

Staged assembly of histone gene expression machinery at subnuclear foci in the abbreviated cell cycle of human embryonic stem cells

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Human embryonic stem (hES) cells have an abbreviated G₁ phase of the cell cycle. How cells expedite G₁ events that are required for the initiation of S phase has not been resolved. One key regulatory pathway that controls G₁/S-phase transition is the cyclin E/CDK2-dependent activation of the coactivator protein nuclear protein, ataxia-telangiectasia locus/histone nuclear factor-P (p220^{NPAT}/HiNF-P) complex that induces histone gene transcription. In this study, we use the subnuclear organization of factors controlling histone gene expression to define mechanistic differences in the G₁ phase of hES and somatic cells using in situ immunofluorescence microscopy and fluorescence in situ hybridization (FISH). We show that histone gene expression is supported by the staged assembly and modification of a unique subnuclear structure that coordinates initiation and processing of transcripts originating from histone gene loci. Our results demonstrate that regulatory complexes that mediate transcriptional initiation (e.g., p220^{NPAT}) and 3'-end processing (e.g., Lsm10, Lsm11, and SLBP) of histone gene transcripts colocalize at histone gene loci in dedicated subnuclear foci (histone locus bodies) that are distinct from Cajal bodies. Although appearance of CDK2-phosphorylated p220^{NPAT} in these domains occurs at the time of S-phase entry, histone locus bodies are formed ≈1 to 2 h before S phase in embryonic cells but 6 h before S phase in somatic cells. These temporal differences in the formation of histone locus bodies suggest that the G₁ phase of the cell cycle in hES cells is abbreviated in part by contraction of late G₁.

HiNF-P | p220^{NPAT} | Cajal body | coilin | G₁/S transition

The abbreviated cell cycle of human embryonic stem (hES) cells represents a unique cellular adaptation that expedites self-renewal and is reflected by a very brief G₁ phase (1, 2). Competency of somatic cells for proliferation is linked to growth factor-dependent passage through the restriction (R) point in G₁ when cells commit toward onset of S phase (3, 4). However, hES cells lack a classical R point and have the capacity for continuous cell division. A principal mechanism that is required for the initiation of S phase in hES cells is the induction of histone gene expression, which is essential for the packaging of newly replicated DNA into chromatin by specific transcription factors (1, 2, 5–12).

In both somatic and hES cells, key histone gene regulatory factors are organized in a limited number of subnuclear foci. For example, recruitment of the coactivator protein p220^{NPAT} (nuclear protein, ataxia-telangiectasia locus) by transcription factor HiNF-P (histone nuclear factor-P) to histone H4 gene promoters, as well as cell cycle-dependent phosphorylation of p220^{NPAT} by cyclin E/CDK2 to induce histone gene transcription occur at these intranuclear sites (7, 8, 13–17). Newly synthesized histone transcripts are not polyadenylated, and their cleavage requires a U7 small nuclear ribonucleoprotein complex (U7 snRNP) that contains U7 snRNA and the protein subunits Lsm10 (U7 snRNP-specific Sm-like protein LSM10) and Lsm11 (U7

snRNP-specific Sm-like protein LSM11), whereas a specific RNA hairpin in histone transcripts interacts with stem loop binding protein (SLBP) (18–22). Studies with somatic cell types have shown that at least some factors mediating 3'-end processing of histone primary transcripts are organized in Cajal body-related foci that contain coilin (23). However, Cajal bodies are not evident in all somatic cell types and are distinct from subnuclear foci that contain p220^{NPAT} (24–27).

