A novel cell response triggered by interphase centromere structural instability

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Introduction

Centromeres are specialized chromosome domains that are responsible for chromosome segregation during meiosis and mitosis. They assemble around repetitive DNA sequences in a complex structure that has yet to be fully elucidated. A simplistic view involves the division of this domain into two areas: the central core region or centromeric chromatin and the flanking heterochromatic regions, which are called pericentromeres. The protein composition of the central core region varies between interphase and mitosis. In this model, constitutive proteins are permanently associated with the centromere even during interphase, whereas facultative proteins are recruited only during mitosis to assemble the kinetochore. A specific feature of the infected cell protein 0 of herpes simplex virus type 1 to induce centromeric structural damage, revealing a novel cell response triggered by centromere destabilization. It involves centromeric accumulation of the Cajal body–associated coilin and fibrillarin as well as the survival motor neuron proteins. The response, which we have termed interphase centromere damage response (iCDR), was observed in all tested human and mouse cells, indicative of a conserved mechanism. Knockdown cells for several constitutive centromere proteins have shown that the loss of centromeric protein B provokes the centromeric accumulation of coilin. We propose that the iCDR is part of a novel safeguard mechanism that is dedicated to maintaining interphase centromeres compatible with the correct assembly of kinetochores, microtubule binding, and completion of mitosis.
The biological function of coilin within CBs remains mysterious, and its additional diffuse staining in the nucleoplasm has been proposed to be the mark of still unrevealed CB-independent activity (Matera and Frey, 1998).

Herpes simplex virus type 1 (HSV-1) infection of cultured cells induces the destabilization of centromeres during interphase, preventing the assembly of the kinetochores and the binding of microtubules during mitosis (Everett et al., 1999a). The factor responsible for this centromere destabilization is the viral protein infected cell protein 0 (ICP0). ICP0 is a RING finger nuclear protein with characterized E3 ubiquitin ligase activity (for review see Hagglund and Roizman, 2004). As soon as it enters the nucleus, ICP0 temporarily localizes to centromeres and induces the proteasomal degradation of CENP-A, -B, and -C (Everett et al., 1999a; Lomonte et al., 2001; Lomonte and Morency, 2007). Thus, ICP0-induced degradation of essential constitutive CENPs during interphase is likely to modify the structure of the central core region extensively, thereby preventing the assembly of the kinetochore. As a consequence, cells that express ICP0 just before entering mitosis are stalled in early mitosis and eventually suffer premature cell division without chromosomal segregation, leading to aneuploidy (Lomonte and Everett, 1999). Although the biological significance of ICP0-induced centromere destabilization is unclear, from the cellular viewpoint, ICP0 is of exceptional interest as a unique tool for studying centromere structure and the putative cell mechanisms implicated in centromere architectural maintenance. Indeed, although it is known that the cell uses kinetochore surveillance mechanisms during mitosis, such as the mitotic checkpoints (for review see Cleveland et al., 2003), it remains unknown whether the cell is able to detect centromeric structural defects in interphase.

In this study, using ICP0 as a tool, we reveal a previously unreported and unexpected cell response to human and mouse centromeres that have sustained structural modifications during interphase. This response is characterized by the accumulation at damaged centromeres of two CB proteins, coilin and fibrillarin, and one CB-associated gemini of CB (gems) nuclear domain protein, the survival motor neuron (SMN) protein. We show that this response does not implicate the entire CBs and/or gems. In addition, we confirm the physical association between coilin and centromere higher order type Iα-satellite (α-SAT) DNA. Using siRNA against CENPs, we demonstrate that depletion

![Image of cellular response to damaged interphase centromeres](image)

**Figure 1. Detection of a cellular response to damaged interphase centromeres.** (a) Coilin distribution in the CBs of control HeLa cells. (b) Coilin distribution in cells infected for 2 h with HSV-1 wt. The localization of ICP0 at centromeres is evidenced by the colocalization of white (ICP0) and red (centromere) dots. (c) Coilin distribution in cells infected for 4 h with HSV-1 wt. Centromeric coilin is clearly evident in the merged image as the colocalization of green (coilin) and red (centromere) dots. For a more representative view of the colocalization, the merged image was processed with the colocalization module of LSM 510 software (coloc.) so that all of the colocalized pixels appear black on a white background. ICP0 is present in the nucleoli (visible by counterstaining), as shown previously (Morency et al., 2005). (d and e) Coilin distribution in cells infected for 4 h with vFXE, a mutant virus that expresses a nonfunctional RING finger–deleted ICP0 protein, or by dl1403, a mutant virus that lacks ICP0 expression. (f) Coilin distribution in cells infected for 4 h with HSV-1 wt in the presence of the proteasome inhibitor MG132. (g) Detection of centromeric coilin in cells transfected with a plasmid that expresses ICP0. As in panel c, the merged image was processed with the colocalization module (coloc.) to show a more representative view of the colocalization. ICP0 and ICP4 (another viral protein that is detected when ICP0 is not present) serve as markers for the identification of infected or transfected cells. Centro, centromeres detected by huACA autoimmune serum. The arrowheads indicate several coilin signals that colocalize with centromeres. hpi, hours postinfection. Bars, 5 μm.
of CENP-B leads to the accumulation of coilin at centromeres. This confirms the existence of a cell response that is triggered by interphase centromere instability, for which we propose the term interphase centromere damage response (iCDR).

