Defective nucleotide excision repair with normal centrosome structures and functions in the absence of all vertebrate centrins

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The principal microtubule-organizing center in animal cells, the centrosome, contains centrin, a small, conserved calcium-binding protein unique to eukaryotes. Several centrin isoforms exist and have been implicated in various cellular processes including nuclear export and deoxyribonucleic acid (DNA) repair. Although centrins are required for centriole/basal body duplication in lower eukaryotes, centrin functions in vertebrate centrosome duplication are less clear. To define these roles, we used gene targeting in the hyperrecombinogenic chicken DT40 cell line to delete all three centrin genes in individual clones. Unexpectedly, centrin-deficient cells underwent normal cellular division with no detectable cell cycle defects. Light and electron microscopy analyses revealed no significant difference in centrosome composition or ultrastructure. However, centrin deficiency made DT40 cells highly sensitive to ultraviolet (UV) irradiation, with *Cetn3* deficiency exacerbating the sensitivity of *Cetn4/Cetn2* double mutants. DNA damage checkpoints were intact, but repair of UV-induced DNA damage was delayed in centrin nulls. These data demonstrate a role for vertebrate centrin in nucleotide excision repair.

Introduction

Centrioles are cylindrical subcellular structures found in eukaryotic cells that consist of nine sets of microtubules, generally triplets, arranged symmetrically to form a barrel-like shape. They nucleate cilia and flagella and anchor the pericentriolar material (PCM) to form centrosomes, the principal microtubule-organizing centers in animal somatic cells. Centrosomes organize the interphase cytoskeleton and the bipolar mitotic spindle and thus contribute to the appropriate segregation of chromosomes during cell division (Nigg and Raff, 2009).

Centrins (caltractin) are small, highly conserved proteins that contain four calcium-binding helix-loop-helix EF hand domains. Originally identified as a major component of the calcium-responsive striated flagellar root structure in the green alga, *Tetraselmis striata* (Salisbury et al., 1984), centrins have been described in a wide range of eukaryotes, including mammals (Huang et al., 1988; Lee and Huang, 1993; Ogawa and Shimizu, 1993; Errabolu et al., 1994). A centriolar localization of centrin was initially established in lower eukaryotes (McFadden et al., 1987; Salisbury et al., 1987, 1988; Salisbury, 1995), with subsequent work in mammalian cells localizing centrin to the PCM as well as to the distal lumen of centrioles (Baron et al., 1992; Paoletti et al., 1996). GFP-tagged centrin2 has become widely accepted as a robust marker for centrioles in live cells (White et al., 2000; Higginbotham et al., 2004; Kuriyama et al., 2007).

Four centrin isoforms have been described to date in mammalian cells. All are related to calmodulin, as was observed in the initial cloning of centrin from the biflagellated green alga, *Chlamydomonas reinhardtii* (Huang et al., 1988). Sequence analysis assigns them either to a subfamily related to budding yeast *CDC31* or to one more homologous to the *C. reinhardtii* centrin (Middendorp et al., 1997, 2000). Centrin1 and centrin2 are closely related to one another (Lee and Huang, 1993; Errabolu et al., 1994), with centrin2 being ubiquitously expressed and centrin1 being more restricted to male germ cells, neurons, and

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Abbreviations used in this paper: CPD, cyclobutane pyrimidine dimer; HU, hydroxyurea; IR, ionizing radiation; NER, nucleotide excision repair; PCM, pericentriolar material; TEM, transmission EM; XPC, xeroderma pigmentosum group C.

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ciliated cells (Hart et al., 1999). *CETN1* is believed to have arisen from a retrotransposition of the *CETN2* transcript (Hart et al., 1999). Centrin3 is of the *CDC31* subfamily and is also ubiquitously expressed (Middendorp et al., 1997). *Cetn4* is an additional centrin2-related gene, with a tissue-restricted expression pattern that has suggested its being limited to ciliated cells (Gavet et al., 2003). All full-length centrin isoforms associate with centrioles, but to varying extents that may reflect differing activities of the centrins during centriole duplication and the formation of cilia (Laoukili et al., 2000; Gavet et al., 2003).

A recent evolutionary analysis of centrosomal proteins assigned centrin2 to a core group of proteins suggested to have been present in the ancestral centriolar structure (Hodges et al., 2010). Consistent with this view of centrin functions, a requirement for centrin in centriolar activities has been described in a range of organisms. The budding yeast centrin orthologue Cdc31p is required for spindle pole body duplication (Baum et al., 1986; Huang et al., 1988). Knockdown of centrin in *C. reinhardtii* led to defects in the duplication and functioning of the flagellar basal body, a structure analogous to the centriole (Koblenz et al., 2003). Depletion of centrin in the water fern, *Marsilea vestita*, inhibited spermiogenesis by blocking basal body formation (Klink and Wolniak, 2001). Analysis of centrins in the multiciliated protozoan, *Tetrahymena thermophila*, revealed the localization of the *CEN1* gene product to basal bodies and its requirement for their duplication, with other centrin gene products also localizing to basal bodies (Guerra et al., 2003; Stemm-Wolf et al., 2005). Ablation of centrin function by gene targeting in the pathogenic trypansosome, *Leishmania donovani*, caused defective basal body duplication, cell cycle arrest, and inhibition of proliferation at the amastigote stage accompanied by cell death, with a similar impact on cytokinesis seen with centrin knockdown in *Trypanosoma brucei* (Selvapandiyan et al., 2004, 2007).

Despite the importance of centrin function in ensuring centriole/basal body duplication throughout evolution (Salisbury, 2007), its role is less clear in human cells. Although partial centrin3 depletion disrupted the radial organization of microtubules in U2OS cells (Dammermann and Merdes, 2002), centriole duplication was inhibited by RNAi depletion of centrin2 in HeLa cells (Salisbury et al., 2002). This treatment caused centriole loss and a marked G1 phase cell cycle delay in nontransformed hTERT-RPE1 cells (Mikule et al., 2007). However, in other experiments reported in HeLa cells, centrin2 depletion using the same siRNA sequence did not impact HsSAS-6 recruitment to nascent procentrioles or the completion of centriole duplication, although centriole assembly rates were delayed (Strnad et al., 2007; Yang et al., 2010). siRNA depletion of centrin2 and centrin3 using different inhibitory sequences had no impact on Plk4-induced centriole duplication in U2OS cells (Kleylein-Sohn et al., 2007).

Noncentrosomal roles for centrins have also been described, supporting the observation that the bulk of cellular centrin is not associated with centrioles (Paoletti et al., 1996). Recent data obtained in budding yeast implicate centrin in proteasome activities (Chen and Madura, 2008), and an association between centrin2 and the vertebrate nuclear pore complex has been described, along with a function for centrins in mRNA and protein export in yeast and vertebrates (Fischer et al., 2004; Resendes et al., 2008). A further role for centrins lies in nucleotide excision repair (NER), a DNA repair process that acts to remove various base lesions from DNA, notably those induced by UV irradiation: 6–4 photoproducts and cyclobutane pyrimidine dimers (CPDs). There are two subpathways of NER: transcription-coupled NER, which acts rapidly to remove lesions that block transcription, and global genome repair, which is less rapid but can act genome wide. Centrin2 is found in a complex with the xeroderma pigmentosum group C (XPC) gene product, a component of the DNA damage recognition complex of global genome repair (Sugasawa et al., 1998). Centrin helps to stabilize XPC in its complex with HRad23B (Araki et al., 2001). This occurs through direct interaction of centrin2 and XPC, with centrin2 stimulating NER in vitro (Nishi et al., 2005). Binding experiments have indicated that amino acids 847–866 in the C terminus of human XPC are sufficient for its interaction with centrin2 (Popescu et al., 2003; Nishi et al., 2005; Thompson et al., 2006). Fibroblasts that express XPC mutants incapable of interacting with centrin2 showed impaired resolution of UV-induced DNA damage (Nishi et al., 2005), and partial centrin knockdown by RNAi delayed the clearance of 6–4 photoproducts in MCF-7 cells (Acu et al., 2010). Mutation of the *Arabidopsis thaliana Cetn2* orthologue caused UV sensitivity and DNA repair defects, which were accompanied by a hyperrecombinogenic phenotype in a reporter assay (Molinier et al., 2004). Collectively, these findings implicate centrins in multiple cellular activities away from the centrosome.

