Phosphorylation Sites on Tyr28 and the C-terminus of Rad9 are Required for Inhibition of Premature Chromosomal Condensation Across the Entire S Phase

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Key Words

Mrad9 • PCC and S/M checkpoint control

Abstract

Rad9 is required for the activity of the genotoxin-induced checkpoint signaling pathway to control cell cycle progress and maintain genomic stability. In the fission yeast *S. Pombe* and chicken cells, the *Rad9* gene is essential for prevention of premature chromosomal condensation (PCC:S/M checkpoint control). However, precise features in the S/M checkpoint controlled by Rad9 in mammalian cells are still not clear. In this study, mouse embryonic stem (ES) cells with a targeted deletion of *Mrad9*, the counterpart of *S. pombe rad9*, were used to evaluate the detailed function of *Mrad9* in S/M checkpoint control. We found that *Mrad9* deletion from ES cells led to failure of S/M checkpoint control across the entire S phase, and was alleviated by introducing mouse or human *Rad9* into the *Mrad9*-deleted cells. We also found that the phosphorylation sites on Tyr28 and the C-terminus of Rad9 are required for this checkpoint control. Moreover, the DNA replication inhibitor hydroxyurea (HU) induced cdc2 Tyr15

phosphorylation and increased 14-3-3 σ protein levels in *Mrad9*^{+/+} ES cells, but failed to do so in *Mrad9*^{-/-} ES cells. Taken together, these results suggest that phosphorylation of Rad9 plays a critical role in the activation of the S/M checkpoint, and that downstream proteins cdc2 and 14-3-3 σ mediate this function.

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Introduction

Errors and damage to genomic DNA arise during normal DNA processing or when cells are exposed to genotoxic agents. Cells manage DNA errors and damage using an intricate network consisting of many proteins including cell cycle checkpoint proteins and DNA repair factors. Mutations in these genes often lead to cell death or cancer development. Two of the core functions of this network are cell cycle checkpoint controls and DNA repair. In *S. pombe*, Rad3, Rad17, Rad1, Hus1, Rad9 and Rad26 are the key molecules in the cell cycle checkpoint network; while Mec1, Rad24, Rad17, Mec3, Ddc1 and Ddc2/Lcd1/Pie1 are key in *S. cerevisiae*.

The function of these molecules is conserved throughout evolution in cell cycle checkpoint control [1, 2]. Recent studies have identified the human and mouse homologues of these molecules. ATM (Ataxia Telangiectasia Mutated) and ATR (ATM-Rad3 Related) are two early DNA damage sensors. ATM primarily responds to double-stranded DNA breaks (DSBs), while ATR is activated by other types of DNA damage such as stalling of the replication fork. ATM and ATR are activated in response to DNA damage and then regulate downstream signaling proteins, including Rad9, Chk1 and Chk2, by phosphorylation [3-7].

Previous studies in yeast and mammal cells have shown that the cell cycle checkpoint proteins Rad9, Rad1 and Hus1 also play an important role in DNA repair [8-10]. These three proteins are evolutionarily conserved in eukaryotes, and can form a ring-shaped heterotrimer, 9-1-1 complex [11-16]. After DNA damage, this PCNA-like complex is localized to the chromatin [4, 11-17]. The 9-1-1 complex interacts with the Rad17-RFC complex, a putative clamp loader composed of Rad17 and four RFC subunits [18, 19]. In vivo, Rad17 is required for association of the 9-1-1 complex with chromatin. In reconstituted reactions, the Rad17-RFC complex can load the 9-1-1 complex to DNA. Both Rad9 and ATR play a critical role in inducing Chk1 activity [18, 20, 21]. Claspin associates with Rad9 and is involved in PCC prevention and intra-S phase checkpoint control in response to DNA damage [22]. Deletion of each of the 9-1-1 complex genes in the fission yeast *S. pombe* inactivates S/M, intra-S and G2/M checkpoint controls, and sensitizes fission yeast cells to killing by UV light, γ -rays and the replication inhibitor hydroxyurea (HU) [23-26]. Disruption of the mouse ortholog of *Rad9* or *Hus1* also sensitizes mouse cells to UV light, γ -rays and HU, and leads to G2/M checkpoint controls as well as genome instability [27, 28].

When chicken *Rad9* was successfully knocked out, chicken *Rad9*-null cells demonstrated PCC and chromosome fragments with HU treatment [29, 30]. It is not clear if Rad9 is required to prevent PCC in mammalian cells.

