

# Decatenation checkpoint deficiency in stem and progenitor cells

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## Summary

**The decatenation checkpoint normally delays entry into mitosis until chromosomes have been disentangled through the action of topoisomerase II. We have found that the decatenation checkpoint is highly inefficient in mouse embryonic stem cells, mouse neural progenitor cells, and human CD34<sup>+</sup> hematopoietic progenitor cells. Checkpoint efficiency increased when embryonic stem cells were induced to differentiate, which suggests that the deficiency is a feature of the undifferentiated state. Embryonic stem cells completed cell division in the presence of entangled chromosomes, which resulted in severe aneuploidy in the daughter cells. The decatenation checkpoint deficiency is likely to increase the rates of chromosome aberrations in progenitor cells, stem cells, and cancer stem cells.**

## Introduction

Differentiated cells in many tissues have a relatively short life span and are continuously replaced by new cells generated from progenitor cells. In human bone marrow, for example, approximately 200 billion erythrocytes and 70 billion neutrophilic leukocytes are produced and released into the bloodstream every day (Gunsilius et al., 2001). There are extensive similarities between progenitor cells, stem cells, and cancer cells, including the central role of Wnt signaling (Reya and Clevers, 2005). This realization has been advanced by the discovery of cancer stem cells, a newly recognized subset of tumor cells responsible for tumor progression (Huntly and Gilliland, 2004; Pardal et al., 2003; Reya et al., 2001). In some cases, cancer stem cells may arise from non-stem cells that acquire self-renewal capability; in other cases they may arise from stem cells that lose proliferative control.

The decatenation checkpoint (Downes et al., 1994) in G2 phase delays entry into mitosis if the chromosomes have not been sufficiently decatenated or disentangled by topoisomerase II (topo II), the enzyme required for chromosome decatenation and condensation (Holm et al., 1989; Uemura et al., 1987). Catenations between sister chromatids result from DNA replication and must be resolved to ensure proper chromatid segregation in mitosis. Nonreplicative catenations, formed incidentally during interphase, also must be resolved to enable chromosome compaction and segregation (Gimenez-Abian et al., 2000). Cells

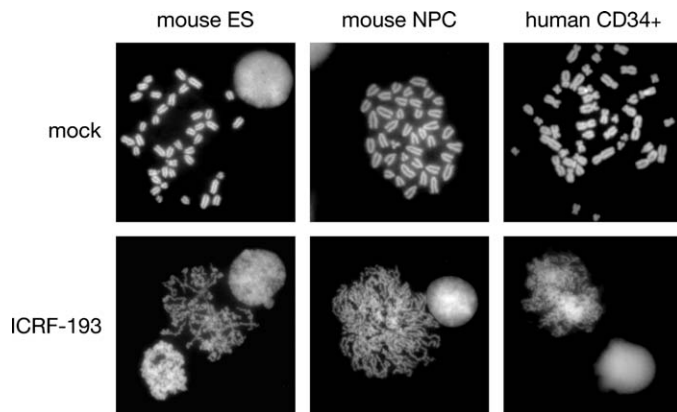
with an inefficient decatenation checkpoint can complete cell division in the presence of entangled chromosomes, which results in aneuploidy in the daughter cells (Gorbsky, 1994; Ishida et al., 1994). Thus, it is possible that a deficiency in the decatenation checkpoint could generate the aneuploidy and tetraploidy that are characteristic of many cancer cells.

The decatenation checkpoint is conserved in the plant and animal kingdoms and has been observed in primary cultured cells, transformed cells, and immortalized cell lines (Deming et al., 2001; Downes et al., 1994; Franchitto et al., 2003; Gimenez-Abian et al., 2002). The checkpoint is deficient in many human bladder cancer and lung cancer cell lines (Doherty et al., 2003; Nakagawa et al., 2004) but has not been reported to be deficient in any normal cell type. The decatenation checkpoint is distinct from the G2/M DNA damage checkpoint (Downes et al., 1994; Nakagawa et al., 2004), and its mediators are thought to include the ATR kinase, Polo-like kinase 1 (Plk1), the BRCA1 tumor suppressor, and the Werner's syndrome helicase (Deming et al., 2001, 2002; Franchitto et al., 2003).