**Results**

**Loss of constitutive CENPs from interphase centromeres induces a specific cell response**

After our work on the ICP0-induced degradation of CENPs and the resulting destabilization of centromere structure, we decided to check whether and how cells were able to respond to centromere instability during interphase. Interphase HeLa cells were infected with HSV-1 wild-type (wt) virus and analyzed by immunostaining at 2 and 4 h after infection. As expected from our previous work, ICP0 transiently colocalized with centromeres at 2 h after infection but not at 4 h after infection (i.e., after degradation of the CENPs; compare ICP0 patterns in Fig. 1, b and c; insets; Everett et al., 1999b). At 4 h after infection (Fig. 1 c), ICP0 accumulated in large, visible foci inside the nucleoli as described previously (Morency et al., 2005). We found that at early stages of infection, the nuclear pattern of coilin underwent profound changes. Fig. 1 a shows noninfected cells in which coilin was localized in CBs. Infection by HSV-1 wt for 2 h did not substantially affect the coilin distribution (Fig. 1 b), whereas at 4 h after infection, coilin adopted a multidotted pattern in >90% of the infected cells (Fig. 1 c).

These coilin foci were more abundant and smaller than those that corresponded to CBs (0.2–0.5 μm for coilin foci vs. 1–1.2 μm for CBs in noninfected cells; compare coilin patterns in Fig. 1, a and c). Upon close examination, it was noticed that the nuclear distribution of these coilin dots looked very much like the pattern of immunostained centromeres. Therefore, we stained the latter together with coilin in ICP0-expressing cells; strikingly, most of the coilin foci indeed colocalized with centromeres (Fig. 1 c, yellow foci in the merged and magnified images). As the centromeric localizations of ICP0 and coilin were temporally separated (2 vs. 4 h after infection), the accumulation of coilin at centromeres is unlikely to be a consequence of an interaction with ICP0. Collectively, these data suggested that coilin accumulates at centromeres as a consequence of ICP0 activity on centromeres. To verify this notion, cells were infected with either the vFXE virus, which expresses the nonfunctional ICP0 RING finger mutant FXE, or the ICP0-null mutant dl1403, whose infection is detectable by ICP4 viral protein staining. We found that infection with these viruses did not induce coilin redistribution (Fig. 1, d and e).

ICP0 has E3 ubiquitin ligase activity that is associated with its RING finger domain. This activity is responsible for the induction of degradation via the proteasome of CENPs, which can lead to the destabilization of interphase centromeres and to defects in kinetochore assembly (Everett et al., 1999a). To verify the implication of proteasome activity in ICP0-induced coilin accumulation at centromeres, cells were infected with the HSV-1 wt virus in the presence of the proteasomal inhibitor MG132 or DMSO alone (unpublished data). In the presence of MG132, HSV-1 wt no longer induced the accumulation of coilin at centromeres (Fig. 1 f). These data suggest that there is a strong correlation between ICP0- and proteasomal-induced protein degradation (and probably CENP degradation) and the accumulation of coilin at centromeres.

Finally, to exclude any effect of the infection, we verified that transfected cells that expressed ICP0 (Fig. 1 g) but not those that expressed FXE (not depicted) showed a centromeric accumulation of coilin similar to HSV-1 wt–infected cells. As expected, 100% of the ICP0-expressing cells showed centromeric coilin. Note that during the course of these experiments, coilin colocalization with centromeres was never seen in mitotic cells. In conclusion, the aforementioned results demonstrate (1) the existence of a cell response that is triggered by the ICP0-induced structural damage of interphase centromeres (i.e., the iCDR) and (2) that coilin is implicated in the iCDR. These observations strongly suggest a role for coilin in a mechanism that is dedicated to the detection and/or repair of unstable interphase centromeric structures.

