Vertebrate cells genetically deficient for Cdc14A or Cdc14B retain DNA damage checkpoint proficiency but are impaired in DNA repair

Citation for published version:
Mocciaro, A, Berdougo, E, Zeng, K, Black, E, Vagnarelli, P, Earnshaw, W, Gillespie, D, Jallepalli, P & Schiebel, E 2010, 'Vertebrate cells genetically deficient for Cdc14A or Cdc14B retain DNA damage checkpoint proficiency but are impaired in DNA repair' The Journal of Cell Biology, vol 189, no. 4, pp. 631-639. DOI: 10.1083/jcb.200910057

Digital Object Identifier (DOI):
10.1083/jcb.200910057

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
The Journal of Cell Biology

Publisher Rights Statement:
available under a Creative Commons License (Attribution–Noncommercial–Share Alike 3.0 Unported license, as described at http://creativecommons.org/licenses/by-nc-sa/3.0/).

General rights
Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.
Vertebrate cells genetically deficient for Cdc14A or Cdc14B retain DNA damage checkpoint proficiency but are impaired in DNA repair

Annamaria Mocciaro,1 Eli Berdougo,2 Kang Zeng,3 Elizabeth Black,4 Paola Vagnarelli,5 William Earnshaw,5 David Gillespie,4 Prasad Jallepalli,2 and Elmar Schiebel1

1Zentrum für Molekulare Biologie der Universität Heidelberg, DKFZ-ZMBH Allianz, 69117 Heidelberg, Germany
2Memorial Sloan-Kettering Cancer Center, New York, NY 10065
3Cancer Research Centre, University of Liverpool, Liverpool L69 3BX, England, UK
4Beatson Institute for Cancer Research, Glasgow G61 1BD, Scotland, UK
5Wellcome Trust Centre for Cell Biology, Institute of Cell Biology, University of Edinburgh, Edinburgh EH8 9YL, Scotland, UK

© 2010 Mocciaro et al. This article is distributed under the terms of an Attribution–Noncommercial–Share Alike–No Mirror Sites license for the first six months after the publication date (see http://www.rupress.org/terms). After six months it is available under a Creative Commons License (Attribution–Noncommercial–Share Alike 3.0 Unported license, as described at http://creativecommons.org/licenses/by-nc-sa/3.0/).

Vertebrate cells genetically deficient for Cdc14A or Cdc14B retain DNA damage checkpoint proficiency but are impaired in DNA repair.
Results and discussion

Cdc14A- and Cdc14B-deficient DT40 cell lines

Chicken DT40 B-lymphoma cells show high efficiency of targeted integration of transfected constructs, allowing the disruption of genes through homologous recombination (Buerstedde and Takeda, 1991). Therefore, we analyzed the function of Cdc14A and Cdc14B in this cell line.

Computational (GNOMON) analysis of the chicken genome predicts orthologues of Cdc14A (chicken Cdc14A [cCdc14A]; GenBank accession no. NC_006095.2) and Cdc14B (cCdc14B; NCBI Protein database accession no. XP_425045.2) on chromosomes 8 and Z, respectively. However, only parts of these sequences are similar to human and mouse Cdc14 proteins. Therefore, we isolated cCdc14A and cCdc14B cDNAs by RT-PCR from total RNA of DT40 wild-type (WT) cells (unpublished data). These cDNA sequences predict cCdc14A and cCdc14B proteins conserved with other species throughout their length (GU550056 and GU550055). The chicken genome does not possess a Cdc14Bretro gene (Rosso et al., 2008).

cCdc14A was detected at the centrosome of cells in interphase and late mitosis (Fig. S1 A). This localization is consistent with that of hCdc14A (Kaiser et al., 2002; Mailand et al., 2002). We were unable to raise antibodies against cCdc14B protein (unpublished data). Therefore, we generated a DT40 cell line stably expressing cCdc14B-GFP. In agreement with the localization of hCdc14B (Kaiser et al., 2002; Mailand et al., 2002), cCdc14B-GFP localized to the nucleus in interphase cells with an enrichment in the nucleolus (Fig. S1 B). In mitosis, cCdc14B-GFP was dispersed throughout the cell (unpublished data).

Next, we generated cCdc14A knockout (KO) and cCdc14B-KO cell lines (Fig. S1, C–G, and S2, A–C). Surprisingly, cells lacking either cCdc14A or cCdc14B were viable. Moreover, the doubling time of the cCdc14B-KO cell lines was indistinguishable from that of WT cells (Fig. S2 D). This indicates that cCdc14B is not essential for viability and proliferation of DT40 cells. hCdc14B has been proposed to regulate mitotic exit by interacting with SIRT2 (Dryden et al., 2003). However, the mitotic index (MI) of cCdc14B-KO cells did not show any significant increase indicative of defects in mitotic exit (Fig. S2 E). This is consistent with the lack of a mitotic exit defect of hCdc14B<sup>-/-</sup> cells (Berdougo et al., 2008).