Here, we report that mouse and human multipotent progenitor cells have an inefficient decatenation checkpoint. Checkpoint efficiency increased when embryonic stem (ES) cells were induced to differentiate, which suggests that the deficiency is a feature of the undifferentiated state. We observed that the decatenation checkpoint deficiency can result in severe aneuploidy in progenitor cells.

## SIGNIFICANCE

Recent findings indicate that tumor progression depends on a subset of tumor cells called cancer stem cells. We have discovered a cell cycle deficiency in normal progenitor and stem cells that gives rise to mutations that could contribute to the development of cancer stem cells. This deficiency in the decatenation checkpoint can result in cell division in the presence of entangled chromosomes and can increase the rates of chromosomal aberrations. Our results suggest that the deficiency is a feature of the undifferentiated state and does not require an extant mutation. Chromosome aberrations that arise in progenitor and stem cells as a result of this checkpoint deficiency are likely to contribute to tumorigenesis.



**Figure 1.** Pseudomitosis in stem and progenitor cells treated with topoisomerase II inhibitor

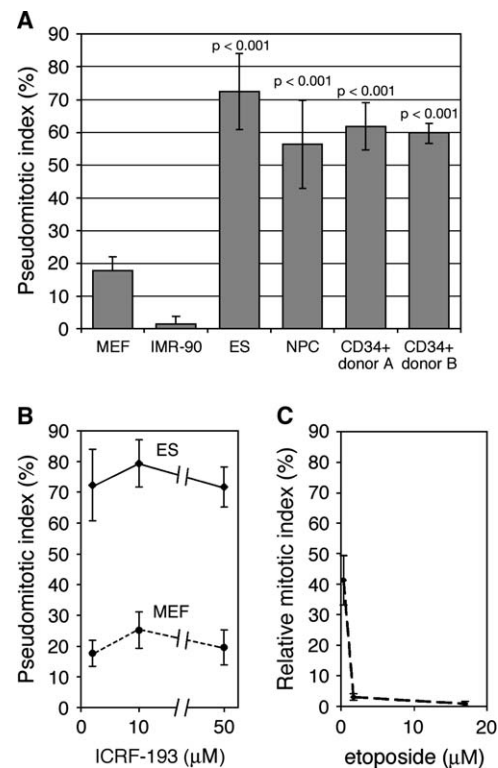
Mouse embryonic stem cells ("mouse ES"), mouse cortical neural progenitor cells (NPC), and human CD34<sup>+</sup> hematopoietic progenitor cells were treated with 2  $\mu$ M ICRF-193 or solvent (mock) and 40 ng/ml colchicine. Metaphase spreads were stained with propidium iodide. A pseudomitosis was distinguished from interphase cells by its condensed, entangled chromosomes.

## Results and discussion

The decatenation checkpoint can be studied with bisdioxopiperazines such as ICRF-193 (Creighton et al., 1969) that specifically inhibit topo II activity without causing DNA chain breaks (Downes et al., 1994; Roca et al., 1994; Tanabe et al., 1991). As shown in Figure 1, we observed pseudomitosis following treatment with ICRF-193 of mouse ES cells, mouse cortical neural progenitor cells (NPCs), and human CD34<sup>+</sup> hematopoietic progenitor cells. With regard to the decatenation checkpoint, the term pseudomitosis (Downes et al., 1994) is used to refer to spindle formation and kinetochore poleward movement in the presence of entangled chromosomes, which results in aberrant chromosome segregation.