**Centromeric coilin interacts with centromeric, chromatin-specific higher order type I α-SAT DNA**

To determine whether coilin physically interacts with the central core region, we performed chromatin immunoprecipitation...
Figure 3. Fibrillarin and SMN but not CB-associated U2 snRNA accumulate at damaged centromeres. HeLa cells were either not transfected (control) or were transfected with an ICP0-expressing plasmid (ICP0) before performing IF or immuno-RNA FISH assays to detect the distribution of CB- and gem-associated proteins and RNAs. Cells that expressed ICP0 were detected by the particular centromeric coilin multidotted pattern described in Fig. 1. (a) IF detection of fibrillarin and SMN. In control cells, fibrillarin and SMN colocalize with coilin in the CBs (arrows in i and iii). Fibrillarin is also present in the nucleoli (large green areas). In ICP0-expressing cells, both proteins show a multidotted pattern similar to that of coilin, with which they colocalize at centromeres (arrowheads in ii and iv). Centro, centromeres detected by the huACA autoimmunserum. (b) Immuno-RNA FISH detection of U2 snRNA. (i) Control cells show a general nucleoplasmic staining for U2, with dense foci colocalizing with coilin. (ii) ICP0-expressing cells show the particular multidotted centromeric coilin without colocalizing U2 foci. The arrows point to a cell with multidotted centromeric coilin in which the classic CB-associated U2 pattern has disappeared. (c) ICP0 does not induce the proteasomal degradation of coilin, fibrillarin, or SMN. Hela cells were either not infected (lane 1) or infected with virus HSV-1 wt in the presence (lane 3) or absence (lane 4) of the proteasome inhibitor MG132, d1403 (ICP0 null, lane 2), or vFEXE (ICP0 mutant that is 44 amino acids shorter than the full-length ICP0; lane 5) for 6 h. 40-μg aliquots of total protein were loaded in each lane to perform WB for the detection of CENP-C (positive control), coilin, fibrillarin, SMN, ICP0, and actin as the loading control. The arrow points to the CENP-C signal. Bars, 5 μm.

(a) Control
(b) Fibrillarin centro collin
(c) ICP0

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Fibrillarin and SMN also accumulate at damaged interphase centromeres

A major issue in the biology of nuclear domains is whether the entire domain is involved in a process or only some components thereof. Thus, there was a need to determine whether components of CBs other than coilin also accumulate at damaged centromeres and, if so, whether whole CB domains are associated with centromeres in ICP0-expressing cells. In addition to coilin, CBs concentrate several small noncoding RNAs as well as an array of proteins, all of which are more or less implicated in transcription (Gall, 2001). Apart from fibrillarin, no other tested CB-associated protein exhibits centromeric accumulation in ICP0-expressing cells (Fig. 3 a, i and ii; Fig. 4, a–e; and Table I). Fibrillarin is one of the most abundant proteins in the fibrillar regions of the nucleolus. It is conserved from yeast to humans and is essential for early development in the mouse, being a catalyst of preribosomal RNA methylation (Omer et al., 2002; Newton et al., 2003). Because CBs also contain RNAs, immuno-RNA FISH assays were performed to analyze the pattern of the major CB-associated U2 small nuclear (ChIP) assays with HSV-1 wt–infected cells (Fig. 2 a) and looked for the association of coilin with centromeric, chromatin-specific higher order type I α-SAT DNA. In each case, we compared the ChIP of noninfected cells with that of cells at 4 h after infection (i.e., the time point at which coilin was found to have accumulated at centromeres; Fig. 1 c). The method and results were validated using an anti–CENP-A antibody as a control. Indeed, as expected, we observed a major decrease in the amount of CENP-A associated with type I α-SAT DNA as a consequence of ICP0-induced CENP-A degradation. We then tested an anticoilin antibody in parallel with the anti–CENP-A and anti-myc antibodies, with the latter being used as a non–specific control (Fig. 2 b). In each experiment, a single batch of chromatin was split into three aliquots, one for each antibody, and the three ChIPs were performed simultaneously.

The results obtained (Fig. 2 b, left) show that more coilin is associated with α-SAT DNA at 4 h after infection compared with noninfected cells, whereas less CENP-A is present at 4 h after infection. In these experiments, the amount of α-SAT DNA associated with the anti-myc control antibody did not notably change. Considering that the 4-h postinfection CENP-A was undetectable at centromeres by immunofluorescence (IF) and almost completely degraded, as shown by Western blotting (WB; Lomonte et al., 2001), a twofold decrease in the amount of α-SAT DNA retrieved (P < 0.05) with CENP-A can be regarded as representative of a major effect. In this context, the 1.7-fold increase in α-SAT DNA retrieved with coilin (P < 0.05) is suggestive of a significant increase in coilin levels at centromeres, which fits with our aforementioned results (Fig. 1). The DNA of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured to control for binding of the anticoilin and anti–CENP-A antibodies to unrelated DNA, and, as expected, no enrichment of coilin was noted (Fig. 2 b, right). From these data, we conclude that ICP0-induced centromere destabilization results in a physical interaction between coilin and centromeric DNA.
RNA (snRNA) in ICP0-expressing cells. No centromeric accumulation of U2 was detected (Fig. 3 b, i and ii). In addition, antibodies raised against both the 5′-terminal caps of the snRNAs and the snRNA-binding Sm proteins did not show any centromeric signals in ICP0-expressing cells (Fig. 4, d and e; and Table I). Collectively, these data indicate that only some components of the CBs, such as coilin and fibrillarin, accumulate at damaged centromeres.