In cCdc14B-KO cells, the levels of the cCdc14A protein were not increased compared with WT cells, and cCdc14A was still associated with the centrosome (Fig. S2, F and G). Thus, it is unlikely that up-regulation or relocalization of cCdc14A compensates for the loss of cCdc14B.

Avian cells lacking cCdc14A and cCdc14B have a functional DNA damage checkpoint

Recently, it was suggested that hCdc14B is an essential component of the G2 DNA damage checkpoint. In response to genotoxic stress in G2, hCdc14B relocalizes from the nucleolus to the nucleus and activates APC/C<sup>Ca</sup>, leading to degradation of Plk1 and stabilization of claspin. This allows for efficient phosphorylation of the checkpoint kinase Chk1 and checkpoint activation. The role of Cdc14A in the G2 DNA damage checkpoint was not investigated (Bassermann et al., 2008).

In agreement with the data of Bassermann et al. (2008), DT40 cells expressing cCdc14B-GFP after synchronization in G2 and exposure to γ irradiation (IR) showed a relocation of cCdc14B from the nucleolus to the nucleus (Fig. S3, A and B), whereas cCdc14A remained at the centrosome (Fig. S3, C and D).

Using cCdc14A-KO and cCdc14B-KO DT40 cell lines, we assayed for a defect in the G2 DNA damage checkpoint. As a control for checkpoint deficiency, we used DT40 Chk1-KO cells (Zachos et al., 2003). To quantify G2 checkpoint proficiency, we first added nocodazole (Noco) to the growth medium to trap cells in mitosis. This allowed us to measure the number of cells that entered M phase from G2 by staining for histone 3 phosphorylated on Ser10 (pH3). WT, cCdc14A-KO, cCdc14B-KO, and Chk1-KO DT40 cells were incubated in medium containing Noco for 8 h with or without prior exposure to IR. Chk1-KO cells accumulate in mitosis to a similar extent regardless of prior IR (Zachos et al., 2003). In marked contrast, IR strongly reduced mitotic accumulation in DT40 WT, cCdc14A-KO, and cCdc14B-KO cells, which is indicative of a functional G2 checkpoint (Fig. 1A, green bars). Similar results were obtained when cells were pulsed for 1 h with the DNA-damaging drug doxorubicin (DXR; unpublished data).

G2 phase–specific activation of APC/C<sup>Ca</sup> after DNA damage was previously reported in DT40 cells (Sudo et al., 2001). To evaluate the importance of Cdh1 for DNA damage–induced cell cycle arrest in G2 in DT40 cells, Cdh1-KO DT40 cells were treated as in Fig. 1A, and the percentage of mitotic cells (MI) was determined by flow cytometry. Cdh1-KO cells were arrested after IR as efficiently as WT cells (Fig. 1A, green bars).

The nature of the G2 arrest after DNA damage varies according to the position of a cell in the cell cycle at the time when damage occurs (Xu et al., 2002). Involvement of hCdc14B and APC/C<sup>Ca</sup> in the G2 checkpoint has been suggested specifically for human cells exposed to damage in G2 (Bassermann et al., 2008). To address the possibility that the discrepancies between the phenotype described by Bassermann et al. (2008) after hCdc14B knockdown and our observations in DT40 cCdc14B-KO cells were a result of irradiating populations of asynchronously growing cells, WT, cCdc14A-KO, cCdc14B-KO, Chk1-KO, and Cdh1-KO DT40 cells were synchronized in early S phase with aphidicolin, released for 4 h to allow progression into G2 (Fig. 1B), and exposed to IR. Under these conditions, cCdc14A-KO, cCdc14B-KO, and Cdh1-KO cells maintained their ability to arrest in G2 after damage as efficiently as WT cells, as shown by the reduction in their MI (Fig. 1C). To exclude the possibility that the G2 checkpoint proficiency in cCdc14A-KO cells was caused by adaptation, we treated transgenic cCdc14A-KO/cCdc14A-HA and cCdc14B-KO/cCdc14B-HA cells with 4-hydroxytamoxifen to activate Cre recombinase and remove the cDNAs encoding cCdc14A-HA or cCdc14B-HA (Fig. S3E). When G2 checkpoint proficiency was assessed immediately after transgene removal, cells arrested efficiently after IR (Fig. S3F).