The efficiency of the decatenation checkpoint in various cell types was measured by the pseudomitotic index, which is defined as the frequency of pseudomitosis in ICRF-193-treated cells divided by the frequency of mitosis in mock-treated cells. This ratio is normalized for variation in growth rate among cell types. A high pseudomitotic index indicates an inefficient decatenation checkpoint because the cells have entered mitosis in the presence of entangled chromosomes. Cultures of unsynchronized cells were treated for 4 hr with 2  $\mu$ M ICRF-193 or solvent; colchicine was added at 40 ng/ml to accumulate cells in mitosis or pseudomitosis. We found a high pseudomitotic index in the stem and progenitor cells (Figure 2A), indicating an inefficient decatenation checkpoint in those cells. In contrast, two non-progenitor cell types, primary mouse embryonic fibroblasts (MEFs) and human lung fibroblasts (IMR-90) (Nichols et al., 1977), exhibited a low pseudomitotic index, indicating an efficient decatenation checkpoint as observed in many other mammalian cell types (Deming et al., 2001; Downes et al., 1994).

As shown in Figure 2B, similar results were obtained over a broad range of ICRF-193 concentrations that exceeded the  $\sim 7$   $\mu$ M concentration required to completely inhibit topo II activity in mammalian cells (Clarke et al., 1993). A normal mitosis was not observed in any cell type after treatment with 2  $\mu$ M ICRF-193, indicating that this was an effective concentration. The



**Figure 2.** Inefficient decatenation checkpoint in stem and progenitor cells

**A:** Unsynchronized cells were treated for 4 hr with 2  $\mu$ M ICRF-193 or solvent (mock) and 40 ng/ml colchicine, and metaphase spreads were prepared. The pseudomitotic index is the frequency of pseudomitosis in ICRF-193-treated cells divided by the frequency of mitosis in mock-treated cells, as described in the text. Stem and progenitor cells were compared to mouse embryonic fibroblasts (MEF) and human lung fibroblasts (IMR-90). Statistics indicate the highly significant difference between progenitor versus non-progenitor cells of the same species. In all panels, error bars represent standard deviation of the mean.

**B:** The experiment in **A** was repeated for mouse ES cells and MEFs treated with 2  $\mu$ M, 10  $\mu$ M, or 50  $\mu$ M ICRF-193 or solvent.

**C:** The experiment in **A** was repeated for mouse ES cells treated with 0.34  $\mu$ M, 1.7  $\mu$ M, or 17  $\mu$ M etoposide or solvent.

observed deficiency was independent of colchicine and independent of the length of treatment (data not shown).

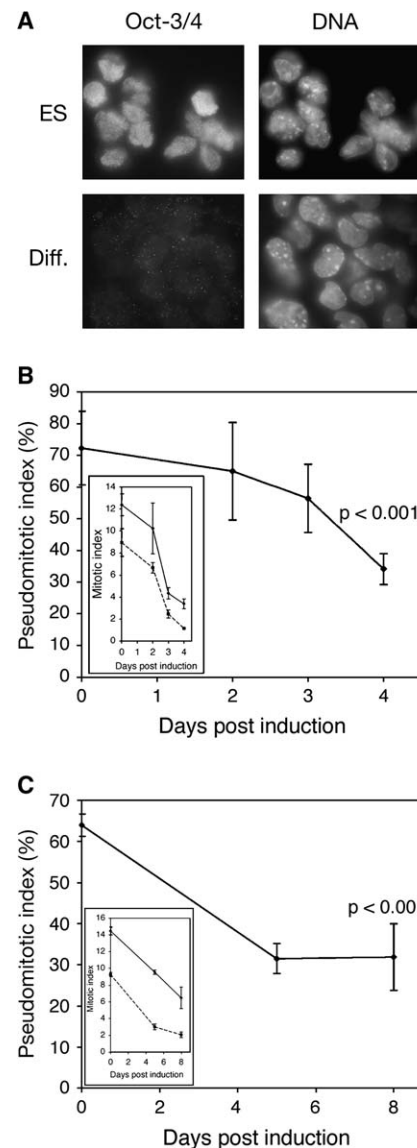
We considered the specificity of the observed deficiency by investigating another G2 phase checkpoint, the DNA damage checkpoint. To this end we compared the effects of ICRF-193 and etoposide, both of which inhibit topo II activity but by distinct mechanisms: etoposide causes double-strand breaks that trigger the G2/M DNA damage checkpoint, but ICRF-193 does not (Downes et al., 1994; Skoufias et al., 2004). When ES cells were treated with etoposide, a dramatic reduction in the mitotic index was observed in a dose-dependent manner (Figure 2C). The sharply contrasting effects of ICRF-193 and etoposide indicate that the decatenation checkpoint deficiency in ES cells represents a specific phenotype, since the DNA damage checkpoint appears to be intact. There is also a clear distinction at the physiological level between the effects of ICRF-193 and etoposide: no significant DNA damage is caused by ICRF-193 in ES cells, otherwise the damage checkpoint would be activated. Our results are consistent with other reports that distinguish the decatenation and DNA damage checkpoints (Downes et al., 1994; Nakagawa et al., 2004).