Table I. Accumulations of CB and gem components at damaged interphase centromeres

| CB Accumulation at centromeres |
|--------------------------------|
| Protein component             | Accumulation at centromeres |
| Collin                        | Yes                         |
| Fibrillarin                   | Yes                         |
| Sm                            | No                          |
| p62 (TFIIH subunit)           | No                          |
| HCF-1 (host cell factor 1)    | No                          |
| FLASH                         | No                          |
| RNA component                 |                             |
| U2 (snRNA)                    | No                          |
| TMG-cap                       | No                          |
| Gems                          |                             |
| SMN                           | Yes                         |
| Gemin 2                       | No                          |
| Gemin 3                       | No                          |
| Other proteins                |                             |
| PML                           | No                          |
| PA28γ                         | No                          |

Sm proteins bind to snRNPs and become concentrated in CBs. FLICE-associated huge protein (FLASH) has recently been described as a component of CBs (Barcaroli et al., 2006). PML is the major component of the nuclear bodies, which are called PML nuclear bodies, ND10, or PML oncogenic domains. PML is degraded in an ICP0- and proteasomal-dependent manner (Everett et al., 1998). Under certain circumstances, PML bodies can be connected to centromeres in the G2 phase (Everett et al., 1999b; Luciani et al., 2006). PA28γ (proteasome activator γ) has recently been shown to colocalize and associate with coilin in UVC–treated cells (Cioce et al., 2006).

As shown by immuno-RNA FISH assays (see Fig. 3 b, ii).

As detected by the 5′-2,2,7-trimethylguanosine (TMG) antibody, which recognizes TMG-capped snRNAs.

In the cell nucleus, gems were originally defined as CB-associated domains on the basis of IF staining with antibodies against the SMN protein (Liu and Dreyfuss, 1996). This protein is the product of the SMN gene, whose loss of function mutations are responsible for the severe inherited disorder spinal muscular atrophy, one of the major genetic causes of infant mortality (Lefebvre et al., 1995). We decided to check the patterns of gem-associated proteins in ICP0-expressing cells. Three gem proteins, SMN, gemin 2, and gemin 3, were tested and showed foci that colocalized with CBs in control cells (Fig. 3 a, iii; arrows for SMN; and Fig. 4, f and g; gemin 2 and 3). In ICP0-expressing cells, SMN but not gemin 2 or 3 was detected in numerous small foci that, similar to coilin and fibrillarin, colocalized with centromeres (Fig. 3 a, iv; arrowheads; Fig. 4, f and g; and Table I). As was the case for coilin, 100% of the ICP0-expressing cells showed centromeric fibrillarin and SMN.

The aforementioned experiments suggest that neither coilin, fibrillarin, nor SMN is degraded in ICP0-expressing cells. To confirm this point, cells were infected with HSV-1 wt in the presence or absence of the proteasome inhibitor MG132, vFXE, or d1403 viruses at a multiplicity of infection of 10 (100% of the cells infected; Fig. 3 c). Coilin, fibrillarin, and SMN did not sustain ICP0-induced degradation.

These data rule out a putative relocation of the entire CB and/or gem to damaged centromeres and strongly suggest the existence of a specific subset of nuclear proteins (containing at least coilin, fibrillarin, and SMN) that accumulates in response to centromere damage. Therefore, coilin, fibrillarin, and SMN...
The iCDR is conserved in mouse cells

Mouse NIH3T3 cells were transfected with the ICP0-expressing plasmid before immuno-DNA FISH assays for the detection of coilin, fibrillarin, and centromere minor satellite sequences. (a) FISH of noninfected cells for the detection of pericentromere and central core regions using probes against the major (major SAT; red) and minor (minor SAT; green) DNA repeat sequences, respectively. The pattern corresponds to that described previously (Pietras et al., 1983; Guenatri et al., 2004). (b and c) Control cells labeled for coilin or fibrillarin, minor satellite repeats, and pericentromeres (DAPI). CB, Cajal body; No, nucleolus. (d) The same area of cells transfected and stained for the detection of ICP0 (i), coilin, minor satellite repeats, and pericentromeres (ii). Coilin is present in the multidots in the ICP0-positive cell, similar to its behavior in human cells. Several of these coilin dots colocalize with minor satellite repeats. (iii) Magnification of the ICP0-expressing cell shown in the boxed area. CB, Cajal body; No, nucleolus. (e) The same area of cells transfected and stained for the detection of ICP0 (i) and fibrillarin, minor satellite repeats, and pericentromeres (ii). Fibrillarin is present in the multidots in ICP0-positive cells, similar to its behavior in human cells. Several of these fibrillarin dots colocalize with minor satellite repeats. (iii) Magnification of the ICP0-expressing cell shown in the boxed area. Bars, 10 μm.