We are interested in the interplay between centrosomes and the DNA damage response (Dodson et al., 2004; Bourke et al., 2007). Disruption of centrosome duplication by centrin deletion appeared to be a useful approach to exploring how centrosomes are involved in controlling this response. Here, we show that ablation of all the centrins in a vertebrate cell line, the chicken lymphoma DT40, had no detectable effect on centrosome duplication or cell cycle progression. However, centrin-deficient cells were markedly sensitive to UV irradiation and required centrin for efficient DNA repair. Our results suggest that the principal functions of centrin lie in the noncentrosomal pool where most of the cellular content resides, with an important caveat being that we were unable to use DT40 cells to assess centrin functions in ciliogenesis.

Results

We set out to define the roles of centrin by targeting centrin genes in chicken DT40 cells. As shown in Fig. S1 A, these proteins are highly conserved between chicken and human. Database analysis indicated the presence of three centrin loci in chicken cells. Using available genome information to compare these loci with their mammalian counterparts, we found that chicken *Cetn2* and *Cetn3* are syntenic with their human orthologues, with a discontinuity 3′ of the *Cetn2* sequence between mammals and the chicken. Chicken *Cetn4* is syntenic with mammalian *CETN4*, which is a pseudogene in human cells. We found no centrin1-coding sequence in the region of chicken

chromosome 2 that is otherwise completely syntenic with the human chromosome 18 region that contains the putatively retroposed, single-exon *CETN1* gene (Hart et al., 1999). Nor was any centrin1-coding sequence found in the corresponding region of the zebra finch (*Taeniopygia guttata*) genome. This suggests that the retroposition that generated *CETN1* occurred after the bird–mammal split of 310 million years ago (Reisz and Müller, 2004). All three centrins are expressed in DT40 cells and in chicken liver (Fig. 1 A), showing no tissue-restricted expression pattern.

To ablate centrin function, we used genomic PCR to generate targeting vectors that would delete the entire coding regions of *Cetn4* and *Cetn2* and all but the last two exons of *Cetn3* (Fig. S2, A–C) and transfected them into DT40 cells. We confirmed the successful targeting of the centrin loci using Southern analysis (Fig. S2, D–F). Our initial expectation had been that centrin loss would be lethal to cells, so we first targeted *Cetn4*, then *Cetn2*, and finally *Cetn3*, separately and in the same cell line. We then verified the loss of expression of the centrins using RT-PCR (Fig. S2 G) and immunoblotting using different antibodies with broad species specificities for centrin1/2/4 and for centrin3 (Fig. 1 B). No full-length or truncated gene products were detected with any of the relevant antibodies. Immunofluorescence microscopy confirmed the absence of all centrin isoforms in our *Cetn4/2/3*-deficient cells (Fig. 1 C).

There is a second centrinlike sequence adjacent to the chicken *Cetn2* locus (LOC422304) that suggests a partial duplication of *Cetn2* in the chicken genome. Although transcripts from LOC422304 can be found in the EST databases, their 5′ ends contain short ORFs that suggest that this is a pseudogene (Fig. S1 B). Even the longest possible ORF from LOC422304 encodes a predicted protein that lacks the centrin-diagnostic N-terminal region including part of the first EF hand and that is predicted to have low homology to other centrins at the C terminus. We cloned the sequence expressed from LOC422304 from DT40 cells by RT-PCR and expressed a myc-tagged version of the longest ORF available in wild-type and *Cetn4/2/3*-deficient DT40 cells. As shown in Fig. S1 C, Myc-LOC422304p localized to centrosomes in wild-type cells but not in centrin-deficient cells, demonstrating that any centrosome localization of this gene product is dependent on endogenous centrin. Finally, as we saw no detectable signal in *Cetn4/2/3*-deficient DT40 cells using four different anti-centrin antibodies (Fig. 1 B), we conclude that LOC422304 does not encode a functional centrin.

We next monitored the proliferative capacity of centrin-deficient cells. As shown in Fig. 1 D, cells that lacked any centrin genes proliferated slightly more slowly than wild-type cells, with the *Cetn4/2/3*-deficient cells proliferating the slowest. However, this was not accompanied by any pronounced cell cycle delay, as determined by flow cytometry for BrdU incorporation (S phase) or histone H3 phosphorylation (M phase) and microscopy analysis of the proportion of the population undergoing cytokinesis (Fig. 1 E and not depicted). From these data, we concluded that centrins are dispensable for the viability of DT40 cells.

We then used microscopy to examine the impact of centrin deficiency on the centrosome. Using antibodies specific for the centriole components Cep135, Ninein, and Cep76, the PCM components pericentrin, γ-tubulin, and PCM1, and the centrosome-associated kinase Aurora A, we saw no impact of centrin deficiency on centrosome composition during interphase (Fig. 2 A). In mitosis, we observed apparently normal spindle pole organization and cytokinetic midbody formation in cells that lacked any or all of the chicken centrin isoforms (Fig. 2 B). To verify these light microscopy observations at higher resolution, we performed EM. As shown in Fig. 2 C, centrioles in centrin-deficient cells were composed of nine triplet microtubules and were arranged orthogonally to one another. Centriole diameters were 211.0 ± 13.6 nm ($n = 42$) in wild-type cells and 208.5 ± 17.0 nm ($n = 34$) in *Cetn4/2/3*-deficient cells, showing no impact of centrin deficiency on the characteristic ultrastructure of the centriole.

We also monitored the stages of centriole duplication in *Cetn4/2/3*-deficient cells using EM. Fig. 2 D shows that *Cetn4/2/3*-deficient cells can form a cartwheel, that the procentriole duplicates orthogonally to the mother, and that paired mother–daughter centrioles associate with one another. These analyses show that centrins are not essential for centrosome assembly.

To test whether centrins are necessary for centrosome function in the mitotic spindle, we used EM to visualize microtubule anchoring by centrin-deficient centrosomes. As shown in Fig. 3 A, centrin-deficient centrosomes can mature and form appendages that are capable of anchoring microtubules. Fig. 3 B shows that these microtubules establish links with mitotic chromosomes and establish mitotic spindles. Metaphase spindle lengths were 6.41 ± 0.59 µm ($n = 55$) in wild-type DT40 cells versus 6.14 ± 0.57 µm ($n = 53$) in *Cetn4/2/3*-deficient cells, showing no significant difference. We then examined the capacity of centrin-deficient centrosomes to organize microtubule regrowth after destabilization of the microtubules with combined cold and nocodazole treatment. We found that *Cetn4/2/3*-deficient cells were as capable as wild-type cells of nucleating microtubule asters (Fig. 3 C). Probably the most demanding test of microtubule nucleation is forming a functional mitotic spindle. Analysis of the percentages of wild-type and *Cetn4/2/3*-deficient cells that entered G1 after release from a nocodazole arrest, where we saw no difference, provided further evidence for the ability of centrin-deficient cells to nucleate microtubules efficiently (Fig. S3). For further analysis of mitosis, we used live cell imaging of cells stably transfected with an expression vector for histone H2B-RFP to define the duration of mitosis in wild-type and *Cetn4/2/3*-deficient cells. As shown in Fig. 3 D, the mean time taken from chromosome condensation to decondensation was 40.5 ± 10.6 min in wild-type cells and 40.2 ± 9.5 min in *Cetn4/2/3*-deficient cells. These data indicate no mitotic impact of centrin deficiency and allow us to conclude that centrosomes are fully functional during mitosis in the absence of centrin.