If the contrasting decatenation checkpoint efficiencies in progenitor cells versus non-progenitor cells reflect differences in developmental state, then the differentiation of progenitor cells should be accompanied by increased checkpoint efficiency. To test this hypothesis, we induced ES cells to differentiate *in vitro* and measured the decatenation checkpoint efficiency over the course of differentiation. In one method of differentiation induction (Smith, 1991), ES cells were plated at low density (day 0) and treated with retinoic acid starting on day 1. Immunostaining against Oct-3/4, a stem cell marker (Rosner et al., 1990; Scholer et al., 1990), indicated that by day 4 the culture consisted almost entirely of differentiated cells (Figure 3A). We found that the pseudomitotic index declined steadily with differentiation (Figure 3B), indicating an increase in the decatenation checkpoint efficiency. The pseudomitotic index by definition is corrected for changes in growth rate; while the frequency of mitosis in mock-treated cells decreased with differentiation, the frequency of pseudomitosis in ICRF-193-treated cells decreased more rapidly (Figure 3B, inset), yielding the declining pseudomitotic index shown in the larger graph.

Similar results were obtained by another method of differentiation induction (Strubing et al., 1995) in which embryoid bodies (EBs) were formed in hanging drops, grown in suspension, and plated on tissue culture plastic on day 4. The decatenation checkpoint assay was performed on days 0, 5, and 8. As shown in Figure 3C, the pseudomitotic index on days 5 and 8 was significantly lower than that on day 0. Again we observed that the frequency of mitosis decreased with differentiation but that the frequency of pseudomitosis decreased more rapidly (Figure 3C, inset). These data are consistent with the above results and indicate an increase in decatenation checkpoint efficiency during the differentiation of ES cells. The pseudomitotic index declined to the same level (~32%) with both methods of differentiation. These results suggest that the inefficient decatenation checkpoint in multipotent progenitor cells is a feature of the undifferentiated state.

An important inference from the data presented in Figures 2 and 3 and Table 1 is that decatenation checkpoint efficiency was independent of growth rate. In general, the progenitor cells grew at a faster rate than the differentiated cells, but there was no obvious correlation between mitotic index and checkpoint efficiency. The lack of correlation was also evident in the differentiation experiments: cells at three points had similar pseudomitotic index (~32%) but a 3-fold range of mitotic index (3.1%, 6.5%, and 9.6%).

We investigated the physiological consequences of the inefficient decatenation checkpoint in progenitor cells by treating ES cells with ICRF-193 and performing immunofluorescence with antibodies to microtubule and kinetochore proteins. We observed that ES cells completed cell division in the presence of entangled chromosomes, which resulted in severe aneuploidy in the daughter cells (Figure 4). Microtubule staining revealed proper spindle formation and the completion of cytokinesis; however, kinetochore staining revealed unequal segregation of centromeres, and DNA staining confirmed that the daughter cells contained vastly disproportionate quantities of DNA. The completion of pseudomitosis could result in many types of aneuploidy, including tetraploidy, the gain or loss of only one chromosome or chromosome fragment, and the induction of chromosomal translocations. Inhibition of topo II activity has been used as a means to drive single chromosome loss in order to generate new cell lines (Clarke et al., 1998).