The iCDR is not induced by DNA breaks

A recent study has described the effect of UV-C irradiation on CB fragmentation (Cioce and Lamond, 2005). Consequently, coilin showed a change of pattern and increased interaction with PA28γ (proteasome activator subunit γ), a protein that is implicated in some aspects of proteasome activity in vitro (Wilk et al., 2000). This interaction resulted in the colocalization of coilin and PA28γ in UV-C-treated cells. Because UV light irradiation induces DNA breaks, the putative participation of coilin in a mechanism that is designed to resolve such damage to DNA has to be considered. Therefore, we investigated whether the response to ICP0-induced damaged centromeres implicated protein complexes involved in DNA break repair. PA28γ did not colocalize with coilin at the centromeres of ICP0-expressing cells (Fig. 4 i and Table I). Likewise, several proteins involved in single- or double-strand DNA break repair pathways (including nucleotide excision, base excision, and double-strand break repair) did not relocalize at the damaged centromeres in ICP0-expressing cells (Fig. 4 i and Table I). Given that irradiation of HeLa cells with γ-rays (from 2 to 10 Gy) does not provoke CB fragmentation (unpublished data) and that ICP0 is not known to provoke DNA breaks, these data do not support the accumulation of centromeric coilin, fibrillarin, and SMN as part of a putative DNA damage response–associated mechanism with activity in resolving DNA breaks.

The loss of CENP-B triggers coilin accumulation at centromeres

The aforementioned results demonstrate that ICP0 provokes the accumulation of at least three proteins at damaged centromeres. However, one could argue that the accumulations of coilin, fibrillarin, and SMN at centromeres are caused by an as of yet unknown activity of ICP0 and are not a direct result of CENP degradation. Therefore, we induced centromere destabilization independently of ICP0 using siRNAs that target the mRNAs of CENP-A, -B, and -C, the three known CENPs that are degraded in an ICP0-dependent manner. We verified by IF (Fig. 7 a) and WB (Fig. 7 b) the effects of the siRNAs on the stability of the targeted proteins. HeLa cells were transfected with single-type siRNAs or a mixture of two or three siRNAs, and the cells were then immunostained to detect (1) decreases in the amounts of the targeted proteins from centromeres and (2) centromeric accumulations of coilin, fibrillarin, and SMN. Cells with multidotted coilin, fibrillarin, or SMN, which were representative of the accumulations of these proteins at DNA, and coilin or fibrillarin (the anti-SMN mAb does not work on mouse cells). In the absence of ICP0, coilin and fibrillarin showed the same patterns in the mouse as in human cells (i.e., present in CBs and/or nucleoli; Fig. 5, b and c). In ICP0-expressing cells, coilin (Fig. 5 d, i–iii) and fibrillarin (Fig. 5 e, i–iii) showed a multidotted pattern that was similar to the pattern in human cells. These dots clearly colocalized with the minor satellite signals and, thus, with the central core region of the centromere. These data show that the iCDR is conserved in mammalian cells, at least between humans and mice, and is not an artifact of a single cell line.

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The iCDR is conserved in mouse cells

We observed the iCDR in additional human cell lines of various origins (e.g., breast carcinoma [T47D], colon carcinoma [SW480, HCT116, and HT29], and skin cancer [HaCaT]) as well as in primary keratinocyte cells. To determine whether this response is conserved in other species, the centromeric accumulations of coilin, fibrillarin, and SMN were analyzed in mouse NIH3T3 cells, which express ICP0. In mouse nuclei, chromosomes are distributed in clusters, and pericentromeric heterochromatin forms chromocenters that can be visualized by DAPI staining. The DNA sequences of the central core and pericentromere regions are based on two different types of DNA repeat, called minor and major satellites, respectively. These two domains are spatially separated and are clearly distinguishable by in situ hybridization (Fig. 5 a; Pietras et al., 1983; Guenatri et al., 2004). We performed immuno-DNA FISH assays on ICP0-expressing cells to detect ICP0 (Fig. 5, d and e), minor satellite
centromeres, were counted in several experiments that were performed independently. A scrambled sequence siRNA never had any effect on the coilin, fibrillarin, or SMN patterns. Similarly, none of the three CENP siRNAs was able to induce multidotted fibrillarin or SMN in a manner similar to ICP0 even when used in combination (unpublished data). Interestingly, coilin showed a multidotted pattern in a small proportion (~5%) of cells that were treated with the CENP-A or -C siRNA and in a large proportion (~30%) of cells that were treated with the CENP-B siRNA (Fig. 7c).