In this study, we used the subnuclear organization of histone gene transcription and processing factors as a paradigm to define mechanistic differences in the G₁ phase of hES and somatic cells. We show first that the Lsm10 and Lsm11 protein subunits of the U7 snRNP, as well as SLBP, are recruited to p220^{NPAT} foci at histone gene loci in both hES and somatic cells. These results establish that cells in G₁ phase preassemble regulatory structures analogous to nucleoli to provide a unique microenvironment for the production of histone mRNAs in S phase. Furthermore, we show that these p220^{NPAT} foci are formed at different stages of the G₁ phase in embryonic versus somatic cells. Cell type-specific differences in the temporal assembly of p220^{NPAT} foci provide insight into the regulatory organization of G₁ and the coordination of transcription and processing of gene transcripts at the G₁/S-phase cell cycle transition in hES cells.

Results

Foci of p220^{NPAT} Associate with the Two Major Histone Gene Clusters at 6p22 and 1q21 in hES Cells. Our studies are directed toward understanding the spatial and temporal organization of the regulatory machinery for histone gene expression during the abbreviated cell cycle in hES cells (H9/WA09). We performed immunofluorescence (IF) microscopy for the histone gene regulatory factor p220^{NPAT} combined with fluorescence in situ hybridization (FISH) using probes spanning genomic segments near histone gene loci on chromosomes 6 and 1. The FISH results show that p220^{NPAT} foci as well as phospho-T1270-p220^{NPAT} foci are associated with the histone gene clusters on 6p22 (Fig. 1) and 1q21 (data not shown) in asynchronous populations of hES cells (Fig. 1A, left column, top and middle rows) and normal fibroblasts (Fig. 1A, right column, top and middle rows). Depending on the stage of the cell cycle, hES cells have either 2 or 4 p220^{NPAT} foci (Fig. 1B). Two of the four p220^{NPAT} foci are always

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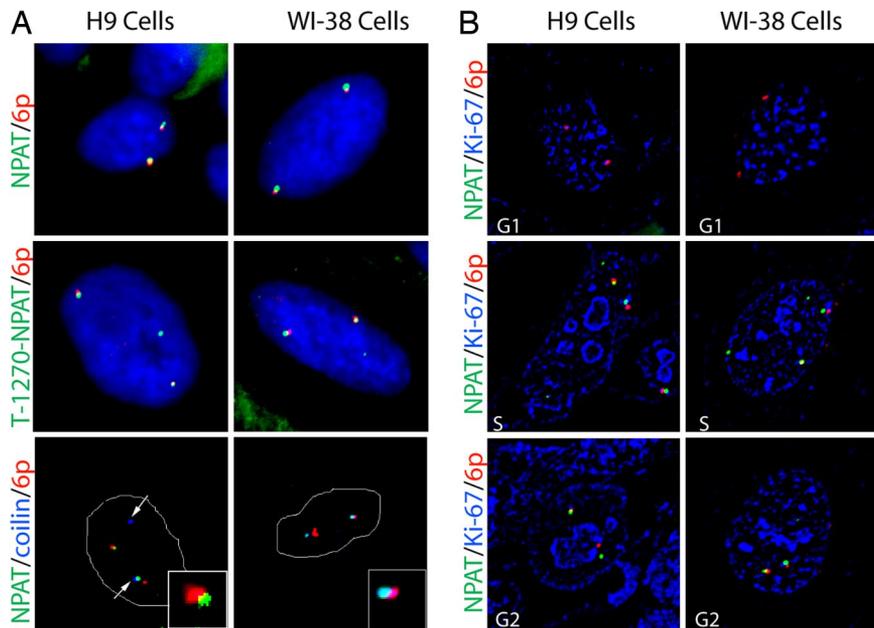


