Poly(ADP-ribose) polymerase 2 localizes to mammalian active centromeres and interacts with PARP-1, Cenpa, Cenpb and Bub3, but not Cenpc

Alka Saxena¹, Lee H. Wong¹, Paul Kalitsis¹, Elizabeth Earle¹, Lisa G. Shaffer² and K.H. Andy Choo¹,*

¹The Murdoch Childrens Research Institute, Royal Children’s Hospital, Flemington Road, Parkville 3052, Australia and ²Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030, USA

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INTRODUCTION

The centromere is the site of organization of a kinetochore on mitotic chromosomes that facilitates attachment and alignment of chromosomes on mitotic spindle microtubules (1). Facultative centromere proteins, such as the mitotic motor protein CENP-E and the spindle assembly checkpoint components CENP-F, Zwint, ZW10, MAD1, MAD2, BUB1, BUBR1 and BUB3, localize to the kinetochore region at specific stages during mitosis and monitor accurate progression from prometaphase to telophase (2–14). Constitutive centromere proteins, such as CENP-A, CENP-B, CENP-C, CENP-G and CENP-H, on the other hand, remain at the centromere at all stages of the cell cycle (15–19).

Poly(ADP-ribose) polymerase 1 (PARP-1) is the first known member of the PARP family. It is a nuclear enzyme and plays an important role in the DNA damage surveillance network. It detects and is activated by DNA strand breaks, and responds by immediate synthesis of poly(ADP-ribose) polymers from NAD. At the site of DNA damage, it catalyses the transfer of poly(ADP-ribose) polymers to protein acceptors. This post-translational modification of acceptor proteins alters their structure and renders them incapable of binding to DNA. Thus PARP-1 plays a significant role in the regulation of their function (reviewed in 20). Proteins participating in a wide range of physiological processes, such as maintenance of chromatin architecture (histones, Topo II and HMG), DNA synthesis and repair (DNA polymerases α and β, DNA ligases I and II, XRCC1), and transcription (RNA polymerases, p53, Yin and Yang, and NFκB), are known acceptors of poly(ADP-ribose) polymers (20–26).

Poly(ADP-ribose) polymerase 2 (PARP-2) is a newly discovered member of the PARP family. We report the association of PARP-2 with mammalian centromeres in a cell-cycle-dependent manner, accumulating at centromeres during prometaphase and metaphase, disassociating during anaphase, and disappearing from the centromeres by telophase. Analysis of a pseudodicentric chromosome and a human neocentromere indicates that PARP-2 binding occurs only at active centromeres in a sequence-independent manner. Centromere binding peaks at the outer centromere region, and is significantly enhanced upon treatment with microtubule-inhibiting drugs. Co-immunoprecipitation assay demonstrates interaction between PARP-2 and its functional homolog PARP-1, constitutive centromere proteins Cenpa and Cenpb, and spindle checkpoint protein Bub3, but not with a third constitutive centromere protein Cenpc. These results, together with our previous demonstration that PARP-1 displays an identical binding pattern with Cenpa, Cenpb and Bub3, but not Cenpc, and that all three proteins undergo significant poly(ADP-ribosyl)ation upon γ-irradiation of cells, point to possible diverse roles of PARP-2 and PARP-1 in modulating the structure and checkpoint functions of the mammalian centromere, in particular during radiation-induced DNA damage.
although chromosomal aberrations and tetraploidy, which may not be attributable to centromere dysfunction, are relatively common in these cells (36,37). The absence of a detrimental centromeric and other cellular phenotype, together with demonstration that cells from the PARP-1-null mice exhibit poly(ADP-ribosyl)ation activity (38) suggests functional redundancy, possibly due to the presence of other members of the PARP family that can modulate a similar poly(ADP-ribose)lation role as PARP-1.

PARP-2 is another member of the mammalian PAR family. Like PARP-1, it has a putative DNA-binding domain and catalytic domain. It catalyses the formation of poly(ADP-ribose) polymers in the presence of damaged DNA in vitro, and has been suggested to be responsible for the poly(ADP-ribose)lation activity observed in PARP-1−/− cells (39). Here we describe the dynamic association of PARP-2 with mammalian centromeres, including a human neocentromere and the active centromere of a human pseudodicentric chromosome. We show that PARP-2 accumulates at centromeres between prometaphase and early anaphase. This association is enhanced with the use of microtubule-inhibiting drugs, suggesting a possible role in the metaphase-to-anaphase transition. We further present evidence that PARP-2 interacts with the centromere proteins Cenpa, Cenpb and Bub3, but not Cenpc, and discuss possible roles of PARP-2 in chromatin organization and checkpoint control.