We confirmed by IF that the multidotted coilin colocalized with centromeres (Fig. 7d). To show a more representative view of the colocalization, the merged images were processed, as in Fig. 1(c and g) with the colocalization module of the LSM 510 software, so that all of the colocalized pixels appeared black on a white background (Fig. 7d, right column). CENP-A colocalization with huACA staining was used as a positive control (Fig. 7d, i). A large proportion of coilin colocalized with centromeres in ICP0-expressing cells (Fig. 7d, iii) as well as in CENP-B siRNA-treated cells (Fig. 7d, iv). Much fewer black spots were visible in the few CENP-A (or CENP-C; not depicted) siRNA-treated cells that showed multidotted coilin (Fig. 7d, v), and almost no black spots were seen in the scrambled sequence siRNA-treated cells (Fig. 7d, ii). In addition, the proportion of centromeres that colocalized with coilin was determined (Fig. 7d, right column; numbers in parentheses). Several images were scanned in each experiment (Fig. 7d, i–v) to determine a mean colocalization coefficient between centromeres and coilin. The coefficient for CENP-A colocalization with centromeres was arbitrarily fixed at 1. The colocalization coefficient in untreated cells or cells transfected with the scrambled sequence siRNA was very low, with a value of 0.046. Cells that were treated with the CENP-A (or CENP-C) siRNA showed a fivefold increase in the colocalization coefficient (0.28). ICP0-expressing cells and CENP-B siRNA-treated cells gave the highest coefficient (0.45) for coilin/centromere colocalization, with a 10-fold increase compared with the normal situation. The specificity of the accumulation of coilin at centromeres that lacked CENP-B and, to a lesser extent, CENP-A and -C renders an off-target effect of the siRNAs highly unlikely. However, we tested another siRNA against CENP-B and obtained similar results (unpublished data). These results confirm that the iCDR is triggered as a direct consequence of the loss of some constitutive CENPs and, thus, by the instability of the centromere structure.

**Discussion**

In this study, we took advantage of the effect of the ICP0 protein on the destabilization of centromere structures to investigate whether cells survive and adapt to such dramatic modifications at domains of major importance for their viability. We reveal a novel cellular response, named iCDR, suggesting the existence of mechanisms dedicated to dealing with structural damage to centromeres during interphase (i.e., before the onset of mitosis). We also describe a role for coilin in the iCDR, which implicates two other proteins, fibrillarin and SMN. In addition, the fact that several human cell lines and mouse cells manifest a similar response suggests a general mechanism that is likely to be conserved, at least in mammals.

**Table II. Involvement of DNA break repair mechanisms in the cellular response to damaged interphase centromeres**

| Proteins involved in DNA break repair | Accumulation at centromeres in ICP0-expressing cells | Remarks |
|--------------------------------------|-----------------------------------------------------|---------|
| γH2AX                                | No                                                  | NA      |
| BLM                                  | No                                                  | NA      |
| Rad51                                | No                                                  | NA      |
| DNA-PKCs                             | No                                                  | Degraded in an ICP0- and proteasomal-dependent manner |
| RPA32                                | No                                                  | NA      |
| P-RPA32                              | No                                                  | NA      |
| p53                                  | No                                                  | NA      |
| p62 (TFIIH)                          | No                                                  | CB-associated protein (see Table I and Fig. 3) |

γH2AX is rapidly phosphorylated on S139 in response to DNA damage. BLM is a helicase from the recQ subfamily that is involved in the cellular response to DNA damage and stalled replication forks. It participates with Rad51 in homologous recombination. DNA-PK plays a central role in the nonhomologous end-joining DNA pathway. DNA-PKcs is the catalytic subunit of DNA-PK. Replication protein A (RPA) is a single-stranded DNA-binding protein that is composed of three subunits of 70, 32, and 14 kD. RPA is essential for the recombination and DNA repair pathways. RPA32 becomes hyperphosphorylated on S4 and S8 in response to DNA damage. p62 is a core subunit of the transcriptional/repair factor TFIIH, which is involved in the nucleotide excision repair pathway. NA, not applicable.

1Nucleotide excision, base excision, and double-strand breaks.
2As shown previously (Parkinson et al., 1999).
Importantly, our data from cells knocked down for CENP proteins confirm the existence of a cellular response that is triggered directly by structural modifications to centromeres. Although efficient for coilin, the single and combined siRNAs were, unlike ICP0, ineffective in stimulating fibrillarin and SMN. Therefore, the centromeric accumulations of fibrillarin and SMN are probably induced by more severe damage than that arising from the absence of CENP-A, -B, or -C or by the absence of other CENPs. Interphase centromeres are complex structures organized into multisubunit protein domains that are associated partly with the CENP-H–I complex and partly with the centromere-specific CENP-A–containing nucleosomes in the distal (CENP-A nucleosome distal) and proximal (CENP-A nucleosome-associated complex) layers (Foltz et al., 2006; Okada et al., 2006). In light of our results, these data are informative in two respects. First, to engineer the collapse of the entire centromeric structure using CENP-directed siRNAs, it is necessary to affect more than one protein. Second, it is anticipated that ICP0 induces the proteasomal degradation of more CENPs than the individual CENP-A, -B, and -C proteins. Therefore, a simple explanation for the differential centromeric accumulations of coilin, fibrillarin, and SMN seen in this study may be the levels of damage caused to the different layers of CENPs.

