked the allelic expression of \textit{SPRY3} gene using two cell lines from ICF male patients, which are polymorphic for a \textit{TaqI} restriction fragment length polymorphism (RFLP), located at its 3'-UTR. The product, 1374 bp in length, was digested with \textit{TaqI} endonuclease, which cuts to yield a 919 bp fragment in all samples and a 455 bp fragment that will be cleaved into 337 and 118 bp fragments in the allele with the polymorphic restriction site (see the map for details).

\textit{SPRY3} monoallelic expression is associated with specific histone changes

We next explored the role of histone modification in the allelic regulation of \textit{SPRY3} expression. Histone H3 methylation was of particular interest given its important DNA methylation-independent role in X-inactivation (2) and genomic imprinting in the placenta (20,21).

Chromatin immunoprecipitation experiments were performed using antibodies against methylated lysines at positions 4, 9 and 27 of histone H3. Experiments on somatic cell hybrids showed specific association of dimethylated H3K9 and di-trimethylated H3K27 with the repressed inactive X and Y alleles, whereas dimethylation at H3K4 and acetylation at H3K9/14 specifically marked the expressed active X allele (Supplementary Material, Fig. S1).

To make the scoring of the results easier when two human sex chromosomes were present, we adopted a strategy for allele-specific PCR, called MAMA–PCR (22). Taking advantage of allele-specific single nucleotide polymorphisms (SNPs) in this way, we examined histone modifications at the \textit{SPRY3} promoter (Fig. 1). We developed specific primers to amplify selectively the active X allele (GA primer), or the Xi or Y alleles (GC primer; see Material and Methods). Chromatin immunoprecipitations followed by allele-specific PCR were carried out on male and female cell lines that are...
polymorphic for the A/C nucleotide at position –585 from +1 (NM_005840) (Fig.1). We have quantified relative levels of immunoprecipitated and input fractions and have presented all results as histograms corresponding to percentage of input (Fig. 3). Allele-specific ratios are shown for all the antibodies tested. It is worth noting the complementary behaviour of H3K4 and H3K27, which mark the expressed and repressed alleles, respectively.

We also analyzed the enrichment of allele-specific histone modifications in the upstream region of the gene, as well as in the coding region and in the long 3'-UTR using polymorphic RFLPs (Fig. 1). The differential modifications were also found in the body of the SPRY3 gene (Supplementary Material, Fig. S3), but were lost outside the gene (data not shown).

To fully understand the role of histone acetylation in maintaining SPRY3 repression, we treated cells with histone deacetylase (HDACs) inhibitors, such as trichostatin A (TSA) and butyric acid (BA). Given that X-linked genes are not often responding to HDACs inhibitors (23), we have chosen as model system cell from fragile X syndrome male patients. In fact, reactivation of FMR1 gene has been linked to both 5aCdr and HDACs inhibitors treatment, with a synergistic effects between these two drugs (24).

S1 cell line, from a fragile X male patient containing roughly 400–500 fully methylated CGG repeats has been used. This cell line is heterozygous for SPRY3 TaqI RFLP at 3'-UTR (discussed earlier), thus permitting again to check allele-specific expression before and after treatment. In Figure 4, the effect of treatment on SPRY3 and FMR1 genes with various drugs is shown. Both genes escape inactivation upon treatment; but, reactivation of SPRY3 is visible only after TSA or BA treatment (see Materials and Methods), whereas FMR1 is reactivated upon TSA, BA or 5aCdr treatment, as previously reported (24).

Recruitment of PRC2 proteins in the maintenance of SPRY3 X- and Y-inactivation
Recent results highlight the involvement of Polycomb group proteins in the early maintenance of both imprinting and random X-inactivation (2). The binding of PRC2 complex components, such as EZH2 (enhancer of zeste homolog-2, which is the histone methyltransferase specific for the H3K27) and EED1 (embryonic ectoderm development) and SUZ12 (suppressor of zeste-12), have also been demonstrated to take part in the repression of placenta-specific imprinted genes by a mechanism possibly involving histone deacetylases (25).