RESULTS

Cell cycle localization of PARP-2 at the centromere

Immunofluorescence analysis of PARP-2 distribution during different stages of the cell cycle was performed on human fibroblast cells grown on coverslips. Centromeres were identified by using CREST#6 antibody (40). Staining of cells in interphase with anti-PARP-2 antibody revealed considerable cytoplasmic signals. No outstanding colocalization of PARP-2 with CREST signals was observed, suggesting the absence of significant accumulation of PARP-2 at the centromeres above background during interphase (Fig. 1A). Similarly, cells in prophase failed to reveal significant enhancement of PARP-2 signals at centromeres (Fig. 1B). Upon breakdown of the nuclear membrane at prometaphase, PARP-2 signals were detected at centromeres, colocalizing with CREST signals (Fig. 1C). As the chromosomes condensed and progressed into metaphase, PARP-2 signals were distinctly visible as discrete dots on centromeres (Fig. 1D). The intensity of PARP-2 signals declined from centromeres during anaphase, becoming barely or non-detectable by late anaphase (Fig. 1E and F). No centromeric localization of PARP-2 could be detected at telophase (Fig. 1G). These results indicate that association of PARP-2 with the centromeres occurs most prominently during the prometaphase and metaphase stages.

PARP-2 localizes broadly across the centromere, peaking at the outer centromere region

We investigated the position of PARP-2 across the centromere–kinetochore region on condensed metaphase chromosomes of human HeLa cells arrested with demecolcine. Figure 2 shows the dual-colour immunofluorescence distribution pattern of PARP-2 relative to the positions of the previously mapped centromere proteins CENP-A, CENP-B and CENP-C. Direct microscopic visualization of the chromosomes under 100× magnification indicated considerable overlap of PARP-2 immunofluorescence signals with those for the constitutive centromere proteins CENP-A, CENP-B and CENP-C at the centromeres (Fig. 2i). Significantly, PARP-2 signals appeared to extend outside the regions occupied by the three constitutive centromere proteins, most prominently seen with CENP-B. Digital image measurement and graphical representation of fluorescence signal intensities were used to more clearly determine the relative positions of the different proteins. The results indicated that PARP-2 signals peaked at positions exterior to those represented by CENP-A, CENP-B and CENP-C, towards the outer centromere regions (Fig. 2ii).

PARP-2 is associated with the active but not inactive centromere of a dicentric chromosome

A human t(X;15) cell line (41) containing a pseudodicentric chromosome with an active centromere derived from a translocated X chromosome and an inactive centromere derived from chromosome 15 was used in this study. Previous reports using pseudodicentric chromosomes have shown that only the active centromere associated with proteins essential for centromere function, whereas both the active and inactive centromeres bound the non-essential CENP-B (41–43). We used CREST#6 antisera to localize the active and inactive centromeres on the dicentric t(X;15) chromosome. As shown in Figure 3A, CREST#6, which cross-reacts with both CENP-A and CENP-B (40), generally gave strong signals on all of the active centromeres (due to the presence of both CENP-A and CENP-B), including that of the chromosome X-derived centromere of the dicentric t(X;15), and a weaker signal on the inactive chromosome 15-derived centromere of the t(X;15) (due to the absence of CENP-B only). More specifically, the results demonstrated colocalization of PARP-2 with the strong CREST#6 signal on the active centromere (showing a primary constriction) but not with the weaker CREST#6 signal on the inactive (non-constricted) centromere. These observations indicate that PARP-2 associates with only the active centromere of a pseudodicentric chromosome.