Coilin accumulation at damaged centromeres is induced by CENP-B depletion, which suggests that the absence of CENP-B, unlike the absence of CENP-A and -C, is sufficient to trigger the response. CENP-B is a DNA-binding protein that has been implicated by in vitro studies in the positioning of nucleosomes (Yoda et al., 1998; Tanaka et al., 2005). It is unclear whether there is an absolute requirement for CENP-B for the preservation of centromere structure and function, as knockout mice for CENP-B are viable (Hudson et al., 1998; Kapoor et al., 1998; Perez-Castro et al., 1998) and CENP-B is essential for the de novo formation of centromeres, control of the epigenetic state of centromeric chromatin, and assembly of CENP-A (Masumoto, H., personal communication; Ohzeki et al., 2002). In any case, CENP-B is highly conserved in higher eukaryotes, which does not fit with the absence of major phenotype reported.

Figure 7. CENP-B depletion induces coilin accumulation at damaged centromeres. (a and b) To validate the efficiency of the siRNAs, HeLa cells were either not transfected (control) or were transfected with a scrambled sequence siRNA (siScr) or siRNAs against CENP-A, -B, or -C. Decreases in the CENP-A, -B, and -C signals were detected by IF (a) or WB (b) using the appropriate antibodies. The numbers in parentheses in panel a are the estimated percentages of cells that showed a substantial decrease in the protein signal. The arrow in panel b points to the CENP-C signal. (c) Counting of the nuclei with multidotted coilin, fibrillarin, or SMN in Hela cells knocked down for CENP-A, -B, or -C. In total, ~800 cells were analyzed for each protein from several independently performed experiments. The graph shows the percentage of nuclei with multidotted proteins. (d) IF showing protein colocalization with centromeres in Hela cells not treated (i), expressing ICP0 (iii), or transfected with siRNAs (ii, iv, and v). Each pixel resulting from the colocalization of coilin (ii–v) or CENP-A (i) with centromeres viewed in the merged images is visualized by black spots using the colocalization module of LSM 510 software. The numbers in parentheses represent the calculated colocalization coefficients (mean). The coefficient for CENP-A colocalization to centromeres is arbitrarily fixed at 1. Centro, centromeres detected by the huACA autoimmune serum. Bars, 5 μm.
in the cenpB\(^{-/-}\) mice studies (Hudson et al., 1998; Kapoor et al., 1998; Perez-Castro et al., 1998). The fact is that in the particular context of mouse cells, centromeres are stable and functional even if the cenpB gene is missing. One explanation is that in cenpB\(^{-/-}\) mice, the centromere structures could have acquired a CENP-B-independent equilibrium that is epigenetically transmissible. This situation is not quite the same as the one described in our present study, in which we hypothesize the rapid disruption of the equilibrium of the centromere structure by the rapid degradation of CENPs. In this regard, we investigated the coilin and fibrillarin distributions in untreated (not expressing ICP0 and not transfected with CENP siRNAs) cenpB\(^{-/-}\) mouse embryonic fibroblast cells derived from CENP-B knockout mice and did not detect any accumulations of these proteins at centromeres (unpublished data).

We know that the accumulation of coilin and fibrillarin at damaged centromeres occurs in ICP0-expressing mouse NIH3T3 cells (this study) and normal mouse embryonic fibroblast cells (unpublished data). Therefore, the absence of the accumulation of these proteins at the centromeres of steady-state cenpB\(^{-/-}\) mouse embryonic fibroblast cells suggests that the centromeric coilin, fibrillarin, and SMN proteins do not act as part of a putative structural complex that replaces the missing proteins but rather as a response of the cell to dramatic modifications of the centromere structure at a given time point. Centromeres possess a very specific organization of their chromatin compared with noncentromeric chromatin, and, thus, they are likely to concentrate unique cellular processes. Our data showing that ICP0-expressing cells lack centromeric accumulations of proteins implicated in DNA break repair mechanisms do not favor the hypothesis of DNA lesions as a direct consequence of the accumulations of centromeric coilin, fibrillarin, and SMN. Therefore, it is likely that the modification of centromere structure itself is responsible for triggering the iCDR. Interestingly, two out of the three known CENP targets of ICP0, CENP-A and -B, are established chromatin-related proteins. This raises the question as to whether the recruitment of centromeric coilin, fibrillarin, and SMN is initiated by the abnormal protein content of the centromere and/or by the abnormal chromatin structure.