**Figure 3.** Increased efficiency of the decatenation checkpoint in differentiating cells

**A:** ES cells were induced to differentiate by plating at  $10^4$  cells/cm<sup>2</sup> at day 0 and treatment with  $10^{-7}$  M all-*trans*-retinoic acid starting on day 1. Immunostaining against the stem cell marker Oct-3/4 was performed on day 0 ("ES") and day 4 ("Diff.").

**B:** ES cells were induced to differentiate by plating at  $10^4$  cells/cm<sup>2</sup> at day 0 and treatment with  $10^{-7}$  M all-*trans*-retinoic acid starting on day 1. The pseudomitotic index was determined on days 0 (before induction), 2, 3, and 4. Inset: frequency of pseudomitosis (dashed line) and mitosis (solid line), the ratio of which is presented in the larger graph. Error bars represent standard deviation of the mean. Statistics indicate the highly significant difference between checkpoint efficiency on day 0 and day 4.

**C:** ES cells were induced to differentiate in embryoid bodies as described in the Experimental Procedures. The pseudomitotic index was determined on days 0 (before induction), 5, and 8. Inset: frequency of pseudomitosis (dashed line) and mitosis (solid line), the ratio of which is presented in the larger graph. Error bars represent standard deviation of the mean. Statistics indicate the highly significant difference between checkpoint efficiency on day 0 and day 8.

We suggest that entangled chromosomes in pseudomitosis allow the bypass of the spindle assembly checkpoint. Tension generated by chromosome entanglements—replicative and

**Table 1.** Mitotic index of cells in this study

Cell type	Mitotic index (%) <sup>a</sup>
MEF	1.6
Human IMR-90	2.3
Mouse ES	12.4
Mouse NPC	3.1
Human CD34 <sup>+</sup> (donor A)	9.4
Human CD34 <sup>+</sup> (donor B)	10.3

<sup>a</sup>Percentage of the cell population in metaphase after 4 hr colchicine treatment; average of at least three samples.

nonreplicative catenations (Gimenez-Abian et al., 2000; Hirano, 1995)—may imitate the tension resulting from amphitelic attachment, thus bypassing the spindle checkpoint and triggering anaphase. This is consistent with the finding in which inhibition of topo II activity reduced activation of the spindle checkpoint in cohesin-deficient cells (Vagnarelli et al., 2004). Additional defects might be required for the completion of pseudomitosis in decatenation checkpoint-deficient cells, but that seems unlikely since it has been observed in many cell types (Downes et al., 1994; Gorbsky, 1994; Ishida et al., 1994).

Chromosome gain and loss have been observed during the ex vivo culture of human and mouse ES cells and could be a consequence of the inefficient decatenation checkpoint. In human ES cells, trisomy 3, isodicentric X chromosome, the recurrent gain of chromosomes 12 and 17q, and several additional chromosome aberrations have been observed (Draper et al., 2004; Inzunza et al., 2004; Kim et al., 2005; Maitra et al., 2005). In mouse ES cells, trisomy 8 is common and significantly reduces the success of genetic manipulations (Cervantes et al., 2002; Liu et al., 1997; Longo et al., 1997). Other aspects specific to the cell cycle in stem and progenitor cells may become additional sources of genetic instability during ex vivo culture. Aladjem and colleagues first reported on differences in the cell cycle between progenitor and non-progenitor cells (Aladjem et al., 1998). The

ex vivo culture of progenitor cells for therapeutic purposes demands more rounds of cell division than these cells typically undergo in vivo, and this could introduce unpredictable genetic variation into the cell population.