We next examined the ability of centrin-deficient cells to produce multiple centrosomes. We used hydroxyurea (HU), ionizing radiation (IR), and Cdk1 inhibition, conditions under which cells are known to overduplicate their centrosomes (Balczon et al., 1995; Sato et al., 2000; Hochegger et al., 2007; Kleylein-Sohn et al., 2007). We also tested the impact of UV irradiation and observed that centrosome amplification arose after this genotoxic stress (Fig. 4, A and B). We found that *Cetn4/2/3*-deficient cells amplified their centrosomes under all conditions tested (Fig. 4, A and B). To confirm that the structures we

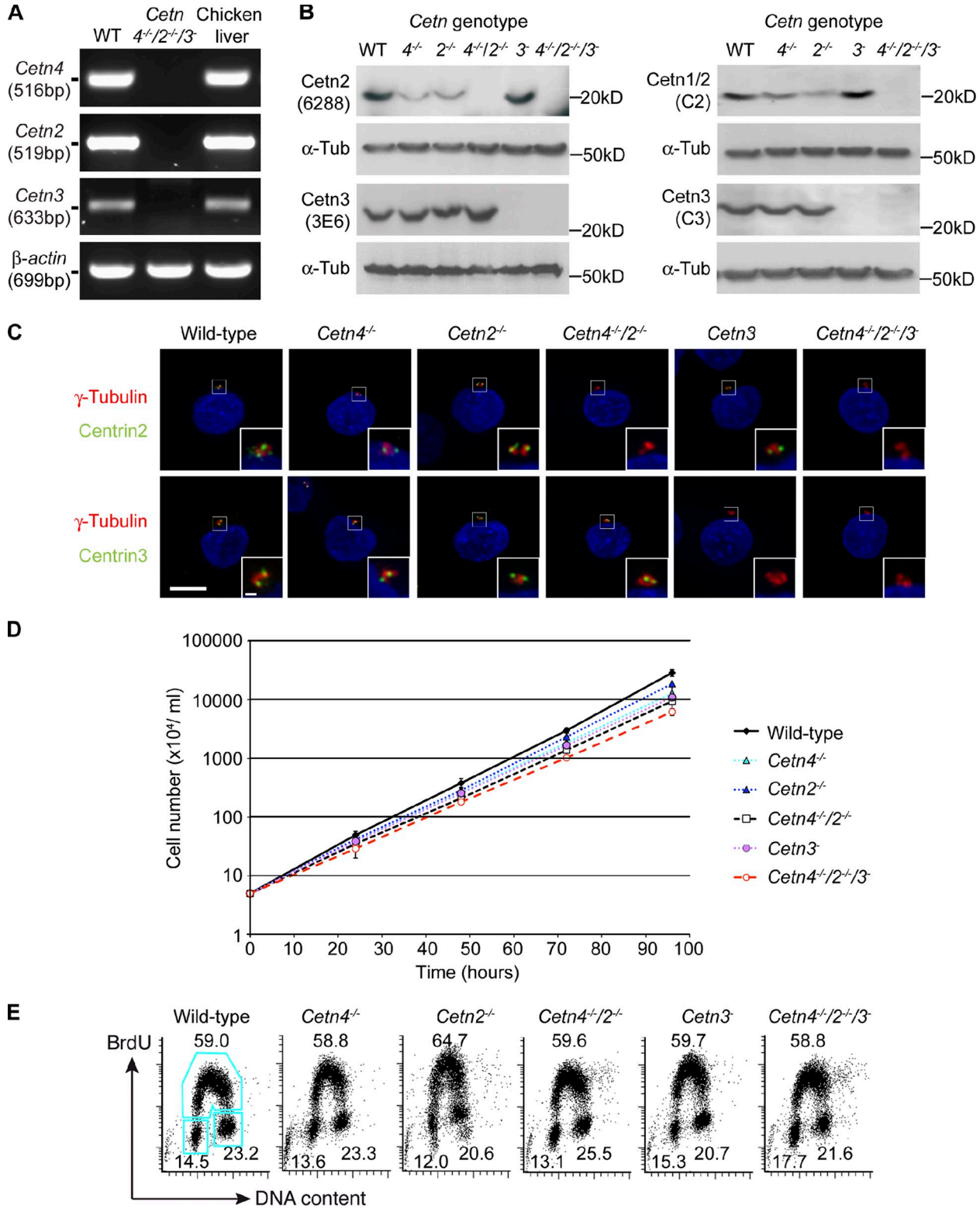

Figure 1. **Centrin-deficient DT40 cells are viable and show no proliferative defect.** (A) RT-PCR analysis of expression of the indicated genes in wild-type (WT) and centrin-deficient DT40 cells and in chicken liver. (B) Immunoblot of centrin in cells of the indicated genotype. Numbers refer to the centrin mutant in each lane. Antibodies used for these analyses are indicated. (C) Immunofluorescence microscopy analysis of wild-type and centrin-targeted DT40 cells stained with antibodies to the indicated centrin orthologue (green) with γ-tubulin (red) as a reference marker. Cells were counterstained with DAPI to visualize the DNA (blue) before imaging. Insets show magnifications as indicated in the main images. Bars: 5 µm; (inset) 0.5 µm. (D) Proliferation analysis of cells of the indicated genotype. Data points show mean ± SD of the results from at least three separate experiments. Doubling times were 7 h 42 min ± 28 min for wild type and 9 h 18 min ± 13 min for *Cetn4/2/3*-deficient cells. (E) FACS plot of cell cycle distributions in asynchronous cells of the indicated genotype. The G1 (bottom left), S (top), and G2/M (bottom right) gates are indicated in blue, and the numbers refer to the percentage of cells detected in each of the gates averaged from two separate experiments.