Localization of PARP-2 on human neocentromere

We have previously described a human chromosome 10q25-derived marker chromosome mardel(10) containing a neocentromere that is devoid of centromeric α-satellite DNA (44). More than 20 proteins that are essential for centromere function have been shown to localize to the neocentromere on this marker chromosome (45). Here, we investigated the localization of PARP-2 to the mardel(10) neocentromere using immunofISH. The results indicated positive colocalization of the anti-PARP-2 antibody with the 10q25 neocentromere-specific FISH probe on the mardel(10) chromosome, but not at the corresponding q25 region of the normal chromosome 10 (Fig. 3B). These results, together with those for the dicentric
Figure 1. Cell cycle distribution of PARP-2 in human fibroblasts: cells in (A) interphase, (B) prophase, (C) prometaphase, (D) metaphase, (E) anaphase, (F) late anaphase and (G) telophase. Results are shown for (i) combined immunofluorescence staining, (ii) chromatin staining with DAPI (blue), (iii) centromere staining with CREST#6 (red) and (iv) anti-PARP-2 antibody staining (green). Note the distinct PARP-2 staining on centromeres at prometaphase and metaphase, the very weak staining at anaphase, and the absence of enhanced centromere staining during the other stages. A moderate to significant amount of background staining is also apparent throughout the cell cycle, especially during mitosis, presumably due to the localization of PARP-2 elsewhere.
chromosome study, further establish a direct association of PARP-2 with functional centromeres.

Comparison of PARP-1 and PARP-2 localization on mouse chromosomes

In an earlier study, we have demonstrated the centromeric localization of PARP-1 on mouse chromosomes, but noted that the PARP-1 signals significantly extended beyond the CREST6 signals along the proximal chromosome arm region, suggesting that PARP-1 probably associated with pericentromeric heterochromatin containing both the minor and major satellite sequences (27). We were therefore interested to determine whether PARP-2 shows a similar pattern of localization on mouse chromosomes. The results indicated that, unlike the broader centromeric and pericentromeric distribution of PARP-1, PARP-2 localizes at the mouse centromeres as discrete doublet signals that do not appear to extend significantly into surrounding pericentromeric heterochromatin (Fig. 3C). These observations suggest that PARP-2 displays a greater degree of specificity for the mouse centromeres compared with PARP-1.

Mitotic arrest induces a build-up of PARP-2 at metaphase centromeres

We were interested in investigating the effects that mitotic arrest may have on PARP-2 binding at the centromeres. Demecolcine and paclitaxel which disrupt microtubule dynamics through different underlying mechanisms (46,47), were used to achieve mitotic arrest. Dual-colour immunofluorescence analysis was used to compare PARP-2 signals against the
signals of CREST#6 antibodies. The results indicated that PARP-2 staining at the metaphase centromeres of drug-treated cells was greatly enhanced compared with those seen in untreated metaphase cells (Fig. 4A, B and C). Reversing the fluorochromes for the detection of PARP-2 and CREST#6 to those used in Figure 4 yielded the same outcome (data not shown). These observations suggested that the significant enhancement of PARP-2 binding at the centromere following mitotic arrest was likely to be specifically related to a delay in progression from metaphase to anaphase.

**Interaction of PARP-2 with Cenpa, Cenpb, Bub3 and PARP-1, but not Cenpc**

To further extend the observed immunofluorescence localization of PARP-2 at the centromere, we investigated whether a physical interaction occurs between PARP-2 and four key centromere proteins: three constitutive proteins CENP-A, CENP-B and CENP-C, and a checkpoint protein Bub3. Possible interaction of PARP-2 with PARP-1 was also investigated. The basic strategy involved immunoprecipitation using antibodies specific for each of these proteins, followed
by western blot analysis with antibody against the protein of interest, to identify co-immunoprecipitated components. In each experiment, the same antibody that was used to immunoprecipitate a protein was also used to probe the western blot to serve as a positive control. These studies were performed in various mouse embryonic stem (ES) cell-based lines (see Materials and Methods).

To study the physical interaction of PARP-2 with the centromere protein Cenpa, we utilized an ES-Cenpa:GFP cell line in which we have previously demonstrated specific localization of its Cenpa:GFP fusion protein to all mouse centromeres (P. Kalitsis et al., manuscript in preparation). The Cenpa:GFP fusion protein was immunoprecipitated from total cell protein extract using anti-GFP antibody followed by immunoblotting with anti-PARP-2 antiserum. An ES:GFP cell line expressing GFP was included as control in the immunoprecipitation assay. A band of 62 kDa corresponding to PARP-2 was observed in the ES-Cenpa:GFP protein extract (Fig. 5B: lane 1), but not in the control ES:GFP protein extract (not shown).