Prolonged G2 arrest after DNA damage induces centrosome amplification in human and DT40 cells, and this amplification is dependent on Chk1 activity (Dodson et al., 2004;
Cdc14-deficient cells have impaired DNA repair

Figure 1. Functional G2 damage checkpoint in DT40 cells deleted for cCdc14A, cCdc14B, or Cdh1. [A] Flow cytometry analysis of the indicated cell lines incubated with Noco for 8 h with or without prior IR (IR + Noco and Noco). Cells were stained with PI and for pH3 to measure the MI. Values normalized to the MI of the corresponding Noco-treated cultures (n = 3). [B] Synchrony in G2 at the time of IR. [C] Cells synchronized in G2 were exposed to IR. Cells were harvested, and MI was measured by flow cytometry (n = 3). [D] Cells were irradiated, fixed (12 h after treatment), and stained for γ-tubulin (green) and centrin (red). The number of cells with more than two centrosomes was scored. Bar, 5 µm. [E] Quantification of phenotype in D (n = 3; 100 cells per each cell line) is shown. [F, top] WT, cCdc14A-KO, and cCdc14B-KO cells were analyzed by IB. [bottom] Quantification of Chk1(S345ph) before (t = 0) and after IR. Chk1(S345ph) was normalized to Chk1. Chk1(S345ph) in the untreated WT sample was set to 1 (n = 2). Error bars indicate mean ± SD.
Figure 2. Defective DNA repair in DT40 cCdc14A-KO and cCdc14B-KO cells. (A) The indicated cell lines were harvested either before (t = 0 h) or after IR, fixed, stained with PI and anti-γ-H2A.X, and analyzed by flow cytometry. (B) Quantification of the γ-H2A.X-positive cells in A (n = 3) is shown. (C) IB analysis of cCdc14A in cCdc14A-Res cells compared with WT. (D) WT, cCdc14A-KO, and cCdc14B-KO cells harvested either before (−IR 0 h) or 0.5 and 3 h after IR. Cells were fixed, stained for pH3 (red) and γ-H2A.X (green), and examined by fluorescence microscopy. Mitotic cells before and after IR. Anti-γ-H2A.X staining of the centrosome (arrowheads) was seen in some unirradiated cells. Bar, 5 µm. (E, left) The proportion of cells positive for pH3 and γ-H2A.X was scored as a percentage of total mitotic cells (n = 3; 100 mitotic cells per genotype). (right) The number of γ-H2A.X foci/cell was counted in projected and deconvolved images of mitotic cells positive for γ-H2A.X (n = 3; 20 mitotic cells). (F) The indicated cell lines were treated ± 1.5 µM DXR for 2 h and analyzed by single-cell gel electrophoresis (comet assay). Representative images are shown. Bar, 5 µm. (G) Tail moments for each time (n = 75; mean of two independent experiments) were quantified with ImageJ software. (H) Cell viability after IR analyzed by MTT assay (n = 3; P < 0.02). Error bars indicate mean ± SD.
To confirm that this phenotype was caused by inactivation of cCdc14A or cCdc14B, we stably reintroduced cCdc14A or cCdc14B cDNA into the parental nullizygous Res cells expressing cCdc14A at close to WT levels (Fig. 2 C). cCdc14A or cCdc14B cDNA was expressed in cCdc14A-Res and cCdc14B-Res, respectively. cCdc14A-Res cells expressed cCdc14A at close to WT levels (Fig. 2 C). Importantly, cCdc14A-Res and cCdc14B-Res cells were essentially indistinguishable from WT in the kinetics of γ-H2A.X signal disappearance (Fig. 2 A and B).

To further investigate the kinetics of IR-induced DSB repair in WT and cCdc14-KO cells, we assessed the presence of γ-H2A.X foci by immunofluorescence (IF). In accordance with the flow cytometry analysis, 3 h after IR, ~80% of the mitotic cCdc14A-KO cells and ~50% of the mitotic cCdc14B-KO cells still showed multiple γ-H2A.X foci. Strikingly, a significant number of mitotic cells in unirradiated cCdc14A-KO or cCdc14B-KO cultures also contained γ-H2A.X foci (Fig. 2, D and E). It is most likely that the DSBs in untreated cells arise from failure to repair damage occurring spontaneously during the cell cycle. This raises the question of how cCdc14A-KO and cCdc14B-KO cells with a functional G2 DNA damage checkpoint are able to enter mitosis with DNA lesions.