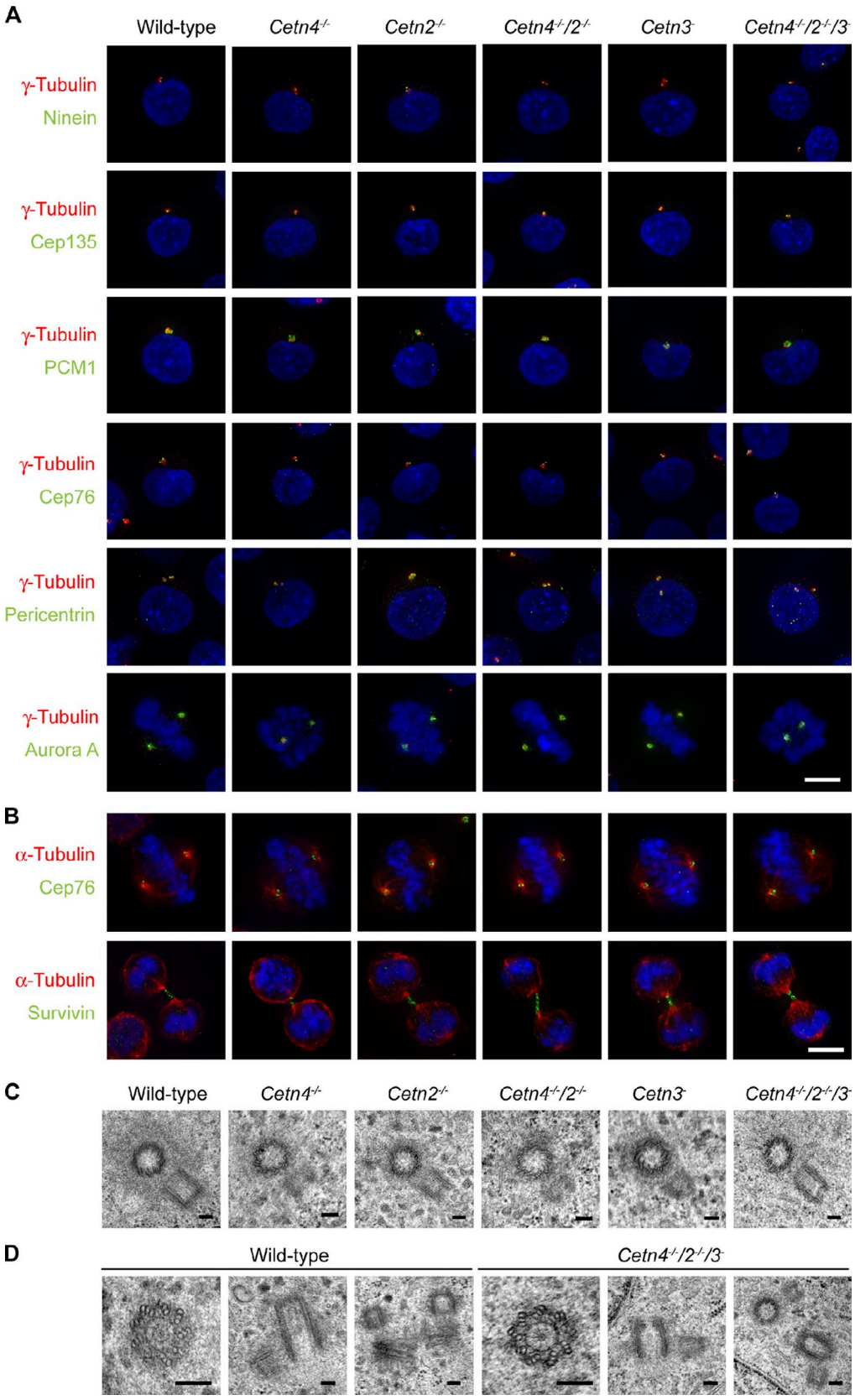

Figure 2. **Structural integrity of centrin-deficient centrosomes.** (A) Immunofluorescence microscopy analysis of wild-type and the indicated *Cetn*-targeted DT40 cells stained with antibodies to the indicated centrosome component (green) with γ-tubulin (red) as a reference marker. Cells were counterstained with DAPI to visualize the DNA (blue) before imaging. Bar, 5 μm. (B) Immunofluorescence micrograph of centrosomes in mitotic cells of the indicated genotype stained as in A but with α-tubulin (red) as the control. Bar, 5 μm. (C) Transmission electron micrographs of centrosomes in cells of the indicated genotype. Bars, 100 nm. (D) TEM comparison of different stages of centrosome cycle in wild-type and centrin-deficient cells. Bars, 100 nm.

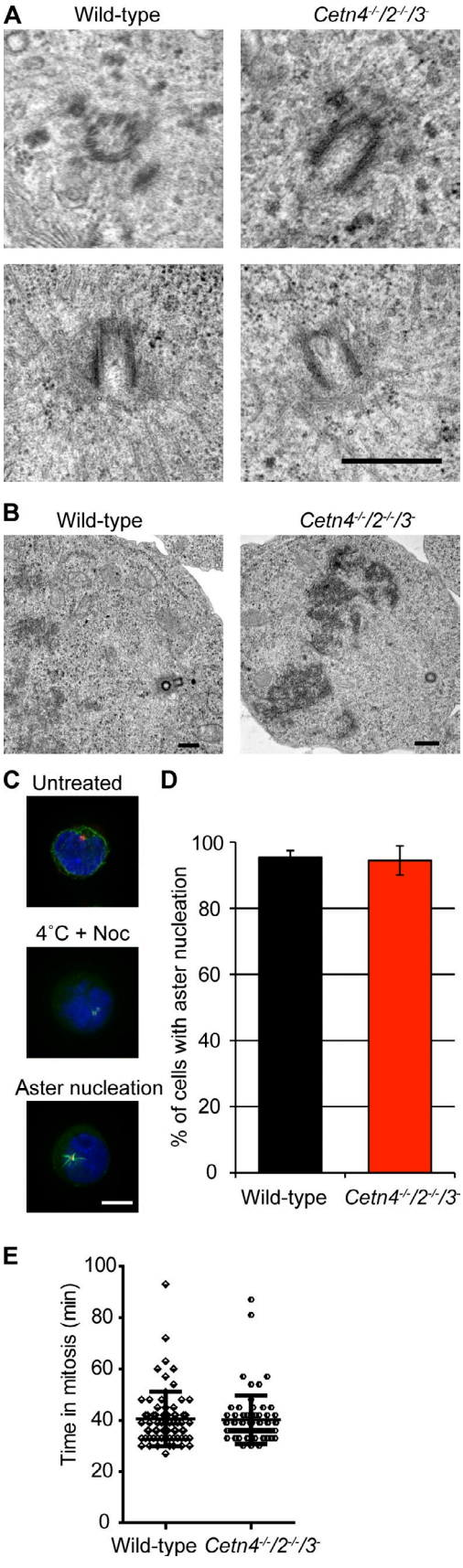

Figure 3. **Normal microtubule nucleation functions of centrin-deficient centrosomes.** (A) Transmission electron micrographs of microtubule anchorage at the centrosomes in cells of the indicated genotype. Bar, 500 nm. (B) Transmission electron micrographs of spindle microtubule association with chromosomes in cells of the indicated genotype. Bars, 500 nm. (C) Immunofluorescence microscopy analysis of microtubule nucleation in DT40 cells before and after release from 1-h arrest in 1 µg/ml nocodazole at 4°C. Cells were stained with antibodies to α-tubulin (green) with γ-tubulin (red) as a centrosomal marker. Cells were counterstained with DAPI to visualize the DNA (blue) before imaging. Bar, 5 µm. (D) Quantitation of the percentage of cells with aster nucleation after microtubule regrowth for 1 min at 39.5°C. Histogram shows mean ± SD of three separate experiments in which at least 200 cells per experiment were counted. (E) Duration of mitosis in centrin-deficient cells. Cells of the indicated genotypes that stably expressed histone H2B-RFP were imaged by time-lapse microscopy, and the time taken from chromosome condensation to decondensation was assessed. Data show mean ± SD for the 70 individual cells analyzed as data points.

counted were, in fact, centrosomes, we also performed EM analyses and observed multiple centrioles in both wild-type and *Cetn4/2/3*-deficient cells that had been irradiated (Fig. 4 C). These findings show that centrin levels are not limiting for centrosome amplification after DNA damage or cell cycle delay.

The elevated levels of centrosome amplification seen in centrin-deficient cells after irradiation suggested some problems in dealing with genotoxic stress. To determine whether centrin plays a role in DNA damage responses, we performed clonogenic survival assays. As shown in Fig. 5 A, *Cetn4/2/3*-deficient cells were no more sensitive than wild-type cells to IR. However, loss of *Cetn4* and *Cetn2* caused a pronounced sensitivity to UV treatment, which was exacerbated by the further loss of *Cetn3* (Fig. 5 B). Although no role for centrin3 in the repair of UV-induced DNA damage has been established, the known interaction of human centrin2 with XPC (Araki et al., 2001) suggests that this sensitivity arises from defective NER. Importantly, this hypersensitivity to UV irradiation was suppressed by transgenic expression of either *Cetn4* or *Cetn2* and partially rescued by *Cetn3* expression (Fig. 5 C), demonstrating a requirement for the chicken centrin2 orthologues and novel potential involvement of centrin3 in dealing with UV irradiation.

We next tested whether the sensitivity of centrin-deficient cells to UV treatment was caused by a defect in checkpoint activation. As shown in Fig. 6 A, the UV-responsive activation of Chk1, as determined by its phosphorylation, occurred with the same kinetics in wild-type and *Cetn4/2/3*-deficient cells. However, the resolution of this phosphorylation signal to normal levels took longer in *Cetn4/2/3*-deficient cells, with the bulk of the phosphorylation being removed by 12 h in wild-type cells, but not in centrin-deficient cells (Fig. 6 A). Cell cycle analysis of the response to UV showed a profile consistent with an attenuated resolution of DNA damage signaling, with the majority of wild-type cells showing an S phase delay by 9 h before accumulating with a 2C DNA content at 12 h and the *Cetn4/2/3*-deficient population showing the S phase delay only by 12 h (Fig. 6 B). An elevated level of cell death was also evident in the centrin-deficient population compared with wild-type cells from 18 h after treatment, consistent with the results obtained in the clonogenic survival assay. To examine the potential cause of the extended DNA damage signaling, we used an immunodot-blot experiment to monitor the kinetics with which DNA damage (CPDs) formed and was repaired after UV irradiation.