Fig. 1. The p220^{NPAT} foci are associated with histone gene loci in H9 hES cells and normal diploid WI-38 fibroblasts. (A) IF microscopy images were obtained using antibodies against p220^{NPAT} (green), phospho-T1270-p220^{NPAT} (green), or coilin (blue), and a DIG-labeled FISH probe adjacent to the histone gene cluster on 6p22 (red). The arrows in the bottom row, left column, indicate the position of coilin foci. The insets in the lower right of each panel in the bottom row indicate colocalization between p220^{NPAT}/coilin/6p. DAPI staining (blue) is used to visualize the nucleus (top 2 rows). There are typically 2 or 4 p220^{NPAT} foci, depending on the cell cycle stage, that are consistently in proximity to histone gene clusters. In 50–60% of cells, coilin foci (Cajal bodies) overlap with at least one p220^{NPAT} foci. (B) IF images as in A, using antibodies against p220^{NPAT} (green) and Ki-67 (blue), a marker for cell cycle position, and the 6p22 FISH probe. Cell cycle stages are indicated at the lower left of each panel.

associated with the histone clusters on chromosome 6 (Fig. 1A), and the remaining 2 foci are associated with the histone gene clusters on chromosome 1 (data not shown). The association of p220^{NPAT} with histone genes indicates that the histone gene transcriptional complexes are architecturally linked with their target genes in hES cells as they are in normal fibroblasts.

The p220^{NPAT} foci are related to Cajal bodies that have been shown previously by our group (26, 28) and others (14, 15, 23) to be associated with histone gene loci. We find that 50–60% of hES cells stain for the Cajal body marker coilin, whereas >95% of cells are stained for p220^{NPAT} (Fig. 1A). Not all p220^{NPAT} foci contain coilin and vice versa, consistent with previous findings (26). Importantly, p220^{NPAT} foci but not coilin consistently associate with histone gene clusters on 6p22 (Fig. 1A, left column, bottom row) and 1q21 (data not shown). These results indicate that Cajal bodies and p220^{NPAT} foci are 2 distinct nuclear domains and that p220^{NPAT} foci are the subnuclear sites where histone loci are organized in hES and somatic cells.

We investigated how histone gene loci are associated with p220^{NPAT} foci and Cajal bodies in different stages of the cell cycle using Ki-67 as a marker. Human ES cells in S phase but not G₁ consistently exhibit costaining of p220^{NPAT} and the histone gene locus at 6p22 (Fig. 1B, left column), whereas a strict correlation is not evident for coilin (data not shown). In somatic WI-38 cells, p220^{NPAT} foci are present in mid to late G₁ and S phases and associate with histone gene loci (Fig. 1B, right column). In contrast, only 15% of WI-38 cells show focal staining for coilin. However, when coilin foci are present, they tend to be in proximity to p220^{NPAT} and histone gene loci (Fig. 1A, right column, bottom row). Thus, hES cells and WI-38 cells exhibit fundamental differences in association of coilin with histone gene-containing p220^{NPAT} foci.

The p220^{NPAT} Foci Colocalize with Factors Mediating 3'-End Processing of Histone Pre-mRNA. Because U7 snRNPs that mediate maturation of histone gene primary transcripts are focally organized

(23), we investigated whether p220^{NPAT} foci and histone mRNA processing factors reside at the same subnuclear locations. We studied Lsm10 and Lsm11, which are integral to U7 snRNPs, as well as the SLBP and 3' histone exonuclease (hExo), which each recognize opposite sides of the histone mRNA-specific 3' hairpin structure.

Costaining of hES cells with either p220^{NPAT} or coilin, along with one of the 3'-end processing factors, reveals that all p220^{NPAT} foci colocalize with Lsm10 and Lsm11 in all cells (Fig. 2, left column, rows 1 and 2). These findings indicate that the machinery for both initiation and processing of histone mRNAs resides in the same subnuclear domains. SLBP compartmentalizes in both the cytoplasm and nucleus of 50–60% of asynchronous hES cells, and a subset of nuclear SLBP is adjacent to or partially overlaps with p220^{NPAT} foci (Fig. 2, left column, row 3). Similarly, Lsm10 and Lsm11 reside at p220^{NPAT} foci in most (>90%) somatic WI-38 cells, whereas SLBP only partially colocalizes with p220^{NPAT} foci (Fig. 2, right column, rows 1–3). These results indicate that 3 principal 3'-end processing factors that recognize histone transcripts are spatially linked to p220^{NPAT} foci.