In this study, *Mrad9*-deleted embryonic stem cells were used to investigate the role of *Mrad9* in S/M checkpoint control. Incubation in a medium containing HU led to premature condensed chromosomes in *Mrad9*-deleted cells throughout the entire S phase. PCC induction rates across the S phase were not uniform in *Mrad9*-null ES cells. Furthermore, HU treatment increased cdc2 Tyr15 phosphorylation and 14-3-3 σ protein levels in wild type cells but failed to do so in *Mrad9*^{-/-} cells. These data

suggest that the S/M checkpoint signaling pathway involving Mrad9 is conserved from yeast to mammals. Moreover we also show that phosphorylation sites on Tyr28 and the C-terminal portion of Mrad9 are critical for S/M checkpoint control.

Materials and Methods

Cell culture

Culture of wild type and *Mrad9*^{-/-} mouse ES cells was described previously [28]. Zeocin at a final concentration of 25 μ g/ml was added to select and maintain stable transfectants. To induce PCC, cells were incubated in a medium containing 1mM HU (or other concentrations as indicated) or 5 μ g/ml alaphidicolin or 5 μ g/ml 1- β -D-arabinofuranosylcytosine (araC). Cell survival was detected after HU treatment with the trypan blue test. Cells were treated with various HU concentrations or incubation times, and stained with 0.1% trypan blue solution for 5 minutes at room temperature. Non-viable cells (positively stained) and viable cells (negatively stained) were observed under a microscope and the percentage of viable cells was calculated.

Cell Synchronization

Cells were incubated in medium containing 50 ng/ml nocodazole (Sigma-Aldrich Co., St. Louis, MO) for 4 hours. Mitotic cells were shaken off at 200 rpm in a mechanical shaker for 5 min. Cells were harvested by centrifugation at 400g for 5 min, rinsed twice with fresh warm medium and then replated in conditioned medium at 37°C. Conditioned medium was obtained by filtering medium collected from exponentially growing cell cultures. Purity of isolated M phase cells was evaluated by determining the percentage of phospho-histone (Ser10) positive cells with 4n chromatin DNA (refer to the Flow cytometry section below).

Western blot

Cells were lysed in 1 $\times$ SDS sample buffer and boiled for 5 minutes. Cell lysates were fractionated on a 10% SDS/PAGE gel and then transferred to a PVDF membrane by electroblotting. Blots were then probed with mouse anti- α -tubulin (Clone DM 1A, Sigma-Aldrich Co., St. Louis, MO) or rabbit anti-Cdc2 or rabbit anti-phospho-Cdc2-Tyr15 (Cell Signaling Technology, Beverly, MA), or mouse anti-14-3-3 σ (Clone CS112-2A8, Upstate Biotechnology, Lake Placid, NY), followed by HRP-conjugated goat anti-rabbit or anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). The ECL Western blotting system (Amersham, Piscataway, NJ) was used to visualize results. Chemiluminescent signals were recorded and analyzed by Phosphoimaging (Molecular Dynamics System, Sunnyvale CA).

Flow cytometry

For the assay of phospho-histone H3 (Ser10), 1 $\times$ 10⁶ cells were suspended in ice-cold PBS, and fixed by dropping cold ethanol into the cell suspension while vortexing. The final

ethanol concentration was 75%. Fixed cells were maintained at 4°C or -20°C for at least 30 min. After ethanol was removed, cells were washed once in PBS and then incubated in 200 µl PBS containing 0.1% Tween-20, 1% fetal bovine serum and 1% bovine serum albumin (PBS-TwBF) at room temperature for 30 min to block nonspecific binding sites. After centrifugation and collection of cells, the cells were incubated in 200 µl PBS-TwBF containing 1 µl rabbit anti-Ser10-phospho-Histone H3 (Upstate Biotechnology, Lake Placid, NY) at room temperature for 2 hour. After centrifugation and discarding of PBS-TwBF, cells were washed once in PBS-TwBF and incubated further in PBS-TwBF containing FITC-conjugated anti-rabbit IgG (ImmunoResearch Laboratories) at room temperature for 1 hour. After washing three times, cells were stained in PBS with 50 µg/ml Propidium Iodide (PI) and 40 U/ml RNase at 4°C for at least 1 hour. Cells were analyzed using FACSCalibur (BD, San Jose, CA). A 488nm laser was used to excite PI and FITC, and the fluorescence emitted by FITC and PI at 530/30 nm and 585/42 nm respectively was recorded.

For detection of BrdU uptake by individual cells, BrdU was added at a 10 µM final concentration to pulse-label cells in culture dishes for 10 min followed by cell fixation as described above. Cells were collected by centrifugation and suspended in 2 mL of 2 N HCl with 0.2 mg/ml pepsin. After incubation at 37°C for 20 min, 3 ml of 2M Tris-HCl buffer was added to neutralize the cell suspension. Cells were collected, rinsed once in PBS and then incubated in 200 µl PBS-TwBF at room temperature for 30 min to block nonspecific binding sites on cells. Cells were incubated in 200 µl PBS-TxwBF containing 10 µl FITC-conjugated anti-BrdU antibody (Clone B44, BD Biosciences, San Jose, CA) in the dark on a rolling nutator for 2 hours at room temperature. Subsequent steps were the same as those described for the flow cytometric assay of phospho-Histone H3-Ser10.