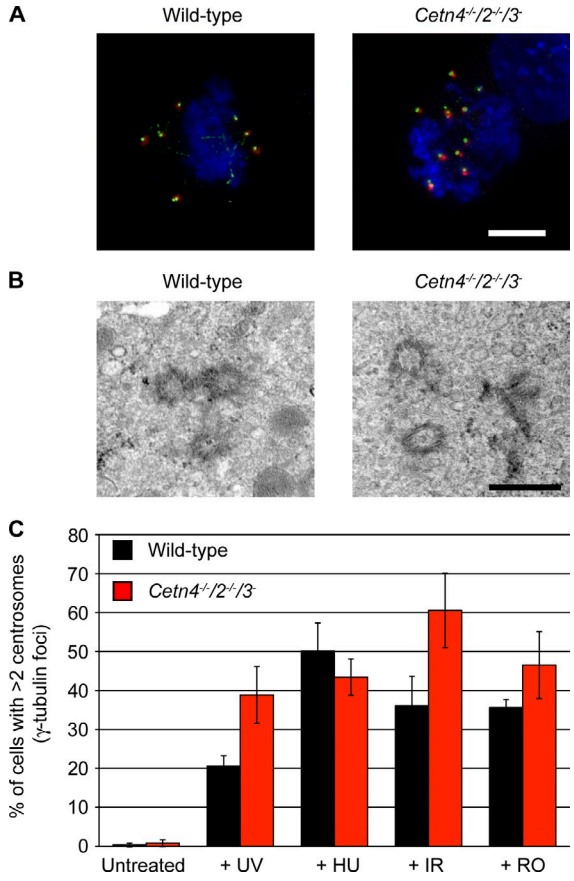

Figure 4. **Efficient DNA damage–induced centrosome amplification in centrin-deficient cells.** (A) Immunofluorescence microscopy analysis showing centrosome amplification in wild-type and the indicated *Cetn*-targeted DT40 cells stained with antibodies to glutamylated tubulin (green) and γ-tubulin (red) at 24 h after treatment with 10 Gy IR. Cells were counterstained with DAPI to visualize the DNA (blue) before imaging. Bar, 5 μm. (B) TEM comparison of amplified centrosomes in wild-type and centrin-deficient cells, seen 24 h after 10 Gy IR. Bar, 500 nm. (C) Quantitation of cells of the indicated genotype with aberrant centrosome numbers 24 h after 5-J/m^2 UV-C treatment (+UV) or 10 Gy IR (+IR) and after 24-h incubation with 4 mM HU (+HU) or 6 μM RO3306 (+RO). Histogram shows mean ± SD of three separate experiments in which at least 300 cells per experiment were counted.

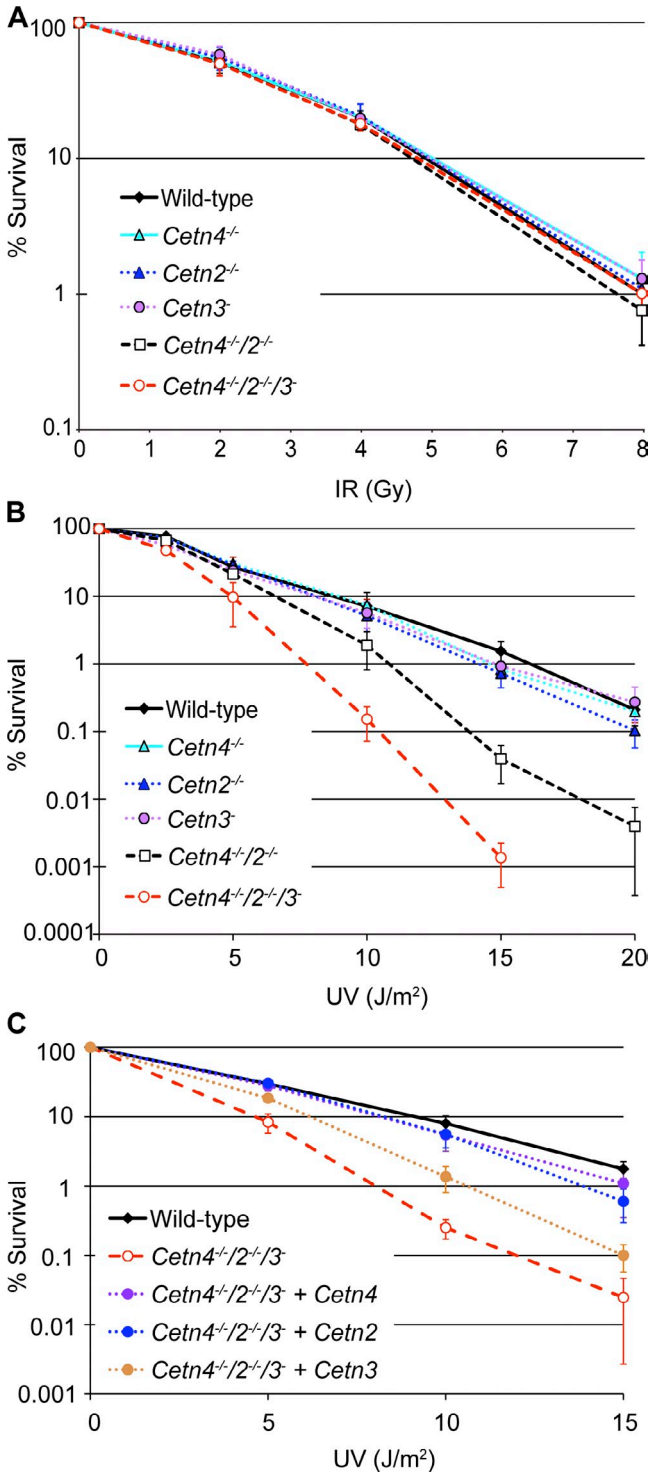

Figure 5. **Hypersensitivity to UV irradiation of centrin-deficient cells and rescue by expression of centrins 4, 2, or 3.** (A) Clonogenic survival assay of cells of the indicated genotype treated with the indicated doses of IR. Data points show mean ± SD of the surviving fractions in at least three separate experiments. (B) Clonogenic survival assay of cells of the indicated genotype treated with the indicated doses of UV-C irradiation. Data points show mean ± SD of the surviving fractions in at least three separate experiments. (C) *Cetn4/2/3*-deficient cells that were stably transfected with the indicated myc-centrin–expressing transgenes were analyzed by clonogenic survival assay. Survival curves show mean ± SD of the surviving fractions in at least three separate experiments.

As shown in Fig. 6 (C and D), UV treatment induces a rapid and massive increase in CPDs, returning to basal levels 24 h after irradiation. *Cetn4/2/3*-deficient cells are much slower to repair the UV-induced lesions, with a notable fraction of the induced damage remaining at 24 h after treatment. Notably, transgenic expression of any of the chicken centrins greatly improved the null cells' ability to repair UV-induced DNA damage (Fig. 6, C and D). These data demonstrate that vertebrate centrins are necessary for the efficient repair of UV-induced DNA damage and that all three centrins are potentially involved in such repair.