To confirm the specificity of this interaction, we performed the reverse experiment using anti-PARP-2 antibody to immunoprecipitate protein extracts from the same two cell lines. Upon

Figure 4. Immunofluorescence analysis of the effects of treatment with microtubule-inhibiting drugs in HeLa cells. (A) Metaphase spread of untreated cells, showing PARP-2 (red) and CREST#6 (green) signals. (B) Metaphase spread of cells treated with demecolcine, where the centromeric signal intensities for PARP-2 (green) relative to those for CREST#6 (red) are significantly increased compared with those of untreated cells. (C) Metaphase spread of cells treated with paclitaxel showing more intense PARP-2 signals (red) compared with the CREST#6 (green) signals at the centromeres. Merged immunofluorescence images (i) and split images for red (ii) and green (iii). Arrowheads point to the chromosomes depicted in the insets, showing extension of PARP-2 signals beyond the outer edges of CREST#6 signals. Chromosomal DNA is stained blue with DAPI.
immunoblotting with anti-GFP antibody, a single band of 43 kDa corresponding to the Cenpa:GFP fusion protein was detected in the ES-Cenpa:GFP cell extract (Fig. 5C: lane 2) but not in the control ES-GFP cell line (Fig. 5C: lane 3). Proteins immunoprecipitated from the ES-Cenpa:GFP cell extract using anti-GFP antibody served as a positive control for the immunoblot, showing the expected 43 kDa band (Fig. 5C: lane 1). These results indicate that PARP-2 interacts specifically with Cenpa.

Next, we immunoprecipitated proteins from wild-type ES cells using anti-Cenpb, anti-Cenpc and anti-PARP-1 antibodies, followed by immunoblotting with anti-PARP-2 antibody. For each of the anti-Cenpb (Fig. 5B: lane 2) and anti-Cenpc (Fig. 5B: lane 4) antibodies, a band of 62 kDa corresponding to PARP-2 was detected. This band was absent from the anti-Cenpc immunoprecipitate (Fig. 5B: lane 3). These results were confirmed by performing the reverse experiments. Firstly, when immunoprecipitates from wild-type ES cell extracts obtained using anti-PARP-2 (Fig. 5D: lane 1) and anti-Cenpb (Fig. 5D: lane 2) antibodies were probed with anti-Cenpb antibody, a band of 80 kDa corresponding to Cenpb was detected in both cases, thus establishing a specific interaction between Cenpb and PARP-2. Secondly, ES extracts immunoprecipitated using anti-PARP-2 antibody failed to show the presence of Cenpc upon probing with anti-Cenpc antibody (Fig. 5E: lane 1), whereas a 140 kDa Cenpc band was observed in the positive control immunoprecipitated using anti-Cenpc antibody (Fig. 5E: lane 2), confirming the absence of interaction between PARP-2 and Cenpc. Thirdly, ES cell extracts immunoprecipitated using anti-PARP-1 (Fig. 5F: lane 1) and anti-PARP-2 (Fig. 5F: lane 2) antibodies, upon western probing with anti-PARP-1 antibody, detected the presence of a 113 kDa band corresponding to the full-length PARP-1 protein in both cases, indicating interactions between PARP-1 and PARP-2.

We further investigated possible interactions between PARP-2 and the spindle assembly checkpoint protein Bub3. Anti-Bub3 (Fig. 5G: lane 1) and anti-PARP-2 (Fig. 5G: lane 2) antibodies were used to immunoprecipitate protein extracts from wild-type ES cells, followed by immunoblotting with anti-PARP-2 antibody. A 62 kDa PARP-2 band was detected in both immunoprecipitates, indicating protein interaction between PARP-2 and Bub3.
Bub3 and PARP-2. Both immunoprecipitates, upon probing with anti-Bub3 antibody, revealed the presence of a 40 kDa band corresponding to Bub3 and confirming the interaction between this protein and PARP-2 (Fig. 5H).