Interestingly, an association of methylated H3K27 to silenced alleles has been revealed for SYBL1 gene (Supplementary Material, Fig. S2). Preliminary results identify
the active X replicate in mid-S (peaks at S2 and S3), whereas
of SYBL1.

Advanced replication time is a key feature of reactivated alleles and ICF cell lines (Fig. 6). In normal male lymphoblasts, SPRY3 and SYBL1 on the active X replicate in mid-S (peaks at S2 and S3), whereas on the Y, they replicate primarily in late S (S4 and G2/M). This pattern can also be seen at the PAR2 DXS1107 locus (Fig. 1), where there is an informative CA repeat polymorphism in this normal male, with the larger band representing the late replicating Y allele (Fig. 6A).

PT5 ICF male lymphoblasts show the normal expression pattern of only the active X allele of SPRY3, but abnormally express both X and Y alleles of SYBL1 (Fig. 6B) (10). Replication of the Y allele of SPRY3 is normal, but that of SYBL1 is advanced to an active X-like pattern. DXS1107 marker, although not informative, is only 33 kb centromeric of the advanced SYBL1 allele and appears to have a somewhat intermediate pattern of advanced replication for the Y allele (decreased G2/M peak, but strong S4 peak). A second ICF cell line from patient PT1, although not informative for SYBL1 XhoI polymorphism, shows the same replication time advance, as in PT5 (data not shown).

We also analyzed replication features for SPRY3 and other PAR2 markers in fibroblasts from normal male and female individuals and from ICF female fibroblasts (Fig. 6C and D). Replication of all the markers analyzed is generally later on the active X in fibroblasts (S3 and S4 peaks) than in lymphoblasts (S2 and S3 peaks), as seen in normal male fibroblast at SPRY3 locus. The Y allele replicates primarily in S4 with some G2/M, similar to the lymphoblast pattern, but resulting in much less resolution between the replication of the active X and Y in fibroblasts. As in lymphoid cells, the normal fibroblast inactive X and Y alleles replicate very late. This pattern remains essentially unaltered in the female PT4 ICF FB clone, with both alleles showing late replication (S4 peak). In PT4 ICF female fibroblasts, the inactive X allele of DXS1107 appears to be somewhat advanced in replication, as seen by the lower G2/M signal. The strong S4 peak, however, indicates that replication remains in the second half of S-phase in PT4 lymphoblasts, similar to the replication of G6PD and several other inactive X loci that remain silent in this cell line (10). The analysis of a second ICF female fibroblast culture (PT3) also suggests late replication for SPRY3 on the inactive X (data not shown).

**DISCUSSION**

Soon after XIST-mediated initiation and propagation of silencing, additional epigenetic mechanisms act to maintain XCI, such as Polycomb 2 group protein association, histone modification, late replication time, macro H2A association and DNA methylation (2). As result, a stable maintenance of the repressive signal is ensured. It is thought that all the elements of this complex array of signals are required to maintain the silencing of genes subject to XCI-inactivation. Failure in the Dnmt1 maintenance methylation system destabilizes embryonic XCI (26). In addition, de novo DNA methylation has been shown to be required for the stable maintenance of XCI, but not for initiation or propagation status (27).