Although p220^{NPAT} foci are clearly linked with active synthesis of histone transcripts, the mechanistic role of Cajal bodies in histone gene expression is less evident. Although only a subset of hES cells and somatic WI-38 cells have focal coilin staining (see above), there is partial or complete overlap of Lsm10, Lsm11, or SLBP with one or more coilin foci in these cells (supporting information (SI) Fig. S1). Thus, some Cajal bodies may have an auxiliary role in maturation of histone mRNAs, whereas others appear to be unrelated to histone gene expression.

In addition to the factors supporting synthesis of mature histone mRNAs, we examined *in situ* localization of the exonuclease 3' hExo that specifically interacts with the stem-loop in histone mRNA and may degrade histone mRNA at the completion of DNA synthesis. This enzyme is present at neither

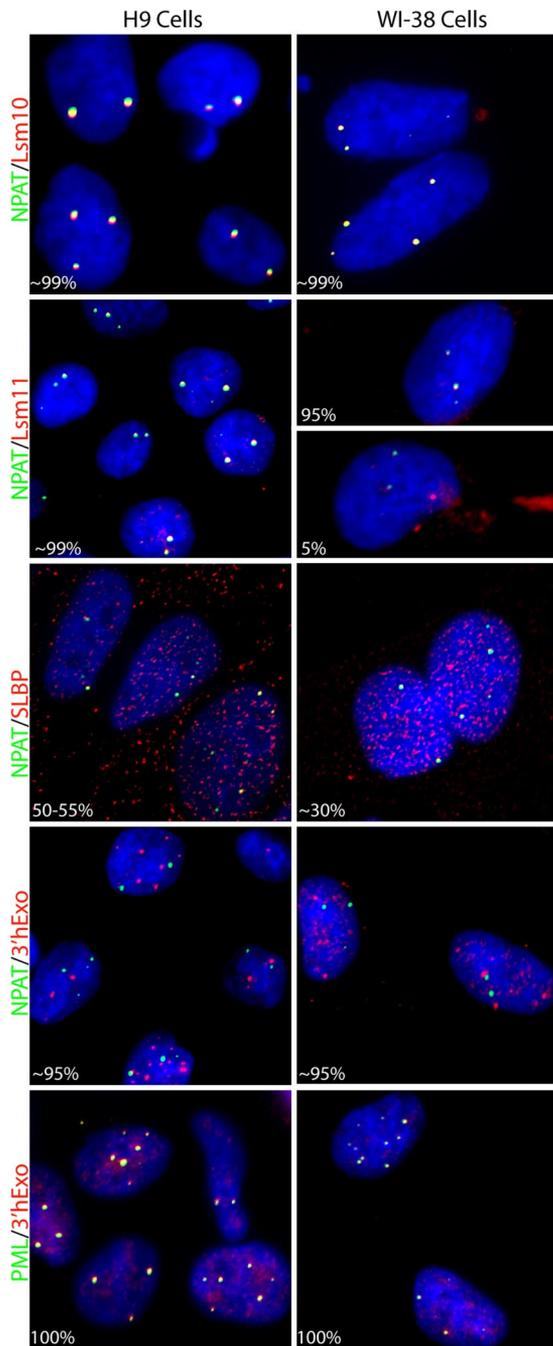


Fig. 2. Colocalization of factors mediating histone gene transcriptional initiation and 3'-end transcript processing of histone mRNA. IF microscopy images were obtained for H9 hES cells (*Left* column) and normal diploid WI-38 cells (*Right* column) using antibodies against p220^{NPAT} (green) and factors that process or interact with histone transcripts (Lsm10, Lsm11, SLBP, or 3' hExo; red). SLBP interacts with the 3' hairpin in histone mRNA; the protein only partially colocalizes with p220^{NPAT} foci. Foci of 3' hExo show no colocalization with p220^{NPAT} foci (green, row 4) and complete overlap with PML/ND10 bodies (green, row 5) in both hES cells and somatic WI-38 cells. The percentages in the lower left of the panels represent positive cells for colocalization of respective factors in each cell type.