Microscopy

For PCC detection, collected cells were subjected to hypotonic swelling in 0.03 M Sodium Citrate (5 to 10 ml) for 15-20 minutes at 37°C, and then fixed in methanol:acetic acid (3:1) (5 to 10 ml). After two or three additional changes in fixative, the cell suspension was placed dropwise onto wet, clean slides and allowed to air-dry. Chromosomes were stained with PI and viewed with a Zeiss Axioplan 2 Imaging microscope equipped with a CCD camera.

To simultaneously examine chromosome status and phosphorylation of histone H3-Ser10 (H3-γ) following hypotonic swelling, cells were spun on a Cytospin 2 centrifuge (Thermo Shandon, Pittsburgh, PA) at 1,000 rpm for 3 min to spread the chromosomes onto slides. The slides were air-dried for 15 min, fixed in 50% methanol and 50% acetone for 10 min, air-dried and then kept at 4°C. Slides were blocked with PBS containing 5% dry milk and 0.2% Tween-20 (PBS-MT) for 30 min at room temperature. 20 µl PBS-MT containing rabbit anti-H3-γ (1/250) was added onto each slide, covered with a coverslip, and incubated at 37°C for 1 hour. The slides were washed twice with PBS containing 0.2% Tween 20 (PBS-T) twice, incubated with PBS-MT FITC-conjugated anti-rabbit (1/500) for 1 hour at 37°C, washed three times with PBS-T, fixed in

Amino acid	Sequence
Ser272	SCSQGP
Ser277	PCSPKP
Thr292	HSTPHL
Ser341	PTSEEE
Thr353	PGTPPP
Ser373	VHSPQG
Ser385	EDSDGE

Table 1. Mard9 mutagenesis

70% ethanol for 5 min and then in 100% ethanol for a further 5 min and then air-dried. One drop of mounting oil containing PI was added to the slides and chromosomes were examined under a microscope.

Site-directed mutation of *Mrad9* and transfection

A GeneTailor kit (Invitrogen, Carlsbad, CA) was used to create a mutant *Mrad9* cDNA insert between HindIII and NotI in pZeoSV2 (Invitrogen). Among phosphorylation sites identified previously [20, 31], seven are conserved between *Mrad9* and *Hrad9* (Table 1). The four phosphorylation sites S272, S277, T292, and T353 were converted into Ala residues in the four *Mrad9* cDNA clones A272, A277, A292, and A353 respectively. All seven phosphorylation sites were changed into Ala residues in one *Mrad9* cDNA clone (7As). Mutant clones were sequenced to confirm the accuracy of mutation sites and that no unintended errors had been introduced. The mutant *Mrad9* cDNA clones were transfected into *Mrad9*^{-/-} mouse ES cells and selected against zeocin at a final concentration of 25 µg/ml. Cell clones expressing mutant *Mrad9* close to wild type endogenous *Mrad9* levels were selected for functional analysis.

Results

Mrad9 deletion enhances PCC induction by replication inhibitors

Histone H3 is phosphorylated on Ser10 when cells enter the M phase, and can be conveniently detected by microscopy and flow cytometry with the help of an extremely specific and high affinity polyclonal antibody against Ser10-phospho-histone H3 [32, 33]. Using anti-Ser10-phospho-histone H3 (H3-γ) in combination with flow cytometry, deletion of *Mrad9* was found to dramatically increase PCC induced by HU, aphidicolin and 1-β-D-arabinofuranosylcytosine in mouse ES cells (Fig. 1A). A significant portion of the *Mrad9* deleted cells with less than 4n DNA moved into the M phase (H3 histone-γ positive), while few wild type S phase cells entered the M phase prematurely. This was confirmed

Fig. 1. *Mrad9* deletion leads to S/M checkpoint control defects. (A) *Mrad9*^{+/+} and *Mrad9*^{-/-} ES cells were mock-treated or treated with 1mM HU or 5μg/ml aphidicolin or 5μg/ml 1-β-D-arabinofuranosylcytosine (araC) for 10 hours. (B) *Mrad9*^{+/+}, *Mrad9*^{-/-} ES cells and *Mrad9*^{-/-} ES cells with the re-introduction of *Mrad9* and *Hrad9* cDNA, were mock-treated or treated with 1mM HU for 12 hours. Cells were collected and labeled with antibodies against the mitotic marker H3-γ, stained with propidium iodide, and analyzed by flow cytometry. Staining intensity for PI (x-axis) is plotted versus intensity of H3-γ staining (y-axis). Cells in the boxed region correspond to normal mitotic cells and the prematurely condensed chromosome mitotic fraction.