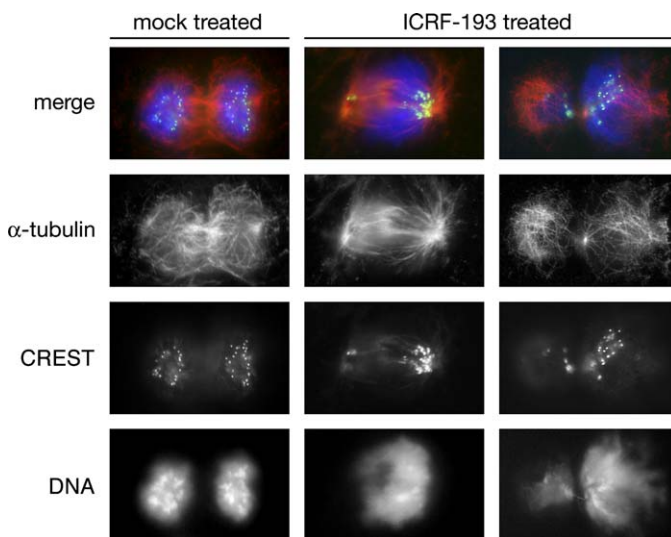
Mutations in progenitor and stem cells that arise from chromosome breakage and nondisjunction due to the inefficient decatenation checkpoint could contribute to the development of cancer stem cells. In the laboratory, certain mutations conferred self-renewal properties to committed hematopoietic progenitor cells and resulted in acute myeloid leukemia in mice (Cozzio et al., 2003; Huntly et al., 2004). Several clinical conditions, including myelodysplasia, juvenile dysmyelopoiesis, and constitutional trisomy mosaicism, have been attributed to mutations in progenitor cells that consisted of gain or loss of chromosomes or chromosome fragments (Heaney and Golde, 1999; Hogge et al., 1987; Parlier et al., 1992; Seghezzi et al., 1996). Pharmaceutical use of topo II inhibitors such as ICRF-193, which has been used in the clinic as a cardioprotectant (Andoh and Ishida, 1998), might enhance the risk of aneuploidy in progenitor cells and cancer stem cells. Wound healing and chronic stress, which are correlated with elevated cancer risk (Hofer et al., 1999), mobilize progenitor cells and might expose inherent cell cycle deficiencies.

## Experimental procedures

### Cell culture

Mouse ES cells derived from day 3.5 embryos (129/SvEv and J1 strain backgrounds) were cultured on gelatinized tissue culture dishes in high-glucose Dulbecco's medium (DMEM; Gibco) supplemented with 15% fetal bovine serum (HyClone), MEM nonessential amino acids (Gibco), 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 0.12 mM β-mercaptoethanol, and leukemia inhibitory factor (LIF). NPCs (Ge et al., 2002) were isolated from day 12 mouse embryos; cultured on dishes coated with 2 µg/ml polyornithine and 15 µg/ml fibronectin in DMEM/F12 with B27 supplement, penicillin, streptomycin, and a daily supplement of 10 ng/ml basic fibroblast growth factor (Peprotech); and used at passage 1 (6–12 days in vitro). The results comprise data from independent preparations of NPCs with triplicate samples in both experiments. MEFs derived from day 13.5–14.5 embryos were cultured on gelatinized dishes in DMEM with 15% serum, L-glutamine, penicillin, and streptomycin, and used at passage 2. IMR-90 cells (Nichols et al., 1977) were obtained from ATCC at passage 25, cultured in Eagle's medium (ATCC) with 10% serum, penicillin, and streptomycin, and used at passages 28 and 30. CD34<sup>+</sup> cells were isolated from peripheral blood of human donors and cryopreserved by the NHLBI PEGT Facility, Fred Hutchinson Cancer Research Center, Seattle, WA. CD34<sup>+</sup> cells were cultured in suspension in Iscove's medium (Gibco) with 20% BIT 9500 serum substitute (Stem Cell Technologies) and 100 ng/ml stem cell factor, 100 ng/ml Flt3 ligand, 10 ng/ml thrombopoietin, 10 ng/ml IL-6, and 10 ng/ml granulocyte colony stimulating factor (all from Peprotech). The cells were cultured for 2 days, during which the cell population approximately doubled, before the experiment.