Our studies of histone modification, DNA methylation and replication timing in the PAR2 region under conditions of DNA hypomethylation suggest that DNA methylation is not important for the maintenance of XCI for the non-CpG island gene, SPRY3. Given that it is also inactivated at
the identical Y allele (14), these experiments further support
the equivalence of the X- and Y-inactivation maintenance
mechanisms, as already demonstrated for SYBL1 gene (11).
The role of DNA methylation has been recently discussed
(28) in the case of the partially escaping X-linked TIMP1
gene. Interestingly, these authors proposed that histone
modifications rather than DNA methylation act to maintain
X-inactivation.
Histone modifications associated with SPRY3 repression
seem to play a major role for the maintenance of repression.
This issue has been further confirmed by the reactivation of
SPRY3 expression upon HDAC inhibitors treatment (Fig. 4).
It is interesting to note that X-linked genes are not prone to
reactivation upon HDACs inhibition alone (23). Thus, FMRI
and SPRY3 are examples of this class of reactivated X-linked
genies, with or without promoter methylation (see also
Supplementary Material, Table SI for further information).
The mechanistic link between DNA and histone methylation
reported by us for SYBL1 (11) and by others (29) is absent in
SPRY3 regulation, supporting the notion that such modifi-
cations may also occur in a DNA methylation-independent way.
Histone methylation in K9 and K27 is common to
promoters of X-linked genes, as elsewhere suggested (30).
Such modifications could be secondary targets of complexes
containing distinct methyltransferases and chromodomains
that are able to distinguish between these methyl marks or
they could be recognized in combination. The recruitment of
the methyltransferase EZH2 and the other members of Poly-
comb group to SPRY3 seems to support the first hypothesis
(Fig. 5). These complexes may act independently to silence
transcription either in a redundant manner or synergistically
such that neither H3K9 nor H3K27 alone can efficiently
repress expression. In our experiments, however, we could
not distinguish the specific contribution of each of two
methyl marks to the silencing.
Because H3K27 methylation is achieved through the func-
tion of the EZH2 histone methyltransferase, this implies the
involvement of Polycomb group protein complex PRC2, the
mammalian ortholog of Drosophila Enc/E(z) complex, com-
posed of EED1, EZH2 and SUZ12 proteins. This complex
has previously been implicated in the maintenance of extra-
embryonic X-inactivation (31); further studies associated the
same complex in the early maintenance random X-inactivation
in the embryo proper (32,33). Transcriptional repression
mediated by PRC2 involves histone deacetylase activity, inter-
acting with the complex (25). Here we demonstrated the
association of PRC2 and HDAC1 to stably silenced alleles
of SPRY3 (Fig. 5). Notably, a similar association has been
established also for the other PAR2 inactivated gene, SYBL1
(Matarazzo, unpublished data).
To study the stability of repression for inactive alleles of
SPRY3, we treated somatic cell hybrids and human lympho-
blastoid cells with the DNA methylation inhibitor 5aCdR and
found no reactivation for SPRY3 (Fig. 2) (data not shown),
but the nearby SYBL1 gene was readily reactivated, as
previously described (9). In addition, inactive alleles of
SPRY3 do not escape in ICF syndrome cells that are deficient
in X- and Y-linked CpG island methylation (Fig. 2B). These

![Figure 5.](https://academic.oup.com/hmg/article-abstract/15/7/1123/715387)
data also match well with those obtained by analyzing SPRY3 expression in uremic/hyperhomocysteinemic (Hcy) patients (13). This metabolic defect causes a plasma increase in the methionine intermediate homocysteine, which leads to a competitive inhibition of cellular methyltransferases (34), resulting in genomic DNA hypomethylation (13,35). SYBL1, as well as H19 and IGF2 allelic expression were profoundly altered in Hcy patients, whereas SPRY3 expression was not (13).

It appears that the DNMT3B deficiency in ICF prevents CpG island promoter methylation associated with XCI (10). Although gene silencing is less stable under these conditions, most genes do not escape XCI, XIST RNA association and repressive histone modifications are normal in ICF for most of the inactive X (36). Spontaneous reactivation only occurred when replication timing was markedly advanced, as seen for SYBL1 and G6PD in specific cell lines (10). Late replication time appears to be an epigenetic silencing mechanism that can be independent of promoter methylation.

Conversely, promoter methylation can silence transcription in the absence of late replication in several systems (12,37–39). It is interesting that the abnormal late–early replication transitions found in ICF cells between the Y-linked reactivated SYBL1 and inactivated SPRY3 genes of the PAR2 region can occur over relatively short distances (30–90 kb; Fig. 6) (data not shown). The rather sharp transition suggests that there may be an insulator element between these loci. The CTCF protein appears to function as a methylation-dependent insulator in imprinted domains (40), as well as in X-linked domains that escape XCI (41). Therefore, CTCF is a good candidate factor for separation of the SYBL1–SPRY3 domains upon demethylation of recognition elements in the intergenic region.