We next tested the possibility that the higher basal level and persistence of Chk1 phosphorylation in cCdc14A-KO or cCdc14B-KO cells (Fig. 1 F) might be related to the presence of damaged DNA, we used flow cytometry to estimate the fraction of cells bearing phosphorylated histone 2A.X (γ-H2A.X), a DNA damage marker (Funuta et al., 2003), before and after IR. The proportion of γ-H2A.X–positive WT cells decreased from nearly 100% to ~35% 3 h after IR, whereas ~80% of cCdc14A-KO or cCdc14B-KO cells remained positive for γ-H2A.X at this time (Fig. 2, A and B). To confirm that this phenotype was caused by inactivation of cCdc14A or cCdc14B, we stably reintroduced cCdc14A or cCdc14B cDNA into the parental nullizygous cells (cCdc14A-Res and cCdc14B-Res, respectively). cCdc14A-Res cells expressed cCdc14A at close to WT levels (Fig. 2 C). Importantly, cCdc14A-Res and cCdc14B-Res cells were essentially indistinguishable from WT in the kinetics of γ-H2A.X signal disappearance (Fig. 2 A and B).

To further investigate the kinetics of IR-induced DSB repair in WT and cCdc14-KO cells, we assessed the presence of γ-H2A.X foci by immunofluorescence (IF). In accordance with the flow cytometry analysis, 3 h after IR, ~80% of the mitotic cCdc14A-KO cells and ~50% of the mitotic cCdc14B-KO cells still showed multiple γ-H2A.X foci. Strikingly, a significant number of mitotic cells in unirradiated cCdc14A-KO or cCdc14B-KO cultures also contained γ-H2A.X foci (Fig. 2, D and E). It is most likely that the DSBs in untreated cells arise from failure to repair damage occurring spontaneously during the cell cycle. This raises the question of how cCdc14A-KO and cCdc14B-KO cells with a functional G2 DNA damage checkpoint are able to enter mitosis with DNA lesions. In yeast, the G2 checkpoint is sensitive to a single DSB (Bennett et al., 1997), whereas higher eukaryotes have a different sensitivity threshold (Löbrich and Jeggo, 2007), which, for mammalian fibroblasts, was calculated to be ~20 DSBs per cell (Deckbar et al., 2007). Indeed, 80% of untreated cCdc14A-KO or cCdc14B-KO mitotic cells had <20 γ-H2A.X foci/cell (Fig. 2 E), thus explaining progression of cells bearing DNA damage into mitosis.

Figure 3. Characterization of hCdc14A-deficient cells. (A) Generation of a conditional-null hCdc14A cell line. (B) Southern blot analysis confirms biallelic mutations of the hCdc14A locus in hTERT-RPE cells. WT (+/+), flox, Δneo, and Δ alleles are marked. (C) RT-PCR analysis confirming expression of WT (asterisks) and exon 2–deleted (asterisks) hCdc14A transcripts. (D) MI of asynchronous populations of Cdc14AΔfloxed/Δfloxed and Cdc14AΔfloxed/Δneo cells. Cells were fixed and stained with Hoechst (n = 3; 300 cells per genotype). (E) Cdc14AΔfloxed/Δfloxed and Cdc14AΔfloxed/Δneo cells were treated with Nocodazole for 12 h followed by shake off into medium without Nocodazole, fixation, and Hoechst (n = 3; 300 cells per time point). (F) Cells were fixed, stained for γ-tubulin, and categorized by centrosome number (n = 3; 200 cells per genotype). Error bars indicate mean ± SD.
Consistent with the γ-H2AX data (Fig. 2, A–E), untreated cCdc14A-KO and cCdc14B-KO cells exhibited longer comet tails compared with WT (Fig. 2 F). Treatment with DXR

To measure DNA damage directly, we used the comet assay (Fairbairn et al., 1995) in which DSBs confer increased electrophoretic mobility to DNA released from single cells. Consistent with the γ-H2AX data (Fig. 2, A–E), untreated cCdc14A-KO and cCdc14B-KO cells exhibited longer comet tails compared with WT (Fig. 2 F). Treatment with DXR
Cdc14-deficient cells have impaired DNA repair

Mocciaro et al.

Cdc14-deficient cells have impaired DNA repair

• Mocciaro et al. demonstrate that hCdc14A is dispensable for viability and proliferation of an untransformed human cell line. Human cells deficient for hCdc14A or hCdc14B have a functional G2 DNA damage checkpoint.