Table 2. Comparison of Chromosome Morphologies of *Mrad9*^{-/-} and *Mrad9*^{+/+} ES cells treated with HU. *Treatment with 1 mM HU for 10 hr. Chromosomes were probed with anti- H3-γ and then stained with PI. The cells counted were those containing chromosomes that were both H3-γ and PI positive.

	<i>Mrad9</i> ^{+/+} no HU	<i>Mrad9</i> ^{-/-} no HU	<i>Mrad9</i> ^{+/+} HU*	<i>Mrad9</i> ^{-/-} HU*
Total number of cells counted	1260	1092	1349	1182
Number of cells with normal M phase chromosomes	31(2.46%)	30(2.75%)	3(0.22%)	2(0.15%)
Number of cells with broken chromosomes	0	0	2(0.15%)	18(1.52%)

Table 3. Ratios of cells bearing broken chromosomes to those containing normal chromosomes after treatment of HU. a. Treatment with 1 mM HU for 10 hr; b. Number of cells containing chromosomes that were both H3-γ and PI positive on all the microscopic fields observed.

	<i>Mrad9</i> ^{+/+} no HU		<i>Mrad9</i> ^{-/-} no HU		<i>Mrad9</i> ^{+/+} HU ^a		<i>Mrad9</i> ^{-/-} HU ^a	
	N ^b	Broken/ normal (%)	N ^b	Broken/ normal (%)	N ^b	Broken/ normal (%)	N ^b	Broken/ normal (%)
Number of cells with normal M phase chromosomes	86	0	71	2.8	22	73	22	332
Number of cells with broken chromosomes	0		2		16		73	

by immunofluorescent microscopy (Fig. 2; Tables 2 and 3). These data suggest that *Mrad9* plays an important role in S/M checkpoint control in mouse ES cells.

Fig. 2. Microscopic view of PCC in HU-treated *Mrad9*^{-/-} mouse ES cells. (A) *Mrad9*^{-/-} ES cells were treated or mock-treated with 1 mM HU for 10 hours. After chromosome spread, the chromosomes were stained with PI. Chromosome spread of treated (right) and mock-treated (left) cells shows that HU-treated *Mrad9*^{-/-} ES cells in S phase are prematurely condensed. (B) *Mrad9*^{+/+} and *Mrad9*^{-/-} ES cells were treated with 1 mM HU for 10 hours. After chromosome spread, the chromosomes were immunostained with anti- H3-γ antibody and PI. Chromosomes of HU-treated *Mrad9*^{-/-} ES cells in S phase are prematurely condensed while chromosomes from the majority of HU-treated *Mrad9*^{+/+} ES cells appear intact.

To confirm the positive role of *Mrad9* in S/M checkpoint control and exclude the possibility that the enhancement of PCC induction by HU in *Mrad9*^{-/-} ES

cells was due to random mutations that occurred during the generation of *Mrad9*^{-/-} ES cell clones, cDNA of *Mrad9* and its human version *Hrad9* was introduced into *Mrad9*^{-/-} ES cells. *Mrad9* and *Hrad9* cDNA significantly reduced HU-induced PCC in *Mrad9*^{-/-} ES cells, indicating that *Mrad9* is indeed required for S/M checkpoint control (Fig.1B).

PCC level in Mrad9^{-/-} ES cells depends on HU concentration and time of HU treatment

PCC from early S phase cells can be easily distinguished from G2 phase cells as they appear granular (Fig. 2), and contain less than 4n DNA (Fig. 1A). In contrast, PCC from late S phase cells is difficult to distinguish from that of G2 phase cells. When assessed by flow cytometry, DNA content per cell from late S phase cells is close to that of G2 phase cells and the two sets of cells cannot be distinguished from each other. An alternative method would be to distinguish cells on the basis of the morphological appearance of chromosomes: (1) chromosomes with a granular appearance are considered to be PCC and (2) chromosomes with an intact appearance are considered to be non-PCC. However, in response to HU treatment, cells reaching the very end of S phase may develop PCC, but their chromosomes may appear normal because prematurely condensed chromatin only comprises an undetectable portion of the chromosomes. Thus, we cannot determine the exact percentage of PCC in H3- γ positive cells with DNA content close or equal to 4n by flow cytometric detection. For this reason, we quantified the two portions of H3- γ positive cells (DNA<4n; DNA $\approx$ 4n) separately to reduce confusion.