Discussion

The viability of centrin-deficient DT40 cells and the integrity of their centrosomes were unexpected, given the wide-ranging requirement for centrins in centriole/basal body duplication throughout evolution. Even though p53-dependent responses are blunted in DT40 cells, so that the p21–p53 checkpoint-driven

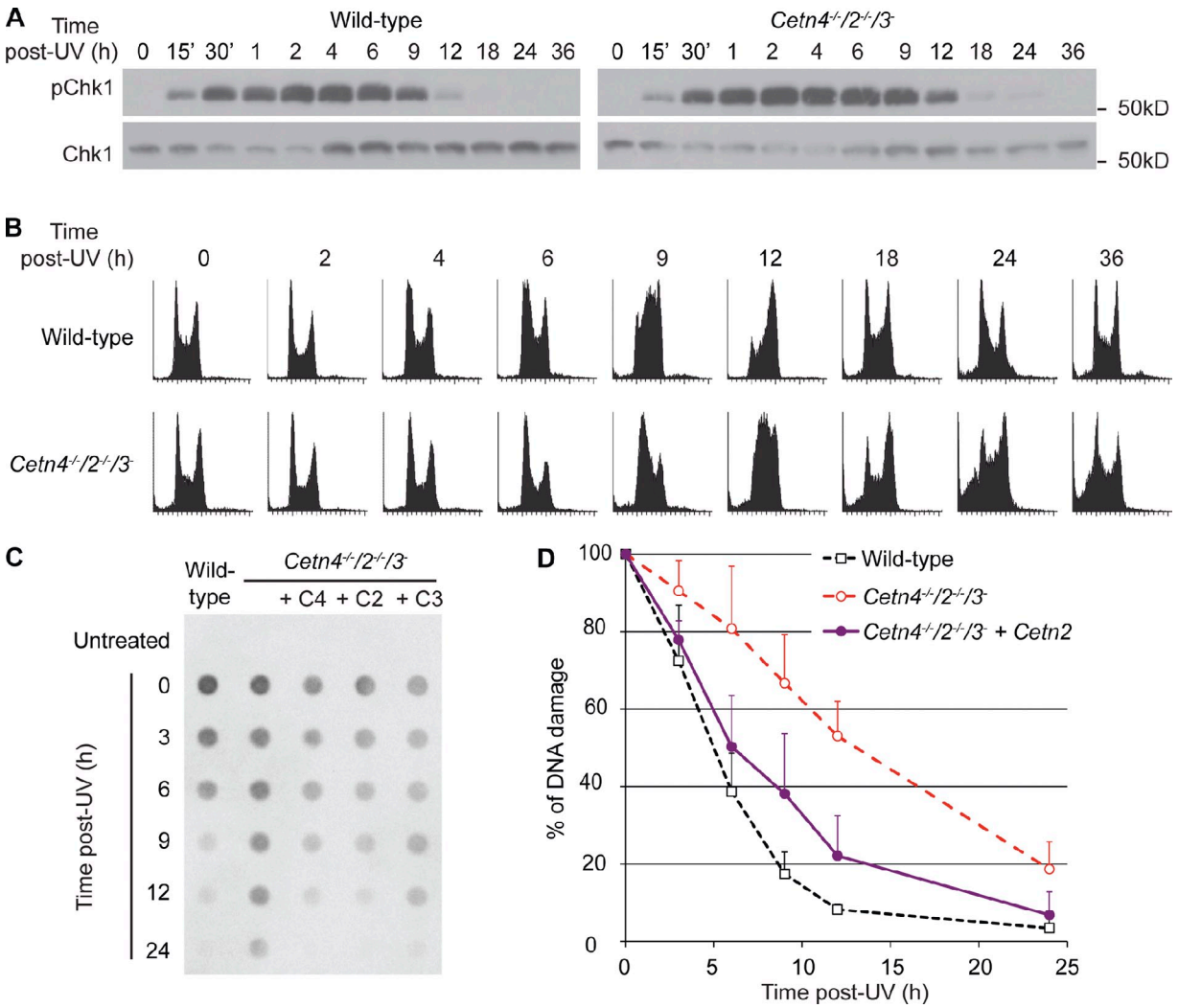

Figure 6. **Defective NER in centrin-deficient cells.** (A) Immunoblot analysis of Chk1 phosphorylation in cells of the indicated genotype was performed before or at different times after 3-J/m^2 UV-C treatment, using anti–phospho-Chk1 S345 with monoclonal mouse anti-Chk1 as loading control. (B) Flow cytometry analysis of the DNA content of wild-type and *Cetn4/2/3*-deficient cells before or at the indicated times after 3-J/m^2 UV-C treatment. (C) Immunodot-blot analysis of CPDs in genomic DNA from wild type, *Cetn4/2/3*-deficient cells, and *Cetn4/2/3*-deficient cells that were stably transfected with the indicated centrin-expressing transgenes (C4, C2, and C3) before or at the indicated times after 5-J/m^2 UV-C treatment. Time 0 is immediately after irradiation. (D) Quantitation of the kinetics of CPD repair in cells of the indicated genotype after 5-J/m^2 UV-C treatment. Data show mean ± SD of the CPD signal normalized to time 0 signals in at least three separate experiments in which each sample was blotted in triplicate.

arrest described after the loss of centrosome components (Mikule et al., 2007) was not anticipated, our prediction had been that we would see the inhibition of centriole duplication seen in RNAi experiments in HeLa cells (Salisbury et al., 2002). However, consistent with the robust centrosome structures and activities we saw in centrin-deficient DT40 cells, siRNA depletion of centrin2 in HeLa cells did not impact HsSAS-6 recruitment to nascent procentrioles or centriole duplication (Strnad et al., 2007; Yang et al., 2010), and similarly, Plk4-induced centriole duplication in U2OS cells occurred efficiently despite siRNA depletion of centrin2 or centrin3 (Kleylein-Sohn et al., 2007). The fact that we have used gene targeting to ablate centrin function allows us to exclude any potential artifact arising from incomplete gene knockdown or off-target effects. Our current data do not indicate a requirement for vertebrate centrins in centriole duplication or mitotic centrosome function.

It has been reported that HU-induced centrosome amplification was suppressed when XPC was overexpressed, and, thus, centrin sequestered in the nucleus of MDA-MB-231 cells (Acu et al., 2010). Furthermore, *Cetn2* knockdown prevented the centriole overduplication that is normally driven by the overexpression of nondegradable Mps1 kinase (Yang et al., 2010). Although we saw no evidence that centrin was rate limiting for the overduplication of centrosomes caused by several genotoxic treatments, a possible reason for the difference between our observations and those reported is that the movement of centrin between the cytoplasm and the nucleus may be involved in the generation of additional centrioles in response to certain conditions that do not use the original centrosome as a template, such as after HU treatment. A model in which nuclear centrin–containing precursors initially assemble in the nucleus has been suggested as one mechanism for centriole overduplication (Prosser et al., 2009), with such aggregates being termed "precentrioles" in the de novo

centriole assembly pathway that generates centrioles after laser ablation of the preexisting structures (La Terra et al., 2005). In terms of templated centriole duplication, we have previously suggested that DNA damage–induced centrosome amplification occurs during an extended G2 arrest (Dodson et al., 2004; Inanç et al., 2010). The elevated UV-induced centrosome amplification seen in centrin-deficient cells thus can be attributed to the extended DNA damage–induced cell cycle delay. The absence of any deficiency in the response to IR in centrin-null cells makes the IR-induced centrosome overamplification difficult to explain. Up-regulation of Cdk activity after IR is a potential mechanism of centrosome amplification (Bourke et al., 2010). As centrin can be phosphorylated in vitro by Cdk1 (Lutz et al., 2001), it is possible that the deletion of centrins may enhance the activity of Cdks on other substrates that drive centrosome overduplication.

An expanding body of work implicates the centrosome as a hub for DNA damage response signaling, with a wide range of DNA repair and checkpoint proteins localizing to the centrosome (Löffler et al., 2006). The sensitivity of centrin-deficient DT40 cells to UV and their defect in resolving CPDs are consistent with several studies linking centrin2 with NER through its interaction with XPC (Araki et al., 2001; Popescu et al., 2003; Nishi et al., 2005). Making this role of centrins more general, a *cdc31* mutant that affects Cdc31p binding of the yeast XPC orthologue, Rad4, is UV sensitive (Chen and Madura, 2008), and an interaction has been described between RAD4 and CENTRIN2 in *A. thaliana* (Liang et al., 2006). Nuclear localization of centrin2 is driven by XPC (Charbonnier et al., 2007). Centrin2 is modified by SUMO2/3, and its efficient binding to XPC and localization to the nucleus depend on SUMOylation activity (Klein and Nigg, 2009). A recent study by Acu et al. (2010) demonstrated that centrin2 relocalization to the nucleus after HU treatment is dependent on XPC. Furthermore, Acu et al. (2010) also observed delayed resolution of UV-induced 6–4 photoproducts where centrin2 was partially depleted in human MCF-7 cells using short hairpin RNA. As the knockdown of centrin2 was toxic in these cells, it was possible that the delayed resolution of DNA damage might not be attributable to an NER defect alone. However, the UV hypersensitivity and DNA repair deficiency seen in our centrin knockout cells provide robust evidence for a function of vertebrate centrins in NER. Interestingly, another centrosomal protein, Cep164, has also been implicated directly in NER (Pan and Lee, 2009), so further examination of the interplay between the centrosome and NER is warranted.