We used Cdc14A^fox/fox and Cdc14B^fox/fox cells together with an hCdc14B^fox/fox HCT116 cell line (Berdougo et al., 2008) to investigate whether hCdc14A and hCdc14B are required for G2 DNA damage checkpoint proficiency. Asynchronously growing Cdc14A^fox/+ and Cdc14A^fox/neo cells were treated with Noco for 6 h with or without prior IR. Quantification of MI revealed that Cdc14A^fox/neo cells arrested in G2 as efficiently as controls (Fig. 4A). Efficient arrest also occurred after DXR treatment (unpublished data). Cdc14B^fox/fox cells were similarly DNA damage checkpoint proficient (Fig. 4B). Nearly identical results were obtained when cells were exposed to DNA damage after synchronization in G2 (Fig. 4, C and D).

We also monitored several markers of the DNA damage checkpoint in human cells by IB. Cdc14A^fox/neo and Cdc14B^fox/fox cells synchronized in G2 activate the main effectors of the DNA damage checkpoint, as indicated by the increased phosphorylation of Chk1 on Ser345 after treatment with DXR (Fig. 4, E and F, lanes 12–14 vs. lanes 8–11). Moreover, inhibitory phosphorylation of Cdk1 on Tyr15 decreased in untreated Cdc14A^fox/neo and Cdc14B^fox/fox cells compared with controls, as indicated by the reduction in comet tails to control levels, whereas comet tails in cCdc14A-KO and cCdc14B-KO cells remained elevated. Based on the comet tail moment value, which quantitatively represents the extent of DNA damage (Helma and Uhl, 2000), we estimate four- to fivefold higher damage in cCdc14A-deficient cells than in control cells 3 h after DXR treatment (Fig. 2G). Thus, cCdc14A-KO and cCdc14B-KO cells repair DSBs slower than WT cells. Consistent with the aforementioned observations, cells lacking cCdc14A or cCdc14B had reduced survival rates after IR compared with WT, cCdc14A-Res, and cCdc14B-Res cells (Fig. 2H).

Homologous deletion of the Cdc14A locus in human cells

To investigate the evolutionary conservation of the role of hCdc14A in the DNA damage responses, we generated a Cdc14A^ââ¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â¬â"
Cdc14B<sup>Δ/Δ</sup> cells after release from synchronization in G2, whereas it persisted at high levels after damage (Fig. 4, E and F; lanes 9–11 vs. lanes 12–14), indicating an arrest in G2. Interestingly, as in cCdc14A-KO DT40 cells, the basal level of Chk1 phosphorylation was already elevated in unirradiated Cdc14A<sup>Δ/Δneo</sup> cells compared with Cdc14A<sup>loxP/loxP</sup> (Fig. 4 E, lanes 1–4 vs. lanes 8–11).

These findings prompted us to investigate whether Cdc14A<sup>Δ/Δneo</sup> or Cdc14B<sup>Δ/Δ</sup> cells also showed an increased number of mitotic cells bearing DNA damage foci in the absence of DSB-inducing treatments (Fig. 5, A and B). The percentage of mitotic cells exhibiting γ-H2A.X foci was significantly higher in Cdc14A<sup>Δ/Δneo</sup> and Cdc14B<sup>Δ/Δ</sup> cultures with controls (Fig. 5, C and D). Also, in human cells, the number of foci was usually <20 per cell (Fig. 5 E), explaining progression of these cells into mitosis. These results suggest that in human cells, Cdc14A and Cdc14B are also likely to be required for efficient DNA repair. The persistence of DNA damage foci in Cdc14A<sup>Δ/Δneo</sup> and Cdc14B<sup>Δ/Δ</sup> cells was associated with lower survival rates than controls after IR (Fig. 5, F and G), indicating a higher sensitivity of these mutants to DNA damage.

Conclusions

In this study, we evaluate the functions of avian and human Cdc14A and Cdc14B in cells lacking these gene products. Surprisingly, Cdc14A-KO and Cdc14B-KO cells are viable and do not show severe cellular defects. With respect to Cdc14B, our findings contrast with the G2 checkpoint defect previously reported in human cells depleted for Cdc14B using siRNA (Bassermann et al., 2008). This is unlikely to be caused by cell type specificity because both avian lymphocytes and human epithelial cells genetically deleted for Cdc14B retained normal G2 checkpoint proficiency. It seems more likely that the effects of complete and permanent loss of Cdc14B function somehow differ from the more short-term and typically less-complete ablation achieved through siRNA depletion.

Although Cdc14A-KO or Cdc14B-KO cells are DNA damage checkpoint proficient, their capacity to repair DNA is diminished, resulting in the presence of a higher number of γ-H2A.X foci compared with controls. This is true even without any treatment with DNA damage–inducing agents and results in an increased sensitivity of the Cdc14-KOs to IR. Thus, these data uncover a new requirement for avian and human Cdc14A and Cdc14B in DNA repair.