The percentages of cells with PCC from early and middle S phase (EM-PCC) depended on HU concentration as well as incubation time with HU (Fig. 3). HU incubation induced a considerable percentage of *Mrad9*^{-/-} ES cells to develop EM-PCC, but only a very low level of *Mrad9*^{+/+} ES cells showed EM-PCC regardless of HU concentration or incubation time. These different PCC induction responses may originate from differences in toxicity of HU to *Mrad9*^{+/+} ES and *Mrad9*^{-/-} ES cells. Cell survival levels were evaluated by trypan blue staining. The survival levels for all treatments were very high, with little difference between wild type and mutant cells (1mM HU for 10 hr: 98.7% for *Mrad9*^{+/+} ES cells and 98.6% for *Mrad9*^{-/-} ES cells; 3 mM HU for 12 hr and 24 hr: 97.7% and 95.9% respectively for *Mrad9*^{+/+} ES cells, and 98.1% and 93.6% respectively for *Mrad9*^{-/-} ES cells), suggesting that the

Fig. 3. PCC versus concentration and time of HU treatment. (A) *Mrad9*^{+/+} and *Mrad9*^{-/-} ES cells were treated with various concentrations of HU for 10 hours. (B) *Mrad9*^{+/+} and *Mrad9*^{-/-} ES cells were treated with 3 mM HU for various times. Cells were collected and labeled with antibodies against H3- γ , stained with propidium iodide, and analyzed by flow cytometry. Cells in the boxed region correspond to H3- γ positive cells from the early-middle S phase (left) or from the later S and G2 phase (right). The percentages of boxed cells were calculated and used to make the curves.

HU treatments are equitoxic for the two cell populations and that differences in PCC induction between HU treatments did not result from differential survival levels, but rather indicate the importance of *Mrad9* in S/M checkpoint control. When HU concentration is raised to 0.5 mM or higher, percentages of both EM-PCC and H3- γ positive cells with DNA content close or equal to 4n leveled off in *Mrad9*^{-/-} ES cells (Fig. 3A), suggesting that a certain concentration of HU is needed to fully induce PCC (probably through complete inhibition of DNA synthesis) and that only a small portion of S phase cells can be triggered into PCC. As HU concentration was increased, the EM-PCC level of *Mrad9*^{+/+} ES cells increased slightly and stayed at a low level, while the percentage of H3- γ positive cells with DNA content close

Fig. 4. Translation inhibitor CHX suppresses PCC. *Mrad9*^{+/+} and *Mrad9*^{-/-} ES cells were mock-treated or treated with 1mM HU for 10 hours and translation inhibitor CHX was added to one set of HU treated cells. PCC of the HU treated cells was suppressed by adding CHX. Cells were collected and labeled with antibodies against H3-γ, stained with propidium iodide, and analyzed by flow cytometry. Staining intensity for PI (x-axis) is plotted versus intensity of H3-γ staining (y-axis). Cells in the boxed region correspond to normal mitotic cells and the prematurely condensed chromosome mitotic fraction.

or equal to 4n decreased. At least a portion of H3-γ positive cells with DNA content close or equal to 4n in both *Mrad9*^{-/-} and *Mrad9*^{+/+} ES cells had PCC, as significantly higher percentages of cells containing broken chromosomes were detected by microscopy than the EM-PCC percentages measured with flow cytometry (Tables 2 and 3; Fig. 3). Calculations based on data from Fig. 3A and Table 3 suggest that more than half of the *Mrad9*^{-/-} ES cells with DNA $\approx$ 4n contain broken chromosomes, thus over 50% of the cells with DNA $\approx$ 4n had PCC. The calculation is as follows. As shown in Fig. 3A, 2.0% of cells from early and middle S phase had PCC and 2.5% of H3-γ positive cells had DNA $\approx$ 4n. From Table 3, the ratio of cells with normal chromosomes to total H3-γ positive cells was 1/(1+3.32). This ratio can be represented as $x/(2+2.5) = 1/(1+3.32)$, thus $x = 1.04$ and the percentage of cells with PCC arising from the late S phase = $(2.5 - 1.04)/2.5 = 0.583 > 50\%$. This quantitative estimation will be used in the next section to describe a feature of the induction of PCC by HU across the S phase in *Mrad9*^{-/-} ES cells.

The EM-PCC of *Mrad9*^{-/-} ES cells was not significantly induced within 6 hr of HU treatment suggesting that the accumulation of some cell components was needed for EM-PCC (Fig. 3B). This was confirmed by the experimental result that the translation inhibitor cycloheximide (CHX) suppressed EM-PCC in *Mrad9*^{-/-} ES cells induced by HU (Fig. 4).