p220^{NPAT} nor coilin foci, but 3' hExo foci show complete colocalization with PML/ND10 (promyelocytic leukemia domain/nuclear domain 10) bodies in both hES cells and somatic WI-38 cells (Fig. 2, rows 4 and 5, and Fig. S1). Hence, 3' hExo is spatially concentrated at domains distinct from p220^{NPAT} foci.

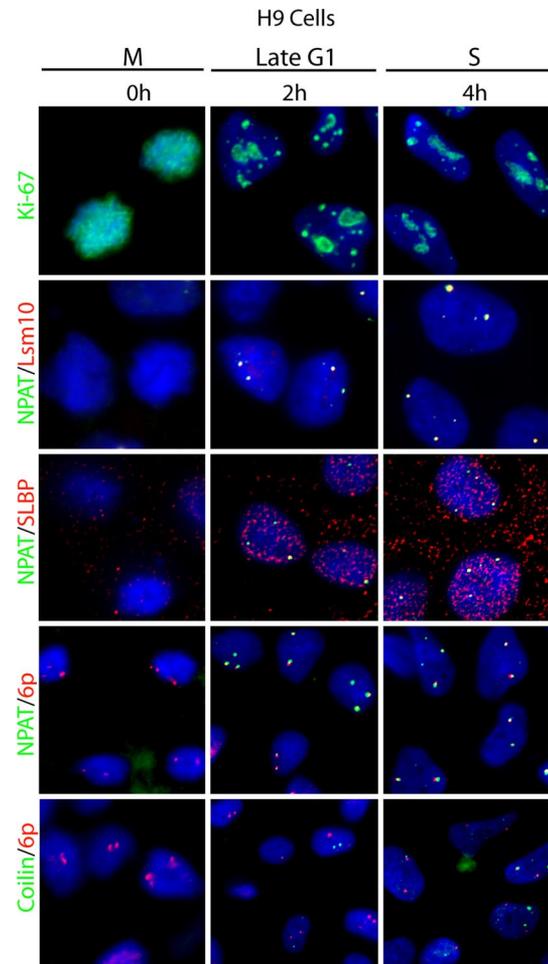


Fig. 3. Association of p220^{NPAT} and 3'-end processing factors with histone gene loci during the hES cell cycle. Mitotically synchronized hES cells at various cell cycle stages were monitored by IF microscopy for association of Lsm10 or SLBP (red) with p220^{NPAT} (green; rows 2 and 3), and spatial linkage of the histone gene cluster at 6p22 (red) with p220^{NPAT} or coilin (green; rows 4 and 5). Ki-67 (green) staining (row 1) was done to establish cell cycle position, and DAPI staining (all rows; blue) was used to visualize the nucleus.

Temporal and Spatial Association of p220^{NPAT} with the Factors Mediating Processing of Histone mRNA at Histone Gene Loci.

To understand the temporal coordination between p220^{NPAT} foci, 3'-end processing factors, and histone loci, we synchronized hES cells in G₂/M phase using nocodazole. Cell cycle entry and progression in synchronized hES cells were monitored using Ki-67 as a marker (Fig. 3, row 1) (1). Cells also were examined for localization of Lsm10 or SLBP to either p220^{NPAT} or coilin foci. Triple labeling by combining double-label IF microscopy with histone gene-specific FISH was performed to determine whether these factors associate with histone chromosomal loci (Fig. 1*B*).