SPRY3 regulation likely shares silencing features with a large number of genes on the Xi that are known to lack CpG islands (4,42). Although we did not observe SPRY3 reactivation, other non-CpG island genes could be envisioned to reactivate under demethylation conditions as a result of domain-wide effects.
such as advanced replication time induced by demethylation of extragenic sequences in the domain. Advanced replication timing alone, however, is not likely to be sufficient for reactivation of non-CpG island genes, as repressive histone modifications and other silencing mechanisms must also be removed, just as in the case of CpG-island genes. In fact, we propose that non-CpG island genes are less prone to reactivation than CpG island genes because the basal transcription of such genes is generally lower.

Genes with CpG island promoters are much more likely to be widely expressed than those without (43). In addition, the maintenance of repression for several tissue-specific genes with CpG islands appears to involve island methylation (44). For many tissue-specific genes with CpG islands that remain unmethylated, the absence of tissue-specific transcription factors prevents high levels of transcription; even in these cases, however, there is evidence that the islands may provide low-level transcription in a variety of cell types (45). Under conditions of promoter hypomethylation on the Y and Xi, such basal transcription is likely to promote full reactivation for CpG island-containing genes.

Genomic imprinting and XCI have been linked in several ways (46,47). Both systems evolved from mammalian-specific pathways, both are controlled by epigenetic mechanisms such as CpG island methylation and histone modification and they both have untranslated RNAs that play key roles in silencing. Stable maintenance of imprinting in placenta has recently been shown to be independent of DNA methylation, relying on specific histone modifications and Polycomb complex PRC2 association (20). Considering that XCI and imprinting in marsupials occur via mechanisms independent of DNA methylation, the authors postulated that the ancestral mechanism of allelic inactivation was dependent only on specific histone modifications. Perhaps, this mechanism was retained in the placenta of eutherian species because the placenta is a transient organ, which may tolerate a less stable inactivation. Indeed, XCI in eutherian species is generally lower.

Stable maintenance of imprinting for several tissue-specific genes including CpG island-containing genes is a fibroblast cell line from a female with highly skewed X-inactivation (>95:5). The ICF cell lines analyzed in this study, all derived from cases with classical ICF features, include lymphoblast cells from PT1 and PT5 males and PT4 female, untransformed PT3 fibroblasts, PT4 fibroblast and h-TERT-transformed PT3 (10,36). Lymphoblasts and fibroblasts were cultured as previously described (10,11).

**Treatments with 5aCdr, TSA and BA**

BA (Sigma, St Louis, MO, USA) was resuspended in sterile water to a concentration of 500 mM and stored at −80°C in aliquots. TSA (Sigma) was resuspended in ethanol to a concentration of 1 mg/ml stock and stored at −20°C in small aliquots. Finally, a 1.75 mM stock solution of 5aCdr (Sigma) was prepared in sterile water and stored at −80°C in aliquots.

For 5aCdr treatment, cells were counted, split and seeded to an initial concentration of 2.5–3 \times 10^5 cells/ml in a total volume of 10 ml per flask. A daily dose of 1 µM of 5aCdr was added to the flasks and was thoroughly resuspended, whereas a control flask was left untreated. Cells were harvested after 120 h treatment.

For 5aCdr treatment, cells were counted, split and seeded to an initial concentration of 2.5–3 \times 10^5 cells/ml in a total volume of 10 ml per flask. A single dose of either BA or TSA was added to the flasks and was thoroughly resuspended. A control flask for each patient cell line was left untreated or received a mock treatment with a comparable volume of ethanol in the case of the TSA treatment. Cells were harvested after 48 h and the RNA extracted as above.