Fig. 5. Quantification of PCC induction rates derived from sub-regions of the S phase. *Mrad9*^{-/-} ES cells were treated with 1 mM HU for 10 hours. S phase was divided into 6 sub-regions (A), and the percentage of H3-γ positive cells in each sub-region was systematically quantified (B), indicating that PCC was more easily induced in early (sub-region 1) and late S phase (sub-region 6) than the middle regions (sub-regions 3 and 4) of the S phase.

PCC induction by HU varies across the S phase

The percentages of *Mrad9*^{-/-} ES cells with PCC derived from various sub-regions of the S phase did not appear uniform (Fig. 1). A systematic quantification of 6 sub-regions revealed that PCC rate (the number of PCC cells divided by the total number of cells in the indicated sub-region of the S phase) was more easily induced in early (sub-region 1) and late S phase (sub-region 6) than during the middle regions (sub-regions 3 and 4) of the S phase (Fig. 5). Although a portion of the H3-γ positive cells in sub-region 6 may have included M phase cells originating from the G2 phase as described in the last section, the majority of these cells contained true PCC. Therefore, a higher level of PCC is induced in early and late S phase than during the middle S phase.

Fig. 6. PCC cannot be induced in G1 phase cells. ES cells were synchronized in the mitosis phase by mitotic shake-off; syn and asyn denote synchronous and asynchronous respectively. (A) ES cells were mock-treated or treated with 1 mM HU at various times (1hr, 2hr, 4hr) after mitotic synchronization and further incubated for 10 hr for PCC induction. Cells were collected and labeled with anti-H3- γ antibody, stained with propidium iodide, and analyzed by flow cytometry. Staining intensity for PI (x-axis) versus intensity of H3- γ (y-axis) is indicated. (B) Quantifying the graphs in Fig A, the percentages of H3- γ positive cells from the early-middle S phase (EM-PCC cells) were calculated and used to make the curves. (C) ES cells were treated with 10 μ M BrdU at various times (1hr, 2hr, 4hr) after the mitotic synchronization and further incubated for 10 min to pulse-label the cells that have moved from G1 phase into S phase. Cells were collected and stained with FITC-conjugated anti-BrdU antibody and PI, then analyzed by flow cytometry. Staining intensity for PI (x-axis) versus for BrdU (y-axis) is indicated. (D) The percentage of BrdU positive cells (the S phase cells) in Fig C was systematically quantified.

PCC is not induced in G1 phase cells

One may argue that the cells with PCC and DNA content close to 2n might arise from G1 phase cells, but not from early S phase cells. To exclude this possibility, we tested whether PCC could be induced in synchronized G1 phase *Mrad9*^{-/-} ES cells. Mitotic cells, prepared by mitotic shake-off (the purity of the mitotic population was 96.5% as described in the Materials and Methods section above) were incubated in conditioned warm medium for 1, 2 and 4 hr, respectively. HU was added to one set of dishes and further incubated for 10 hr for PCC induction.

BrdU was added to another set of cells and incubated further for 10 min to pulse-label the cells that had moved from the G1 phase into S phase. After 1 hr incubation, very few cells had moved into the S phase and cells with PCC were at background levels (Fig. 6A). At 2 hr, cells began to enter S phase as shown by the presence of BrdU positive cells (Fig. 6C). At 4 hr, more cells moved into the S phase and PCC was induced by HU in more *Mrad9*^{-/-} ES cells (Fig. 6A, C). We used *Mrad9*^{+/+} ES cells as a control, and no significant PCC induction occurred when the cells moved into the S phase.

It is worth noting that H3- γ negative cells with 4n DNA appeared at 1 hr and these cells were not contaminant G2 phase cells, but anaphase or telophase cells which were H3- γ negative (Fig. 6) [32]. These results indicate that HU-induced PCC was not from G1 phase cells.

Phosphorylation sites on Tyr28 and the C-terminus of Mrad9 are required for S/M checkpoint control