**DISCUSSION**

We have previously described the localization of PARP-1 at mammalian centromeres (27). PARP-2 has a modular structure similar to that of PARP-1, and has been shown to be capable of binding DNA and poly(ADP-ribosyl)ation following DNA damage in vitro (39). Our results show that, like PARP-1, PARP-2 localizes at centromeres in mouse and human cells. However, unlike PARP-1, which associates with a much broader centromeric/pericentromeric heterochromatic region presumably covering both the minor and major satellite sequences on mouse chromosomes, PARP-2 appears to be more specific to centromeric chromatin, localizing as highly discrete paired dots corresponding presumably to the minor satellite positions of the mouse centromeres. Furthermore, on both mouse and human metaphase chromosomes, PARP-1 shows considerable binding throughout the arms (27), whereas relatively little or no binding of PARP-2 on metaphase chromosome arms is observed.

Two types of data indicate that PARP-2 is associated only with functional centromeres. Firstly, analysis of a human pseudodicentric chromosome containing two widely separated regions of α-satellite DNA has demonstrated the presence of the protein on the active but not the inactive centromere. Secondly, we have shown that PARP-2 binds specifically at a 10q25-derived functional human neocentromere that is completely devoid of α-satellite DNA, but not at the corresponding 10q25 region of a normal chromosome 10. The combined pseudodicentric and neocentromere data indicate that PARP-2 binding is linked with centromere activity rather than the underlying DNA sequences.

We have compared the position of PARP-2 binding relative to those for CENP-A, CENP-B and CENP-C at the human centromeres. CENP-B has previously been localized diffusely across the inner centromere region (18). Other studies have pointed to CENP-A and CENP-C colocalizing at the inner domain of the kinetochore trilaminar structure (15,16), although more recent data have indicated CENP-A localization interior to CENP-C (48). Our dual-colour immunofluorescence data demonstrate that PARP-2 signals, whilst showing considerable overlap with those of CENP-A, CENP-B and CENP-C, peak at positions exterior to the domains occupied by these three proteins, suggesting significant binding of PARP-2 at the outer centromere region. A similar binding pattern has been described for the spindle checkpoint proteins Bub1 and hBUBR1, which concentrate at the outer kinetochore plate, extending in some instances into the inner plate (8), and Bub3, which binds to the entire kinetochore (10).

Cell cycle study shows that a significant level of PARP-2 is first noticeable at the centromeres during prometaphase, intensifying during metaphase, diminishing at anaphase, and becoming undetectable at the centromeres by telophase. Unlike the dynamic centromeric distribution pattern of a subclass of transient centromere proteins (e.g. INCENP and Survivin) that relocate to the spindle midzone during metaphase–anaphase transition and to the midbody during cytokinesis (49–51), no significant relocation of PARP-2 onto such spindle position or structure is observed, although a significant amount of background staining is detectable. The pattern of transient centromeric association seen with PARP-2 is therefore more akin to those previously described for the spindle assembly checkpoint proteins ZW10, MAD1, MAD2, BUB1, BUBR1 and BUB3 (2–6,8,10,52).

We further tested the behaviour of PARP-2 binding at the centromeres when cells are arrested at mitosis using demecolcine and paclitaxel. These chemicals disrupt microtubule dynamics via different mechanisms, the former by inhibiting microtubule polymerization while the latter actively blocking depolymerization of microtubules. The presence of these chemicals is believed to activate spindle checkpoint at mitosis (46,47). Our results indicate that cells arrested at metaphase display a significantly enhanced binding of PARP-2 at the centromeres. Enhanced staining of checkpoint proteins such as BUB1 and BUBR1 at human centromeres has been reported after treatment of cells with nanomolar concentrations of microtubule-inhibiting drug vinblastine (53). BUB1 concentration also increases at centromeres following treatment with nocodazole and paclitaxel (54). MAD2 is also known to associate with metaphase centromeres in cells arrested by nocodazole (55). Similarly, BUB3 staining is enhanced at the centromeres in nocodazole-treated HeLa cells (10). This recruitment of checkpoint proteins to the metaphase centromeres of arrested cells is generally attributed either to loss of spindle tension or microtubule attachment. Our results demonstrating an enhancement of PARP-2 binding at the centromeres using a drug (paclitaxel) that induces loss of spindle tension and one (demecolcine) that induces loss of microtubule attachment is therefore reminiscent of the behaviour observed with the spindle checkpoint proteins.