On the basis of the data in the literature, it is difficult to come up with a convincing explanation for the interactions of coilin, fibrillarin, and SMN with damaged centromeres. First, this association was unexpected. Second, there is a general lack of information concerning the architecture of the central core centromere, particularly during interphase. However, coilin, fibrillarin, SMN, and centromeres may be linked by RNAs, especially small noncoding RNAs. Indeed, coilin, fibrillarin, and SMN are components of nuclear bodies, CBs, and gems, whose best-characterized functions remain the maturation of small noncoding RNAs that are implicated in the processing of larger transcripts. The association of small RNAs through the RNA interference mechanism with the epigenetic modifications of centromeric heterochromatin is now well documented, particularly in the yeast Schizosaccharomyces pombe (for reviews see Pidoux and Allshire, 2005; Verdel and Moazed, 2005). Importantly, this RNAi-dependent heterochromatization is directly linked to transcriptional activity in the pericentromeric heterochromatin. Although the existence of a similar mechanism in higher eukaryotes has not yet been demonstrated, it is clear that centromeric heterochromatin–associated transcriptional activity is conserved at least in humans, maize, rice, and chickens (Saffery et al., 2003; Fukagawa et al., 2004; Topp et al., 2004; Zhang et al., 2005). Moreover, recent studies have shown that transcription and small RNAs can participate in the architecture and function of centromeres in murine cells (Maison et al., 2002; Bouzinba-Segard et al., 2006). From these data, it is tempting to speculate that the presence of coilin, fibrillarin, and SMN at damaged centromeres reflects the involvement of RNAs in maintaining the centromeric chromatin structure. Future studies should provide new information that is relevant to this hypothesis.

At the molecular level, we anticipate a close link between the iCDR and the need to reform a functional centromere structure that has been accidentally damaged during interphase. This response could trigger a mechanism that eventually results in the reformation of a fully functional prekinetochore. This inevitably raises questions as to the consequences of centromere instability for general chromosomal instabilities that result in aneuploidy and cancer development (Jallepalli and Lengauer, 2001). Several types of genetic alteration are responsible for chromosomal instabilities, including those that affect kinetochore functions (Bharadwaj and Yu, 2004). Although there is a clear need for correct kinetochore structures to prevent chromosomal instabilities, not much is known concerning the capacities of interphase centromeres to serve as platforms for kinetochore nucleation. If interphase centromeres are unable to build functional kinetochores as a result of structural problems, it is likely that the mitotic spindle checkpoint will be weakened and its activity bypassed, forcing mitosis and provoking aneuploidy (for review see Rieder and Maiato, 2004). This is precisely what has been described in a recent study of late embryonic development in Drosophila cid/cenp-A–null mutants (Blower et al., 2006). Therefore, it is reasonable to propose the existence of a cellular response that acts as a sensor mechanism to signal the emergence of structural problems to interphase centromeres.

In retrospect, it is not surprising that cells are sensitive to defects at interphase centromeres considering the major roles of these genetic loci. To date, it has been unknown whether cells have developed mechanisms during interphase to check the functionalities of these structures. Our present results clearly show that this is probably the case, and they raise questions as to the existence of pathways that are committed to sensing, signaling, and repairing centromeres before the cell enters mitosis. In this framework, future studies will need to address the importance and functions of proteins, such as coilin, fibrillarin, SMN, and other proteins, in these types of pathways.

Materials and methods

Cell lines, plasmids, and viruses

HeLa and NIH3T3 cell lines were cultivated in BHK-21 and DME, respectively, which were supplemented with 10% FBS, l-glutamine (1% vol/vol), 10 U/ml penicillin, and 100 μg/ml streptomycin. The pc110 plasmid, which expresses ICP0, has been described previously (Everett et al., 1993). The wt strain HSV-1 syn + (17+) is the parental strain (referred to as HSV-1 wt in this study). The d1403 virus, which was deleted of ICP0 (Stow and Stow, 1986), the vfaE virus, which expresses a nonfunctional ICP0 isoform...
mutated in its RING finger domain, and the standard infection procedures have been described previously (Everett, 1989).

**Transfection, infection, and confocal microscopy**

Cells were seeded at 1.5 × 10^5 cells per well for transfection and at 2.5 × 10^5 cells per well for infection in 24-well plates that contained round coverslips. 24 h later, the cells were transfected with the pCI110 plasmid containing the virus to the manufacturer’s recommendations (Effectene Transfection Reagent; QIAGEN) or infected. Cells were treated for IF as described previously (Lomonte et al., 2001). With the exception of Fig. 5, in which a CCD camera (CoolSNAP HQ; Roper Scientific) was used for the analysis (Metaview software; Molecular Devices), all of the samples were examined under a confocal microscope (LSM 510 Meta; Carl Zeiss Microlmaging, Inc.). The data from the channels were collected sequentially with fourfold oversampling at a resolution of 512 × 512 pixels using optical slices of 0.8-1.0-μm thickness. A macro (Axiovert 200M; Carl Zeiss Microlmaging, Inc.) was used at either 63× (NA 1.25) or 100× (NA 1.3) magnification by oil-immersion objective lenses (Carl Zeiss Microlmaging, Inc.). Datasets were processed using LSM 510 software (Carl Zeiss Microlmaging, Inc.) and exported in preparation for printing using Photoshop (Adobe).

**IF in situ hybridization**

**Immuno-
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