Roos-Mattjus *et al.* 2003 reported that when *Hrad9* was ectopically expressed in *Mrad9*^{-/-} ES cells, multiple phosphorylation sites on the Hrad9 C-terminus were essential for the cells to resist HU and for Chk1 phosphorylation to occur [20]. We questioned whether phosphorylation sites were necessary for S/M checkpoint control. Seven of the phosphorylation sites identified previously are conserved between Mrad9 and Hrad9 (Table 1) [20, 31]. Among these seven, phosphorylation on Ser272, Ser277 and Thr292 is radiation-inducible, and the rest of the sites are phosphorylated constitutively [31]. We made five Mrad9 mutants by changing the following sites to alanine: (1) the three inducible sites, (2) the one constitutively phosphorylated site Thr353 and (3) all seven sites together (S272, S277, T292, S341, T353, S373 and S385). Stable clones expressing these mutant *Mrad9* cDNAs at a level nearly identical to the endogenous Mrad9 level (data not shown) were selected to examine their S/M checkpoint control status. To avoid the complication of evaluating the ratio of cells with PCC and DNA content close to 4n as described previously, we only quantified the ratios of cells containing EM-PCC. Each of the individual mutations did not cause significant loss of S/M checkpoint control, but the mutation of all seven sites abrogated S/M checkpoint control (Fig. 7), suggesting that phosphorylation on S341, S373 or S385 is responsible for S/M checkpoint activation. Delacroix *et al* recently reported that human Rad9 Ser387 (equivalent to mouse Rad9 Ser385) is critical for Chk1 phosphorylation, thus suggesting that Mrad9 Ser385 is responsible for preventing PCC [34].

Yoshida and colleagues reported that c-Abl phosphorylated human Rad9 Tyr28 and this phosphorylation was required for apoptosis induced by DNA damage [35]. Replacement of Tyr28 with Ala dramatically enhanced HU-induced PCC (Fig. 7), indicating the important role of the Tyr28 in Mrad9 in S/M checkpoint control.

It is worth noting that ectopically expressed wild type Mrad9 did not fully restore S/M checkpoint control to *Mrad9*^{-/-} ES cells. This may be because one or more

Fig. 7. Phosphorylation of the Mrad9 Ser28 and C-terminus is required for S/M checkpoint control. *Mrad9*^{+/+}, *Mrad9*^{-/-} ES cells and *Mrad9*^{-/-} mouse ES cell lines re-constructed with *Mrad9* cDNA or various mutants of *Mrad9* cDNA were treated with 1mM HU for 12 hours. The ratios of cells containing EM-PCC were systematically quantified. Each of the individual mutations except Ser28 did not cause significant loss of S/M checkpoint control, but the mutation of all seven sites abrogated S/M checkpoint control. 272, 277, 292, 353 and 7As denote Ser272, Ser277, Thr292, Thr353 mutants and the mutant with all seven sites mutated (S272, S277, T292, S341, T353, S373, and S385) respectively.

endogenous 9-1-1 protein components vary at phosphorylation levels through the cell cycle or in response to HU treatment. This variation may contribute to PCC prevention.

Deletion of Mrad9 leads to reduced phosphorylation of the Tyr15 of Cdc2 and 14-3-3 σ protein level induced by HU treatment

Previous evidence has shown that increased Cdc2 (Tyr15) phosphorylation and enhancement of 14-3-3 σ expression are required for S/M checkpoint control [36, 37]. Our study of *Mrad9*^{+/+} ES cells is consistent with these previous experimental results (Fig. 8 and Table 4). Incubation of *Mrad9*^{+/+} ES cells with 1 mM HU for 10 hr increased 14-3-3 σ and Cdc2 protein levels and significantly induced Cdc2 (Tyr15) phosphorylation. Deletion of *Mrad9* not only eliminated the enhancement of 14-3-3 σ and Cdc2 protein levels, but also suppressed their levels even lower than that of the untreated cells (Fig. 8 and Table 4). A lower Cdc2-Tyr15 phosphorylation level in *Mrad9*^{-/-} would lower the efficiency of S/M checkpoint control. 14-3-3 σ was reported to increase levels of Cdc2 in the nucleus and the phosphorylation of Cdc2 Tyr15 [38-42].

Fig. 8. In *Mrad9*^{-/-} ES cells HU-induced Cdc2 Tyr15 phosphorylation and the expression of 14-3-3σ are defective. *Mrad9*^{+/+} and *Mrad9*^{-/-} ES cells were mock-treated or treated with 1mM HU or nocodazol (Noc) for 10 hours. Whole cell lysates were subjected to western blotting using anti-cdc2, anti-phospho-cdc2 (Tyr15) or anti-14-3-3σ antibody, and tubulin served as a loading control. The signal intensity ratio of the indicated gene was quantified in Table 4.

	<i>Mrad9</i> ^{+/+}			<i>Mrad9</i> ^{-/-}		
	Ctl	Noc	HU	Ctl	Noc	HU
CDC2-γ/α-tublin (%)	47	13	100	53	13	29
CDC2-γ/ CDC2 (%)	57	18	100	58	25	51
CDC2/ α-tublin (%)	84	72	100	91	50	51
14-3-3σ/ α-tublin (%)	42	43	100	53	47	6

Table 4. The quantified ratios of protein expression levels

Discussion

In this study we demonstrated that *Mrad9* plays an indispensable role in preventing S phase cells from prematurely moving into mitosis (premature chromosome condensation, PCC), and present detailed features of PCC throughout the S phase. We also found that deletion of *Mrad9* restrained HU-induced enhancements of the levels of γ-Cdc2 and 14-3-3σ levels. Furthermore, phosphorylation of *Mrad9* was shown to be essential for preventing PCC.