Four potential explanations for the high level of centrin conservation are compatible with our observations. The first is that there exists cell type– or developmental stage–specific requirements for centrins in centriole duplication. Differing effects of centrin2 overexpression were observed in various human cell lines (Yang et al., 2010), but the ubiquity of centrin2 and centrin3 expression suggests a more general role for the centrins. Second, a further possible centrosomal function for centrins is that of establishing a primary cilium. As DT40 cells are lymphocytes and thus do not bear primary cilia (Alieva and Vorobjev, 2004), we are unable to examine this activity directly. Although the absence of normal primary cilia in several human genetic disorders is not cell lethal (Goetz and Anderson, 2010), a role for centrins in ciliogenesis may nevertheless provide evolutionary pressure for centrin conservation in terms of the whole organism. A third possibility is that centrins play a predominantly regulatory role in the control of centriole duplication or functioning that is dispensable during the cell cycle. Ser170 phosphorylation of centrin2 occurs early during mitosis, and, when stimulated during interphase by increased protein kinase A activity, such phosphorylation was accompanied by centriole separation (Lutz et al., 2001). Centrin2 has been described as an Mps1 substrate, and recent work implicated the three Mps1 target sites identified in the control of centriole duplication (Yang et al., 2010). In keeping with a regulatory role, overexpression of centrin drives centriole overduplication (Yang et al., 2010). The final possibility for the evolutionary pressure that has maintained the high degree of conservation in centrin sequences is that their key role in the cell is not at the centrosome. Although centrins are sufficiently conserved to have been present in the last common ancestor of the eukaryotes (Hodges et al., 2010), their presence in other ancient cellular structures such as the nuclear pore may indicate coevolution with macromolecular complexes other than the centrosome (Neumann et al., 2010). Centrin activities in NER and the observation that the bulk of the cellular centrin is not at the centrosome (Paoletti et al., 1996) provide support for this view. Gene-targeting experiments in mammals may allow the resolution of these various possibilities in the entire organism.

Materials and methods

Cloning

Genomic sequences for the *Cetn*-targeting arms were amplified by PCR with KOD Hot Start (EMD), cloned into pGEM-T Easy (Promega), and sequenced. The following primer sets and strategies were used: *Cetn4* 5′ arm (5′-GCACAGGCTTGCTGAAAGGATGCACTGC-3′ and 5′-CTGCTTGCGGCCGCACCGCGCCGGGATC-3′) and 3′ arm (5′-GGATCCGATTTGTTGCCTTACAGCTGTTCC-3′ and 5′-CAGTATCCATTCCAGCCTTGCCTTGAGT-3′). An NdeI–ScaI fragment containing the 5′ arm was cloned using AatII–ScaI sites upstream of the 3′ arm and histidinol and blasticidin resistance cassettes inserted into a BamHI site. PvuI and SalI digests were used for linearization of the completed targeting vectors.

We also used *Cetn2* 5′ arm (5′-CCTAAGAATCCGGAGCCACCAATCA-3′ and 5′-TGCTCAACACTACCCTTCCCCATTG-3′) and 3′ arm (5′-GGATCCGTTGCTTGTTTGCTGCGCTCGTAAG-3′ and 5′-GTCGACTGCCTTTATTCACCTCACGCACAGG-3′). A NotI–BamHI fragment containing the 5′ arm was cloned with a BamHI–SalI fragment containing the 3′ arm into pBluescript II SK. LoxP-puromycin (Arakawa et al., 2001) and hygromycin resistance cassettes were inserted into the BamHI site, and a NotI digest was used for linearization.

Lastly, we used *Cetn3* 5′ arm (5′-CTTTGTGCAACGGCTCATTGTC-3′ and 5′-GGATCCAGACTACTGCGCTGCCACTCAAG-3′) and 3′ arm (5′-GGATCCGCAGCTTGACCAGGAGTACTTTG-3′ and 5′-TGAAGGGTAAACTCAGAATGACAGA-3′). The 5′ arm was cloned as a SpeI–BamHI fragment upstream of the 3′ arm in pGEM-T Easy. LoxP-puromycin and loxP-neomycin resistance cassettes were cloned into the BamHI site, and MluI digests were used for linearization.

For cDNA cloning, RNA was extracted from DT40 cells and chicken liver using TRI reagent (Invitrogen). Reverse transcription was performed using SuperScript First-Strand (Invitrogen) and PCR with KOD Hot Start. cDNAs were cloned into the pGEM-T Easy, sequenced, and then subcloned into pCMV-3Tag-2 (Agilent Technologies). The primers used to amplify cDNA were as follows: *Cetn4* (5′-CGGAATTCGATGGCGTCCAACTATAGA-3′ and 5′-CGGGATCCCTAATATAAGCTCGTTTTCTT-3′), *Cetn2* (5′-GAAGATCTTCTATGGCCTCCAGCTTCAAGAAG-3′ and 5′-CGGGATCCTCAGTAAAGGCTGGTCTTCTTC-3′), *Cetn3* (5′-CGGAATTCGATGAGCGTGTGAAGGGG-3′ and 5′-CGGGATCCTTAAATATCTCCAGTCATAATAG-3′), and *LOC422304* (5′-GAATTCTATGGTGAGGCCCGCTGGGCAC-3′ and 5′-TCTAGATTACAGGTCAGTCAATGTCAGAATC-3′). All expression constructs, including

pmRFP-N1-H2B (Dodson et al., 2007), were linearized with MluI or ApaLI before transfection into DT40 cells.

Cell culture and gene targeting

DT40 cells were cultured as described in RPMI media (Lonza) and supplemented with 10% fetal calf serum (Lonza), 1% chicken serum (Sigma-Aldrich), and 1% penicillin/streptomycin (Sigma-Aldrich) at 39.5°C with 5% CO_2 (Takata et al., 1998). Electroporations for gene targeting were performed using 15 µg of linearized and purified DNA and 300 V/600 µF or 550 V/25 µF in a GenePulser (Bio-Rad Laboratories), as previously described (Takata et al., 1998). Cells were transiently transfected with 5 or 15 µg endotoxin-free plasmid DNA using nucleofection (program B-23; Lonza). Clonogenic survival assays were performed as described previously (Takata et al., 1998), using irradiation with a ^{137}Cs source at 23.5 Gy/min (Mainance Engineering). For UV-C irradiation, cells were irradiated in PBS using a 254-nm UV-C lamp at 23 J/m^2/min (NU-6 lamp; Benda). Unless otherwise stated, cell culture reagents were purchased from Sigma-Aldrich. Cdk1 Inhibitor IV (RO-3306; EMD) was dissolved in DMSO. Selection with antibiotics was with 1 mg/ml histidinol, 25 µg/ml blasticidin (InvivoGen), 2.5 mg/ml hygromycin (InvivoGen), 0.5 µg/ml puromycin, and 2 mg/ml geneticin (Invitrogen).

DT40 cells were targeted sequentially to generate *Cetn4*$^{-/-}$, *Cetn4*$^{-/-}$ *Cetn2*$^{-/-}$, and finally the triple knockout *Cetn4*$^{-/-}$*Cetn2*$^{-/-}$*Cetn3*$^{-}$. A separate triple knockout was generated after transiently expressing Cre recombinase (Arakawa et al., 2001) in *Cetn4*$^{-/-}$*Cetn2*$^{-/-}$ cells to remove the puromycin resistance cassette. The *Cetn3* locus was then targeted with the puromycin construct.