Materials and methods

Generation of cCdc14A-KO and cCdc14B-KO cells

cCdc14A and cCdc14B cDNAs were isolated by RTPCR using total RNA extracted from DT40 WT cells as template. For cCdc14A, two separate targeting vectors containing a 2.3-kb left and 3-kb right arm of homology were synthesized and cloned into Bluescript (Agilent Technologies) flanking either puromycin or blastidin selection cassettes (Sonoda et al., 1998). After one round of targeting with the puromycin-targeting vector, drug-resistant clones were genotyped by PCR to identify cCdc14A<sup>+/+</sup> cells. After excision of the puromycin cassette by induction of Cre recombinase, one hemizygous chicken was electroporated with a rescue vector containing the HA-tagged cCdc14A cDNA under control of the chicken β-actin promoter and a puromycin-resistant cassette (plasmid derived from pBluescript; provided by J.M. Buerstedde, Institute for Molecular Radiobiology, Munich, Germany) and selected in 0.5 μg/ml puromycin (InvivoGen). Clones expressing cCdc14A at close to endogenous levels were electroporated with the targeting vector as described previously (Sarbasak and Arakawa, 2006) and selected in 30 μg/ml blastidin to obtain a nullizygous clone. To generate the cCdc14A rescue cell line, cCdc14A-KO cells were transfected with the cCdc14A vector and selected in 0.5 μg/ml puromycin. Drug-resistant clones were screened for cCdc14A expression by IB, and a cell line with nearly endogenous levels of cCdc14A was identified and designated cCdc14A-Res.

For cCdc14B, a targeting vector containing a 3.3-kb left and 4.8-kb right arm of homology was synthesized by long-range PCR and cloned into Bluescript flanking a blastidin selection cassette (Sonoda et al., 1998). To generate cCdc14B-deficient DT40 clones, DT40-Cre-ER cells expressing cCdc14B-HA were transfected with the targeting construct, using initially PCR and subsequently Southern blotting to genotype drug-resistant clones after selection in 30 μg/ml blastidin (InvivoGen). To generate the cCdc14B rescue cell line, cCdc14B-KO cells were transfected with a rescue vector containing the HA-tagged cCdc14B cDNA under control of the chicken β-actin promoter and a puromycin-resistant cassette and selected in 0.5 μg/ml puromycin. Drug-resistant clones were screened for cCdc14B-HA expression by IB, and the positive clones designated cCdc14B-Res.

Generation of hCdc14A-KO cells

to generate a conditional KO of the hCdc14A locus, 5′ and 3′ homology arms were amplified from a human BAC clone (RP11-976I7) and cloned into a vector containing a central FRT-neoFRT-HoeP cassette. A secondary loxP site was introduced downstream of exon 2 via QuikChange mutagenesis. The entire Cdc14A insert was subcloned into pAAV. Transfection of HEK293 cells, isolation of AAV particles, and infection of hTERT-RPE cells were performed as described previously (Berdougo et al., 2009). G418-resistant colonies were screened by PCR. The neo cassette was excised from Cdc14A<sup>neo+/+</sup> transfected by pCAAGS-FLPe followed by puromycin selection and limiting dilution. Individual colonies were tested for neo excision by genomic PCR and reacquisition of G418 sensitivity. Targeting of the second allele was achieved with a Cdc14A vector lacking exon 2 (pAAV/Cdc14A ΔΔ) Cdc14A<sup>Δ/Δneo</sup> cells were converted to Cdc14A<sup>Δ/Δneo</sup> cells by infection with a recombinant adenovirus expressing Cre recombinase. Targeted clones were confirmed by Southern blotting. The transcript from the exon 2-deleted hCdc14A gene contains a frame-shift and does not code for a functional hCdc14A protein.

Cell culture and treatments

DT40 B-lymphoma cells DT40 B-lymphoma cells were grown in DME (Invitrogen) containing 10% FBS, 1% chicken serum, 1% glutamine, 1% sodium pyruvate, 10−5 M β-mercaptoethanol, penicillin, and streptomycin at 37°C. HCT116 cells were grown in McCoy’s 5A medium (Invitrogen) supplemented with 10% FBS (Invitrogen) at 37°C. hTERT-RPE1 cell lines were grown in DME/F-12 medium supplemented with 10% FBS, 1% glutamine, and 0.348% sodium bicarbonate at 37°C.