To assess the expression status of *SPRY3* gene after 5aCdr treatment in hybrid cells, we performed RT–PCR using the following primers: F7 5'-ATGCCCATACAGGTGGTGGAC TCG-3' and RT2 5'-GACACTTGAGGAACAAAAGA-3'. PCR amplification for the control genes *SYBL1* and *M1C2* was performed using primers described previously (9).

PCR amplification of *SPRY3* has been carried out after TSA, BA and 5aCdr treatment of cells derived from fragile-X S1 patient with primers F5 5'-GTGGGGGCTAGTTCTTGAC TTT-3' and RT2 (described above). Here, PCR product, 362 bp in length, was digested with TaqI restriction enzyme, giving the following fragments, 264 and 98 bp: primers

**MATERIALS AND METHODS**

**Sequence analysis and SNPs searching**

We obtained the human sequence for *SPRY3* mRNA (NM_005840) and 5′ and 3′ sequences from the UCSC Genome Bioinformatics Server (http://www.genome.ucsc.edu/) using the May 2005 freeze assembly. For the SNPs analysis, we used the SNP track of the UCSC genome browser and sequences were obtained from dbSNP at NCBI (http://www.ncbi.nlm.nih.gov/SNP).

**Cells culture conditions**

Epstein–Barr virus-transformed lymphoblast cell line AB was derived from a normal male individual. GM07693 (NIGMS) is a fibroblast cell line from a highly skewed X-inactivation (>95:5). The ICF cell lines analyzed in this study, all derived from cases with classical ICF features, include lymphoblast cells from PT1 and PT5 males and PT4 female, untransformed PT3 fibroblasts, PT4 fibroblast and h-TERT-transformed PT3 (10,36). Lymphoblasts and fibroblasts were cultured as previously described (10,11).

**Cells culture conditions**

Epstein–Barr virus-transformed lymphoblast cell line AB was derived from a normal male individual. GM07693 (NIGMS) is a fibroblast cell line from a highly skewed X-inactivation (>95:5). The ICF cell lines analyzed in this study, all derived from cases with classical ICF features, include lymphoblast cells from PT1 and PT5 males and PT4 female, untransformed PT3 fibroblasts, PT4 fibroblast and h-TERT-transformed PT3 (10,36). Lymphoblasts and fibroblasts were cultured as previously described (10,11).

**Treatments with 5aCdr, TSA and BA**

BA (Sigma, St Louis, MO, USA) was resuspended in sterile water to a concentration of 500 mM and stored at −80°C in aliquots. TSA (Sigma) was resuspended in ethanol to a concentration of 1 mg/ml stock and stored at −20°C in small aliquots. Finally, a 1.75 mM stock solution of 5aCdr (Sigma) was prepared in sterile water and stored at −80°C in aliquots.

For 5aCdr treatment, cells were counted, split and seeded to an initial concentration of 2.5–3 \times 10^5 cells/ml in a total volume of 10 ml per flask. A daily dose of 1 µM of 5aCdr was added to the flasks and was thoroughly resuspended, whereas a control flask was left untreated. Cells were harvested after 120 h treatment.

For 5aCdr treatment, cells were counted, split and seeded to an initial concentration of 2.5–3 \times 10^5 cells/ml in a total volume of 10 ml per flask. A single dose of either BA or TSA was added to the flasks and was thoroughly resuspended. A control flask for each patient cell line was left untreated or received a mock treatment with a comparable volume of ethanol in the case of the TSA treatment. Cells were harvested after 48 h and the RNA extracted as above.

To assess the expression status of *SPRY3* gene after 5aCdr treatment in hybrid cells, we performed RT–PCR using the following primers: F7 5'-ATGCCCATACAGGTGGTGGAC TCG-3' and RT2 5'-GACACTTGAGGAACAAAAGA-3'. PCR amplification for the control genes *SYBL1* and *M1C2* was performed using primers described previously (9).