At 1 h after release from mitotic block, when cells progress through early G₁, we do not observe focal organization of either p220^{NPAT} or Lsm10, nor is there evidence of association with histone loci (data not shown). However, in mid to late G₁ (2 h after release), p220^{NPAT} foci show complete colocalization with both Lsm10 and histone loci, while coilin foci do not (Fig. 3, rows 2, 4, and 5). This colocalization is maintained throughout S phase and the remainder of the cell cycle until mitosis (data not shown). Because Lsm10 colocalizes with histone genes containing p220^{NPAT} foci as soon as these foci form, our results demonstrate that 5'-end transcriptional initiation and U7 snRNP required for

and 1q21, as well as the histone transcript processing components Lsm10, Lsm11, and SLBP in both somatic and embryonic stem cells. Our findings indicate that the factors supporting synthesis and processing of histone transcripts are architecturally organized to form unique subnuclear domains. Previous studies in somatic cells have demonstrated that Cajal bodies are enriched for U7 snRNP components (23) and are often in proximity to histone gene loci (14, 15, 26, 28). Consistent with these data, we found that Cajal bodies are sporadically associated with histone gene loci in hES cells. Foci of p220^{NPAT} and coilin exhibit limited overlap. In contrast, p220^{NPAT} foci consistently associate with the 2 major histone gene clusters on 6p22 and 1q21 in hES cells. Because our data show that p220^{NPAT} foci are distinct from Cajal bodies and PML/ND10 bodies, and they contain histone genes and mRNA processing factors, we designate these domains histone locus bodies (HLBs), a term originally proposed for similar domains in *Drosophila* (24, 25).

Our findings support the concept that formation of p220^{NPAT} foci at histone gene loci, the recruitment of Lsm10 and SLBP, and the phosphorylation of p220^{NPAT} by CDK2 at HLBs are staged events that mediate histone gene expression. We have applied this staged spatial assembly of HLBs to define mechanistic differences between the cell cycles of hES cells and normal human diploid fibroblasts. In this and previous studies (26), we have shown that p220^{NPAT}, the defining resident protein of HLBs, rapidly forms detectable foci (≈ 1.5 h) following mitosis in hES cells. However, the assembly of these foci and their associated processing factors still occurs just ≈ 1 –1.5 h before the G₁/S transition, reflecting the abbreviated G₁ phase (≈ 2.5 –3 h) of hES cells (2). In contrast, results presented here and in previous studies (14, 15) for normal human somatic cells (with a G₁ phase of ≈ 8 –12 h) establish that HLBs assemble at least 6 h before S phase. Thus, the striking differences in cell cycle times between somatic and ES cells are directly reflected by in situ differences in the spatiotemporal assembly of the histone gene expression machinery.

HLBs contain CDK-phosphorylated p220^{NPAT} immediately preceding and following the G₁/S-phase transition in both somatic and embryonic stem cells. Hence, the shortened interval between formation of p220^{NPAT} foci and their phosphorylation indicates that the mid-to-late G₁ cell cycle stage is dramatically contracted in hES cells. In addition, because p220^{NPAT} foci are detected only by mid G₁ in somatic cells but appear rapidly following exit from mitosis in hES cells, the truncated G₁ phase of hES cells may also eliminate events coupled with early G₁. In conclusion, we show that both embryonic stem cells and somatic cells use a novel dedicated subnuclear domain (HLB) to coordinate synthesis and processing of histone gene transcripts. The temporal differences in the assembly of this domain between the 2 cell types provide insight into the abbreviated cell cycle of hES cells.

Materials and Methods

Cell Culture and Cell Synchronization. Human ES cells were cultured on a mitotically inactivated mouse embryonic fibroblast layer as described previously (1, 26). The cells were allowed to grow for 3–5 days after passaging

before use. WI-38 cells were grown in complete medium containing MEM, penicillin–streptomycin, L-glutamine, sodium pyruvate, nonessential amino acids, and 10% FBS (Atlanta Biologicals). The hES cells were synchronized using Nocodazole (200 ng/mL) (Sigma) for 16 h, and samples were taken at different time points after release. WI-38 cells were synchronized by serum depletion. Cells were allowed to grow for 7 days without changing the media and were released by adding fresh media containing 20% serum. Samples were collected at different times after serum stimulation.