We have investigated the physical interaction between PARP-2 and a number of centromere-binding proteins. The results indicate that PARP-2 interacts with Cenpa, Cenpb, Bub3 and PARP-1, but not with Cenpc. Cenpa is an essential, DNA-binding, constitutive centromere protein that replaces a subpopulation of histone H3 at the centromere to provide the foundation for centromeric chromatin assembly (48,56–58). Cenpb is also a DNA-binding protein, and is presumably involved in the organization of centromeric satellite DNA repeats. The absence of this protein from neocentromeres, and the viable phenotype of Cenpb null mice, suggest functional redundancy of this protein (44,45,59–62). It is interesting that PARP-2 does not localize to the inactive, CENP-B-binding, centromere of a pseudodicentric chromosome, suggesting that in situ interaction between PARP-2 and CENP-B is dependent on centromere activity.

In previous work using an immunoprecipitation and western blot strategy similar to that described here, we have demonstrated that PARP-1 also interacts with Cenpa, Cenpb and Bub3, but not Cenpc (63). We have further shown that whilst no significant poly(ADP-ribosyl)ation of these proteins can be detected in normal cells, Cenpa, Cenpb and Bub3, but not Cenpc, undergo significant poly(ADP-ribosyl)ation upon DNA damage induced by γ-irradiation of the cell (63). Poly(ADP-ribosyl)ation adds negatively charged ADP-ribose polymers
that generally render acceptors such as chromatin-associated proteins incapable of DNA binding, resulting in the relaxation of the chromatin to allow access to the DNA (20,22,64–68). The observed poly(ADP-ribosyl)ation of at least two of the constitutive centromere proteins (Cenpa and Cenpb) upon γ-irradiation therefore suggests a possible mechanism whereby through the action of PARP-1 and PARP-2, the normally highly compact chromatin structures of the centromere can be decondensed to allow access for DNA repair enzymes.

Bub3 is a mitotic checkpoint protein, and is known to interact with the other checkpoint proteins Mad2, Mad3 and Cdc20 (69). Disruption of the Bub3 gene in mice leads to complete mitotic disarray and early embryonic lethality (70). Bub3 is also a component of the mitotic checkpoint complex that inhibits the anaphase-promoting complex/cyclosome (APC/C) from targeting key proteins for proteolytic degradation—an essential step for transition into anaphase (71). The demonstration of binding between Bub3 and PARP-2, together with the similar cell cycle distribution pattern, subregional localization at the outer centromere region, and accumulation of both proteins at the centromeres following colcemid or paclitaxel treatment, suggest that PARP-2 may play a role in modulating the activity of the spindle checkpoint, and thus metaphase-to-anaphase transition. The observed poly(ADP-ribosyl)ation of Bub3 upon γ-irradiation (63) further implicates possible involvement and functional alteration of the spindle checkpoint system during DNA damage. How this occurs remains to be investigated.

The results of this and the earlier study (63) have demonstrated the association of both PARP-1 and PARP-2 with the mammalian metaphase centromeres, and interaction of these proteins with each other as well as with two key structural centromere proteins Cenpa and Cenpb, and a mitotic spindle checkpoint protein Bub3. We have further shown that Cenpa, Cenpb and Bub3 are all acceptors of (ADP-ribose) polymers in the event of DNA damage (63). These results suggest that PARP-1 and PARP-2 may normally function as heterodimers and that the roles of these two proteins may be redundant. Such functional redundancy provides a viable explanation for the absence of severe centromere-specific phenotype in PARP-1 gene knockouts (30,32,34–37). Further work aimed at studying PARP-2 gene knockout and the generation of PARP-1/PARP-2 double knockouts will be illuminating.

MATERIALS AND METHODS

Antibodies

Antiserum used in this study included rabbit polyclonal antibody against PARP-2 (a gift from Dr. Gilbert de Murcia, CNRS, Strasbourg). The specificity of this antibody was confirmed by western blot analysis of crude nuclear extract (Fig. 5A: lane 1) and cytoplasmic proteins (Fig. 5A: lane 2). Upon probing with this antibody, a predominant band corresponding to the size of PARP-2 was detected in both extracts. Cross-reactivity with PARP-1 was not detected, as evidenced by the absence of a band at the 113 kDa region (Fig. 5A: lanes 1 and 2).