PCR amplification of *SPRY3* has been carried out after TSA, BA and 5aCdr treatment of cells derived from fragile-X S1 patient with primers F5 5'-GTGGGGGCTAGTTCTTGAC TTT-3' and RT2 (described above). Here, PCR product, 362 bp in length, was digested with TaqI restriction enzyme, giving the following fragments, 264 and 98 bp: primers
described previously have been used to amplify the control gene FMRI (9). GAPDH has been amplified to normalize cDNA templates with the following primers: GAPDH-F 5'ACATGTCTTCCAATAATTGATT-3' and GAPDH-R 5'TGGACTCCACGACGTATCTCAG-3'.

To assess the allelic SPRY3 expression status in ICF cell lines, cDNAs derived from males polymorphic for a TaqI RFLP in the 3' UTR of the gene (G/T; dbSNP rs306889) were amplified with primers F7 and RT2 (described earlier). PCR amplification was done at 64°C and the product, 1374 bp in length, was digested with TaqI endonuclease, which cuts in two different positions, nt 919 and nt 1256, being polymorphic only in the latter. As a result, the digestion of every allele will produce a 919 bp fragment. The remaining 455 bp band will be cleaved into 337 and 118 bp fragments in the allele with the polymorphic site.

**ChIP assay, allele-specific PCR (MAMA) and 5'–3' allele-specific modifications**

Chromatin immunoprecipitation has been performed as previously described (11). Polyclonal antisera have been used, according to the manufacturer's instructions (Upstate), to detect the following modifications of histone H3 tails at PAR2 loci: acetylation of lysines 9 and 14, dimethylation of lysine 4 and 9, di-trimethylation of lysine 27. Moreover, commercial antisera against Eed, Ezh2 and Suz12 were used to analyze PAR2 associations (Abcam anti-Ezh2, Upstate anti-Eed, Abcam anti-Suz12). A rabbit IgG antisera (Upstate, Inc.) was used as a negative control.

After immunoprecipitation, allele-specific semi-quantitative PCR was performed as described previously (22,53). Briefly, using MAMA technology, we have designed two primer pairs spanning the known rs306882 SNP (C/A), the first one annealing specifically to the ‘A’ allele and the other one to the ‘C’ allele. The primers and annealing temperatures were MAMA-GA: 5'-AGCTACAGCTCGGAAAAAG-ga-3'/ MAMA-rev: 5'-GTTGGCAGTGTGTTCAGCTTAG-3' at 62°C and MAMA-GC: 5'-AGCTACAGCTCGGAAAA Ggc-3'/MAMA-rev at 67°C. For semi-quantitative PCRs we quantified, after electrophoretic separation, the intensity of each immunoprecipitated and input band using QuantityOne (BioRad) software. We performed each immunoprecipitation in each immunoprecipitated and input band using QuantityOne software. We performed each immunoprecipitation in each immunoprecipitated and input band using QuantityOne software. We performed each immunoprecipitation in each immunoprecipitated and input band using QuantityOne software. We performed each immunoprecipitation in each immunoprecipitated and input band using QuantityOne software.

**Replication timing analysis**

Replication time was determined by analysis of newly replicated DNA labeled with bromodeoxyuridine (BrdU), which was isolated from cells sorted into specific cell cycle stage by flow cytometry as previously described (54,55). The BrDU-labeled DNA was isolated with an anti-BrdU antibody, and the newly replicated DNA fractions were then analyzed for the presence of specific loci by semi-quantitative PCR (54,55).

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG Online.

**ACKNOWLEDGEMENTS**

The authors gratefully acknowledge M. Cuccurese and G. Chirico for help in the initial phases of the work, R. Feil and S. Gartler for comments, D. Reinberg for the gift of H3K27 di-trimethylated antibody. Also, we acknowledge the help of Genome Browser available at UCSC (http://genome.ucsc.edu). Research in M'D'E laboratory is sponsored by Telethon grant GGP02308 and EEC contract no. LSHB-CT-2004-503243, (SAFE NoE). R.S.H. was supported by grant HD16659 from the NIH. G.N. and P.C. gratefully acknowledge Telethon for funding.

**Conflict of Interest statement.** None declared.

**REFERENCES**