Immunofluorescence microscopy

Cells were left to attach to poly-L-lysine–coated slides for 15 min and then plunged into 95% methanol with 5 mM EGTA (prechilled to –20°C) for 10 min. Alternatively, cells were fixed in 4% PFA for 10 min and permeabilized in 0.15% Triton X-100 in PBS for 2 min. Thereafter, the cells were blocked in 1% BSA in PBS and incubated with primary antibodies for 1 h at 37°C followed by a 45-min incubation at 37°C with secondary antibodies. Secondary antibodies were labeled with FITC and Texas red (Jackson ImmunoResearch Laboratories, Inc.). Slides were mounted with DABCO (2.5% DABCO [Sigma-Aldrich], 50 mM Tris-HCl, pH 8, and 90% glycerol) and supplemented with 1 µg/ml DAPI. Cells were imaged at 37°C on an integrated microscope system (DeltaVision) controlled by SoftWorx software (Applied Precision) mounted on a microscope (IX71; Olympus) with a PlanApo N100× oil objective (NA 1.40). Images were taken using a camera (CoolSnap HQ2; Photometrics) and deconvolved in SoftWorx using the ratio method, and maximal intensity projections were saved as Photoshop CS version 8.0 files (Adobe Systems). Cell counting was performed with a microscope (BX51; Olympus), using 60× oil (NA 1.4) and 100× oil (NA 1.35) objectives.

The primary antibodies used in this study and their dilutions were as follows: mouse monoclonal anti–Aurora A (35C1; Abcam) at 1:500; rabbit polyclonal anti-centrin2 (poly6288; BioLegend) at 1:250; monoclonal anti-centrin3 (3E6; Abnova) at 1:1,000; rabbit anti-Cep76 (a gift from W. Tsang and B. Dynlacht, New York University School of Medicine, New York, NY; Tsang et al., 2009) at 1:200; mouse anti-Cep135 (a gift from R. Kuriyama, University of Minneapolis, Minneapolis, MN; Ohta et al., 2002) at 1:1,000; mouse anti–myc 9E10 at 1:1,000; rabbit polyclonal anti-ninein (ab4447; Abcam) at 1:100; rabbit polyclonal anti–PCM-1 (817; a gift from A. Merdes, Centre National de la Recherche Scientifique and Laboratoires Pierre Fabre, Toulouse, France; Dammermann and Merdes, 2002) at 1:5,000; rabbit polyclonal anti-pericentrin (ab4448; Abcam) at 1:100; mouse monoclonal anti–α-tubulin (B512; Sigma-Aldrich) at 1:2,000; mouse monoclonal anti–γ-tubulin (GTU88; Sigma-Aldrich) at 1:150; anti–γ-tubulin (C-20; Santa Cruz Biotechnology, Inc.) at 1:250; mouse monoclonal anti-glutamylated tubulin (GT335; a gift from C. Janke, Institut Curie, Paris, France; Wolff et al., 1992) at 1:300; and rabbit polyclonal anti-survivin (WCE43D; a gift from B. Earnshaw, Wellcome Trust Centre for Cell Biology, Edinburgh, Scotland, UK; Yue et al., 2008) at 1:300. Labeled secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc.

Live cell imaging

For live cell imaging, cells that stably expressed H2B-RFP were allowed to attach to poly-D-lysine–coated dishes (MatTek) for 2–3 h, and the media were supplemented with 12.5 mM Hepes, pH 7.5. Images were taken every 3 min for 3 h on an integrated microscope system (DeltaVision) using a PlanApo N60× oil objective (NA 1.42) and a 39.5°C environmental chamber (WeatherStation; Precision Control).

Microtubule regrowth assays

DT40 cultures were treated with 1 µg/ml nocodazole for 3 h, incubated for an additional hour on ice, and subsequently spun down at 250 *g* for 5 min and washed three times in ice-cold PBS for 15 min each. Cells were adhered to poly-L-lysine slides for 30 min at 4°C, submerged in PBS/1% FCS at 40°C for 1 min, and immediately fixed in methanol. Immunofluorescence microscopy with antibodies to α- and γ-tubulin was then used to assess microtubule growth.

EM

DT40 cells were processed for transmission EM (TEM) using an established protocol (Liptrot and Gull, 1992) in which they were pelleted at 250 *g* for 5 min and fixed with a combination of 2% glutaraldehyde and 2% PFA in 0.1 M cacodylate buffer. After posfixation in a solution of 2% osmium tetroxide/0.1 M cacodylate buffer, pH 7.2, cell pellets were dehydrated through a graded series of ethanol (30, 60, 90, and 100%) that was then replaced by propylene oxide. Subsequently, cell pellets were embedded in Agar Low Viscosity Resin. Sections were cut on a microtome (Reichert-Jung Ultracut E; Leica), stained with uranyl acetate and lead citrate, and then viewed on an electron microscope (H-7000; Hitachi). Images were taken with a camera (ORCA-HRL; Hamamatsu Photonics) and processed using AMT version 6 (AMT Imaging).

Immunoblotting experiments

Whole-cell extracts were prepared with radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, and protease inhibitor cocktail). Proteins were transferred to nitrocellulose membranes for analysis using the following primary antibodies: rabbit anti-centrin2 (poly6288; BioLegend) at 1:1,000; monoclonal anti-centrin3 (3E6; Abnova) at 1:1,500; rabbit anti-centrin2 and anti-centrin3 at 1:1,500 and 1:250, respectively (C2 and C3; gifts from M. Bornens, Institut Curie, Paris, France; Laoukili et al., 2000); anti–phospho-Chk1 S345 (Cell Signaling Technology) at 1:500; anti-Chk1 (Cell Signaling Technology) at 1:1,000; anti-thymine dimer antibody (H3/ab10347; Abcam) at 1:1,000; and anti–α-tubulin (B512; Sigma-Aldrich) at 1:5,000.

Flow cytometry

Cells were fixed with 70% ice-cold ethanol overnight at 4°C, washed in PBS, and incubated in 40 µg/ml propidium iodide and 200 µg/ml RNase A in PBS for 1 h. Where indicated, cells were cultured with 20 µM 5-bromo-2′-deoxydine for 15 min. After fixation in 70% ice-cold ethanol overnight at 4°C, cells were washed in PBS with 1% BSA, treated with 2 M HCl/0.5% Triton X-100 for 30 min at 37°C, and washed again. Cells were then incubated with 30% anti-BrdU (B44; BD) in PBS with 1% BSA/0.5% Triton X-100 for 1 h with shaking at 37°C followed by washing and incubation with FITC-conjugated anti–mouse (Jackson ImmunoResearch Laboratories, Inc.) at 1:50 in PBS with 1% BSA. Finally, cells were resuspended in propidium iodide/RNase A solution as described previously. Flow cytometry was performed on a FACSCalibur (BD).

Immunodot-blot analysis

Cells were harvested after 3-J/m^2 UV-C irradiation and washed in PBS, and genomic DNA was extracted using a Genomic DNA Miniprep kit (Sigma-Aldrich). DNA was quantified using a spectrophotometer (NanoDrop 2000c; Thermo Fisher Scientific). Triplicates of 250 ng DNA from each time point were denatured in 0.3 M NaOH at 60°C for 1 h, cooled on ice, and neutralized in 2× salt–sodium citrate buffer. Samples were then transferred to a nylon membrane (Hybond-N; GE Healthcare) using a microfiltration dot-blot apparatus (model 170-6545; Bio-Rad Laboratories). The membrane was then baked at 80°C for 1 h, immunoblotted with thymine dimer antibody, and visualized with HRP-conjugated secondary antibody. Signals were acquired using an Imager (LAS-3000; Fujifilm), quantified with Multi-Gauge v.2.2 (Fujifilm), and normalized to time 0 after UV-C irradiation.

Online supplemental material

Fig. S1 shows the conservation of the centrin proteins between human and chicken and the analysis of LOC422304. Fig. S2 shows the gene-targeting strategies for the three chicken centrins with their respective diagnostic Southern blots and RT-PCR results. Fig. S3 shows the integrity of spindle assembly checkpoint and robust recovery in *Cetn*-deficient cells after nocodazole treatment and washout. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201012093/DC1.

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