Antibodies. The antibodies, their working dilutions, and their supplier are as follows: monoclonal p220^{NPAT} (mouse monoclonal; 1:1000; BD Biosciences), polyclonal p220^{NPAT} (rabbit polyclonal; 1:1000) (14, 15), coilin (pdelta; mouse monoclonal; 1:500; Santa Cruz Biotechnology), coilin (rabbit polyclonal; 1:500; ref. 29), PML (H-238; rabbit polyclonal; 1:250; Santa Cruz Biotechnology), PML (mouse monoclonal; PG-M3; 1:250; Santa Cruz Biotechnology), Lsm10 (mouse monoclonal; 1:500; Biomatrix Research), Lsm11 (rabbit polyclonal; 1:500), SLBP (rabbit polyclonal; 1:250), 3' hExo (rabbit polyclonal; 1:200; refs. 22 and 30, and Z.D., X.Y., and W.F.M., unpublished data). Secondary antibodies were goat anti-mouse Alexa 488, goat anti-rabbit Alexa 488, goat anti-mouse Alexa 594, goat anti-rabbit Alexa 594, goat anti-mouse Alexa 350, goat anti-rabbit Alexa 350 (all 1:800 dilution in 1 \times PBSA) (0.5% bovine serum albumin (BSA) (Sigma) in 1 \times phosphate buffered saline (PBS)). For FISH secondary antibody we used anti-digoxigenin (anti-DIG) rhodamine [1:500 in 4 \times standard saline citrate (SSC)/1% BSA; Roche].

IF Microscopy. Human ES cells or WI-38 cells were grown on gelatin-coated coverslips. IF was carried out as described previously (26). Briefly, cells were fixed with 3.7% formaldehyde for 10 min, permeabilized by 0.25% Triton X-100 for 20 min, and then treated with primary antibody for 1 h at 37°C, followed by detection using appropriate fluorescently-tagged secondary antibody. The nuclei were counterstained with DAPI.

FISH. Probes were made of BAC clones spanning a region at or very near to the histone gene loci on chromosome 6p22 (RP11-2p4) and 1q21 (CTD-2018M10) (Children's Hospital Oakland Research Institute, Oakland, CA). BAC DNA was purified by using Qiagen columns, and probe DNA was labeled using the DIG Nick translation kit (catalogue no. 11745816910; Roche Diagnostics) according to the manufacturer's protocol. The probe mixture was prepared by adding 50–100 ng probe DNA, 70% formamide, 10 μ g human Cot-1 DNA, and 10 μ g salmon sperm DNA. FISH was performed after hES cells or WI-38 cells were subjected to IF as described above. After IF, cells were fixed by passing through ethanol grades (70%, 85%, and 100%) and were briefly air dried. Cells were then codenatured with the probe mixture at 80°C for 8 min and allowed to hybridize overnight at 37°C in a moist chamber. Cells were washed by using 50% formamide/4 \times SSC for 15 min at 37°C, followed by washes in 2 \times SSC for 15 min at 37°C and 1 \times SSC for 15 min at room temperature. Probes were detected by incubation with appropriate secondary antibody, followed by 3 washes with 4 \times SSC, 0.1% Triton/4 \times SSC, and 4 \times SSC at room temperature with shaking. Some cells then were counterstained with DAPI and mounted in Prolong-Gold (Invitrogen). Cells were viewed under an epifluorescence Zeiss axioplan 2 microscope and images were captured using a Hamamatsu (C4742-95) charged coupled device (CCD) camera and analyzed by Metamorph imaging software (Universal Imaging). All images were captured at 100 \times magnification unless noted otherwise.

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