Cell lines were irradiated with 10 Gy IR using a caesium source (Gamma Cells 1000S Atomi Energy of Canada Ltd) and treated with 0.5 µg/ml nocodazole (Sigma-Aldrich), 5 µM aphidicolin (Sigma-Aldrich), 2 mM thymidine (Sigma-Aldrich), 2 mM thymidine (Sigma-Aldrich), 2 mM thymidine (Sigma-Aldrich), and 0.1 mM 4-hydroxytamoxifen (Sigma-Aldrich) as appropriate.

Flow cytometry

Cells were fixed in 70% ethanol in PBS overnight. For DNA content analysis, cells were pelleted and resuspended in PBS containing 1 mg/ml RNase (Sigma-Aldrich) and 10 mg/ml propidium iodide (PI) incubated at room temperature for 30 min then analyzed using a flow cytometer (FACScan; BD).

For MI determinations, fixed cells were incubated with polyclonal anti–phospho histone H3 antibodies followed by FITC-conjugated secondary antibody (Invitrogen). Cells were counterstained with propidium iodide and analyzed for FITC fluorescence and DNA content by flow cytometry. For determination of γ-H2A.X foci, fixed cells were incubated with monoclonal anti–γ-H2A.X antibody followed by FITC-conjugated secondary antibody and counterstained with propidium iodide.

IB

Cell extracts were prepared in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% Na deoxycholate, 0.1% SDS, 50 mM Tris-Cl, pH 8.0, 1 mM PMSF, complete protease inhibitor cocktail [Roche], and PhosStop phosphatase inhibitor cocktail [Roche]), resolved by SDS-PAGE, and blotted onto nitrocellulose membranes (GE Healthcare). Antibodies against Chk1 (S345ph) (Cell Signaling Technology), Chk1 (S45), Santa Cruz Biotechnology, Inc., Chk1[Y15ph] (Ik-15; Santa Cruz Biotechnology, Inc.), and CDK1 (c17; Santa Cruz Biotechnology, Inc.), and analyzed by IB.
Cdc14-deﬁcient cells have impaired DNA repair and mitotic exit in human cells.

Berdougo, E., M.E. Terret, and P.V. Jallepalli. 2009. Functional dissection of mitotic regulators through gene targeting in human somatic cells. Methods Mol. Biol. 545:21–37. doi:10.1007/978-1-60327-993-2_2

Bourke, E., H. Dodson, A. Merdes, L. Cuffe, G. Zachos, M. Walker, D. Gillespie, and C.G. Morrison. 2007. DNA damage induces Cdk1-dependent centrosome ampliﬁcation. EMBO Rep. 8:603–609. doi:10.1038/sj.emborep.7400662

Bourke, E., J.A. Brown, S. Takeda, H. Hochegger, and C.G. Morrison. 2010. DNA damage induces Cdk1-dependent threonine-160 phosphorylation and activation of Cdk2. Oncogene. 29:616–624. doi:10.1038/onc.2009.340

Buerstedde, J.M., and S. Takeda. 1991. Increased ratio of targeted to random integration after transfection of chicken B cell lines. Cell. 67:179–188. doi:10.1016/0028-0842(91)90251-I

Cho, H.P., Y. Liu, M. Gomez, J. Dunlap, M. Tyers, and Y. Wang. 2005. The dual-speciﬁcity phosphatase CDC14B bundles and stabilizes microtubules. Mol. Cell. Biol. 25:4541–4551. doi:10.1128/MCB.25.11.4541–4551.2005

Deckbar, D., J. Birraux, A. Krempler, L. Tchouaoudong, A. Beucher, S. Tschik, P. Peggo, and M. Löbrich. 2007. Chromosome breakage after G2 checkpoint release. J. Cell Biol. 176:749–755. doi:10.1083/jcb.2006112047

Dodson, H., E. Bourke, J.L. Jeffers, P. Vagnarelli, E. Sonoda, S. Takeda, W.C. Earnshaw, A. Merdes, and C. Morrison. 2004. Centrosome ampliﬁcation induced by DNA damage occurs during a prolonged G2 phase and involves ATM. EMBO J. 23:3864–3873. doi:10.1038/sj.emboj.7600393

Dryden, S.C., F.A. Nahitas, J.E. Nowak, A.S. Goustine, and M.A. Tainsky. 2003. Roles for human SIRT2 NAD-dependent deacetylase activity in control of mitotic exit in the cell cycle. Mol. Cell. Biol. 23:3137–3145. doi:10.1128/MCB.23.9.3137-3145.2003

Fairbairn, D.W., P.L. Olive, and K.L. O’Neill. 1995. The comet assay: a comprehensive review. Mutat. Res. 339:37–59.