Features of PCC induced in *Rad9*-null ES cells

Rad9 is required for S/M checkpoint control in fission yeast and chicken cells [26, 29, 43], but detailed dynamics of PCC induction have never been described. In this study, we used *Mrad9*-null ES cells to carry out this analysis. First, PCC induction was not uniform throughout the S phase, and this unevenness did not depend on inhibition mechanisms because all three different reagents (HU, aphidicolin and 1-β-D-arabinofuranosylcytosine) caused identical PCC induction patterns throughout the S phase (Fig. 1). Second, the level of PCC induced was limited, and not all cells in the S phase could be triggered into PCC (Fig. 3). Third, the ratios of PCC induction

(PCC induction rate in sub-regions of the S phase) were not uniform throughout the S phase (Fig. 5), being high in the early and late S phases but low in the middle S phase. This phenomenon may reflect variation in the replication status at different stages through the S phase. It has been shown that there is a transition in replication from the early stage to the late stage in S phase [44-46]. DNA replication of the mammalian chromosome starts from many replication origins and adjacent replicons initiate at the same time. Clusters of replicons are activated at distinct times in the S phase; GC-rich and gene-rich zones replicate at the early S phase, whereas GC-poor and gene-poor zones replicate later in the S phase. Most replicons are always located in different isochors and either all replicate early or all later [44-49]. It is likely that the most active replicating process enhances PCC, and newly synthesized protein(s) is required for it as the addition of the translation inhibitor cycloheximide prevented PCC (Fig. 4). The level-off of PCC induction in *Rad9*-deletion cells by either an increase in HU concentration or incubation time with HU suggests that only a subpopulation of S phase cells were PCC inducible. One possibility to explain this phenomenon is that only a portion of S phase cells accumulate sufficient amounts of certain protein factor (s) for PCC.

Phosphorylation of Tyr28 and the C-terminus of Mrad9 in preventing PCC

Human Rad9 protein is phosphorylated at Tyr28 and multiple sites of its C-terminus [31, 35, 50, 51]. Mutation of the C-terminal multiple phosphorylation sites leads to heightened sensitivity to HU [20, 52]. Replacement of Ser387 with Ala resulted in the inactivation of the Chk1 signaling checkpoint pathway [34], and an intact Chk1 signaling pathway is required for S/M checkpoint control [53-55]. The Tyr28 on human Rad9 is phosphorylated by cAbl and this phosphorylation is enhanced when cells are exposed to genotoxic agents [35]. The phosphorylation was also shown to be required for Rad9-induced apoptosis [35]. Sequence alignment between human and mouse Rad9 proteins indicates that Tyr28 and Ser389 in human Rad9 correspond to Tyr28 and Ser385 in mouse Rad9 and are evolutionally conserved (data not shown). We have demonstrated that phosphorylation of either C-terminal multiple sites including Ser385 or Tyr28 of mouse Rad9 is essential for preventing PCC. The PCC phenotype resulting from mutation of the multiple mouse Rad9 C-terminal phosphorylation sites is consistent with the role of these phosphorylation sites in the Chk1 signaling checkpoint pathway. The PCC phenotype of the Tyr28 mutation is surprising since the mutation has only been reported to be related to deficiency in human Rad9-induced apoptosis [35]. Nevertheless, our experimental results suggest that Tyr28 is essential for S/M cell cycle checkpoint control.

Mrad9 is required for the phosphorylation of Cdc2 and enhancement of 14-3-3 σ expression induced by HU treatment

Chk1 and 14-3-3 proteins play important roles in G2 checkpoint control in both yeast and human cells [56, 57]. The endpoint of G2 checkpoint control is the activity of Cdc2/CyclinB. Cdc2/cyclinB1 complex triggers the transition from G2 phase to M phase. Either relocation from the nucleus to the cytoplasm or the phosphorylation of the Tyr15 of Cdc2 blocks the transition. Both Chk1 phosphorylation and 14-3-3 σ induction caused by DNA damage are critical contributors to the Tyr phosphorylation of Cdc2, and the phosphorylated form of Cdc2 is inactive for the transition from G2 to M phase [38, 42, 58-60]. In this study, we have shown that Mrad9 is required for the phosphorylation of Cdc2 and 14-3-3 σ induction in response to HU [20, 30]. Therefore, Rad9 may mediate S/M checkpoint control through Chk1 phosphorylation and 14-3-3 σ induction. How Rad9 induces 14-3-3 σ is unknown and merits further study.

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