ES cells were induced to differentiate by two methods. Differentiation by plating at low density (Smith, 1991) was achieved by plating ES cells at 10<sup>4</sup> cells/cm<sup>2</sup> in the medium described above in the absence of LIF and treating cells with 10<sup>−7</sup> M all-trans-retinoic acid (Sigma) starting 24 hr after plating. Differentiation by EBs was based on Strubing (Strubing et al., 1995). EB differentiation medium was Iscove's medium (IMDM; Gibco) with 20% fetal bovine serum, MEM nonessential amino acids, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 400 µM monothioglycerol (Sigma). Twenty microliter droplets containing ~600 ES cells in medium with 10<sup>−7</sup> M all-trans-retinoic acid (Sigma) were incubated for 2 days on the underside of the lids of bacteriological petri dishes filled with PBS; retinoic acid was included to increase the efficiency of differentiation. EBs were then incubated in suspension for 2 days in differentiation medium without retinoic acid. On day 4, the EBs were plated on 12 gelatinized 60 mm tissue culture dishes. EBs were dissociated thoroughly by pipetting before harvesting.



**Figure 4.** Aneuploidy in ES cells after challenge to the decatenation checkpoint

Mouse ES cells were treated with 2 µM ICRF-193 or solvent (mock) and then analyzed by immunofluorescence with antibodies against kinetochore proteins (CREST serum) and microtubules (α-tubulin).



## Chromosome preparations

ICRF-193 (MP Biomedical) was dissolved in dimethyl sulfoxide and stored in aliquots at  $-20^{\circ}\text{C}$ . Unsynchronized cells were treated for 4 hr with 40 ng/ml colchicine (Sigma) and 2  $\mu\text{M}$  ICRF-193 or solvent, unless otherwise noted. When necessary, cells were detached with trypsin and dissociated by pipetting. Hypotonic treatment consisted of either 75 mM KCl for 20 min at room temperature or prewarmed diluted serum (1:6 in water) for 20 min at  $37^{\circ}\text{C}$ . Cells were fixed in four changes of freshly mixed methanol:acetic acid (3:1) and spread on cold wet slides. Slides were stained with propidium iodide, and cells were counted using the 63 $\times$  objective of a fluorescence microscope. The number of cells counted per sample ranged from 1200 for cell types with higher mitotic index to 5000 for cell types with lower mitotic index. Experiments were performed with triplicate samples. The pseudomitotic index is the frequency of pseudomitosis in ICRF-193-treated cells divided (normalized) by the frequency of mitosis in mock-treated cells and is expressed as a percentage. The calculation of standard deviation for pseudomitotic index accounted for the normalization procedure. Student's *t* test modified for small sample size was used to compare cell types and also accounted for the normalization.

## Immunofluorescence

All solutions were based on phosphate-buffered saline (pH 7.2) with 1.5 mM  $\text{MgCl}_2$  and 0.9 mM  $\text{CaCl}_2$ . For Oct-3/4 staining, cells were cultured on gelatinized glass coverslips and fixed in 3.7% formaldehyde. Cells were permeabilized and blocked in 10% goat serum/0.1% BSA/0.5% Triton X-100; incubated with anti-Oct-3/4 monoclonal antibody (BD Transduction Laboratories) diluted 1:20 in block solution; and incubated with Cy3-conjugated goat anti-mouse antibodies (Jackson ImmunoResearch). Slides were mounted in Vectashield with DAPI (Vector Laboratories).

For CREST and tubulin costaining, cells were treated with 2  $\mu\text{M}$  ICRF-193 or solvent for 4.5 hr, harvested, centrifuged onto glass slides, and fixed in absolute methanol at  $-20^{\circ}\text{C}$  for 8 min. Cells were permeabilized in 0.2% Triton X-100 (Sigma) for 12 min, washed, and blocked in 5% goat serum/0.2% Tween 20 (both from Sigma). Cells were incubated with CREST serum (Moroi et al., 1980) (gift of P. Warburton and W.C. Earnshaw) and anti- $\alpha$ -tubulin monoclonal antibody (DM1A; Sigma) each diluted 1:1000 in blocking solution, and then with FITC-conjugated donkey anti-human and Cy3-conjugated goat anti-mouse antibodies. DNA was stained with 10 ng/ml Hoechst 33258. Slides were mounted in *n*-propyl gallate.

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