Furuta, T., H. Takeamura, Z.Y. Liu, G.J. Aune, C. Redon, O.A. Sedelmakova, D.R. Pilch, E.P. Rogakou, A. Celeste, H.T. Chen, et al. 2003. Phosphorylation of histone H2AX and activation of Mre11, Rad50, and Nbs1 in response to replication-dependent DNA double-strand breaks induced by mammalian DNA topoisomerase I cleavage complexes. J. Biol. Chem. 278:20303–20312. doi:10.1074/jbc.M300198200

Helma, C., and M. Uhl. 2000. A public domain image-analysis program for the single-cell gel-electrophoresis (comet) assay. Mutat. Res. 466:9–15.

Kaiser, B.K., Z.A. Zimmerman, H. Charbonneau, and P.K. Jackson. 2002. Disruption of centrosome structure, chromosome segregation, and cytokinesis by misexpression of human Cdc14A phosphatase. Mol. Biol. Cell. 13:2289–2300. doi:10.1091/mbc.01-11-0535

Löbrich, M., and P.A. Jeggo. 2007. The impact of a negligent G2/M checkpoint on genomic instability and cancer induction. Nat. Rev. Cancer. 7:861–869. doi:10.1038/nrc2248

Mailand, N., C. Lukas, B.K. Kaiser, P.K. Jackson, J. Bartek, and J. Lukas. 2002. Deregulated human Cdc14A phosphatase disrupts centrosome separation and chromosome segregation. Nat. Cell Biol. 4:317–322. doi:10.1038/ncl777

Queralt, E., and F. Uhlmann. 2008. Cdk-counteracting phosphatases unlock mitotic exit. Curr. Opin. Cell Biol. 20:661–668. doi:10.1016/j.ceb.2008.09.003

Rosso, L., A.C. Marques, M. Weier, N. Lambert, M.A. Lambot, P. Vanderhaeghen, and H. Kaessmann. 2008. Birth and rapid subcellular adaptation of a hominoid-speciﬁc CDC14 protein. PLoS Biol. 6:e140. doi:10.1371/journal.pbio.0060140

Saribasak, H., and H. Arakawa. 2006. Targeted transfection of DT40 cells. Subcell. Biochem. 40:419–421.

Sonoda, E., M.S. Sasaki, J.M. Buerstedde, O. Bezzubova, A. Shinohara, H. Ogawa, M. Takata, Y. Yamaguchi-Iwai, and S. Takeda. 1998. Rad51-deficient mice are viable but exhibit multiple checkpoint and survival defects. EMBO J. 17:595–608. doi:10.1093/emboj/17.2.595

Steigemann, F., and A. Amon. 2004. Closing mitosis: the functions of the Cdc14 phosphatase and its regulation. Annu. Rev. Genet. 38:203–232. doi:10.1146/annurev.genet.38.072902.093051

Sudo, T., Y. Ota, S. Kotani, M. Nakao, Y. Takami, S. Takeda, and H. Saya. 2001. Activation of Cdc1-dependent APC is required for G1 cell cycle arrest and DNA damage-induced G2 checkpoint in vertebrate cells. EMBO J. 20:6499–6508. doi:10.1093/emboj/20.22.6499

Wu, J.H., P.C. Cho, D.B. Rhee, D.K. Johnson, J. Dunlap, Y. Liu, and Y. Wang. 2008. Cdc14B depletion leads to centriole amplification, and its overexpression prevents unscheduled centriole duplication. EMBO J. 27:1970–1980. doi:10.1038/sj.emboj.7600962

We are grateful to Dr. J.-M. Buerstedde for plasmids, the DT40 CreEB cell line, and advice. This work was supported by the Deutsche Forschungsgemeinschaft (grant Schi295-3).

Submitted: 8 October 2009 Accepted: 20 April 2010

References

Bassermann, F., D. Frescas, D. Guardavaccaro, L. Busino, A. Peschiaroli, and M. Pignataro. 2008. The Cdc14B-Chk1-Pik1 axis controls the G2 DNA-damage-response checkpoint. Cell. 134:256–267. doi:10.1016/j.cell.2008.05.043

Bennett, C.B., J.R. Snipe, and M.A. Resnick. 1997. A persistent double-strand break destabilizes human DNA in yeast and can lead to G2 arrest and lethality. Cancer Res. 57:1970–1980.

Berdougo, E., M.V. Nachury, P.K. Jackson, and P.V. Jallepalli. 2008. The molecular phosphatase Cdc14B is dispensable for chromosome segregation and mitotic exit in human cells. Cell Cycle. 7:1184–1190.