Other antibodies used were monoclonal anti-PARP-1 antibody C2-10 (Trevigen, Gaithersburg, MD), goat polyclonal anti-GFP antibody (Rockland, Gilbertsville, PA), mouse monoclonal Cenpb antibody 2D-7 (72), rabbit polyclonal anti-Cenpe antibody (73) and rabbit polyclonal anti-Bub3 antibody (45). Secondary antibodies labelled with HRP, Texas red and FITC were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Cell culture

HeLa cells and human fibroblast cells were grown in Dulbecco’s modified Eagle’s medium (DMEM), and lymphoblast cells were grown in suspension in RPMI 1640 in the presence of 5% CO2 at 37°C. All mouse ES cell lines, including wild-type ES cells W9.5, ES-GFP and ES-Cenpa: GFP, were grown in ES medium supplemented with LIF (ESGRO Chemicon International) and β-mercaptoethanol in the presence of 5% CO2 at 37°C. All media used were supplemented with 10% fetal calf serum (Trace Biosciences).

The ES-GFP cell line was generated by transfecting ES cells with the plasmid pEGFP-C1 (Clonetech) containing a neoymycin-resistance gene and a gene expressing green fluorescent protein (GFP). Cells were allowed to recover for 24 h before the addition of 250 μg/ml G418 (GIBCO Life Technologies) to select for resistant colonies. ES-Cenpa: GFP was a heterozygous ES cell line in which one of the endogenous Cenpa alleles has been replaced by a mouse Cenpa-GFP fusion protein gene. This cell line was generated using a targeted knock-in strategy, where GFP was placed at the C terminus of full-length Cenpa (P. Kalitsis et al., manuscript in preparation).

Immunofluorescence analysis

Cells used for immunofluorescence were either harvested and cytospun onto a slide or cultured directly on sterile coverslips. Cells grown on coverslips were prefixed, whereas cytospun cells were treated with primary and secondary antibodies before fixation and immunofluorescence performed with relevant antibodies as described previously (40,45). In some experiments, cells in culture were treated with microtubule-inhibiting drugs for 2 h at 37°C before harvesting. The drugs used were paclitaxel (P Sigma) at a final concentration of 10 μm, or colcemid (Karyomax, Gibco, Life Technologies) at a final concentration of 100 μm in culture medium.

ImmunoFISH of neocentromeric chromosome

Simultaneous immunofluorescence detection of chromosomal proteins and fluorescence in situ hybridization (immunoFISH) was performed as previously described (40). Since this procedure led to considerable depletion of PARP-2 signal after FISH, cells were first prepared for immunofluorescence with anti-PARP-2 antibody, after which chromosome images were captured before FISH analysis was performed.

Preparation of protein extracts

For protein extracts, cells were lysed in ice cold radiolabeled immunoprecipitation assay (RIPA) buffer containing 1% NP40, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl
0.01 M sodium phosphate, 2 mM EDTA, 50 mM NaF with protease inhibitors and 0.2 mM sodium vanadate added fresh. The cell lysate was pelleted by centrifugation at 2500 g at 4°C, and supernatant was collected as total protein extract and stored at −70°C. Cytoplasmic and nuclear protein extracts were prepared as described previously (27).

**Immunoprecipitation**

Protein extracts (500 μl) were incubated with 5 μg of antibody overnight at 4°C with gentle agitation. 125 μl of 50% protein G sepharose resuspended in incubation buffer (50 mM NaCl, 20 mM Tris, 5 mM EDTA and 0.1 mM PMSF) containing cocktail protease inhibitors (Complete, Roche) was added, and the mixture was further incubated for 3 h at room temperature with constant agitation. The sepharose beads were then pelleted by centrifugation and washed in IP wash buffer (50 mM Tris, 10 mM EDTA and 150 mM NaCl), prior to elution with incubation buffer containing 1% SDS.

**Western blotting**

Protein samples were subjected to PAGE on 10–15% gel, and were transferred to Hybond C (Amersham) by standard western blotting technique. Blots were incubated with the relevant primary antisera at dilutions recommended by the manufacturers. After washing in PBS containing 0.1% Tween-20, blots were incubated with relevant HRP-conjugated secondary antisera and developed using a chemiluminescence detection kit (Amersham Pharmacia Biotech).

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