Mutations in *CDCA7* and *HELLS* that respectively encode a CXXC-type zinc finger protein and an SNF2 family chromatin remodeler cause immunodeficiency, centromeric instability, and facial anomalies (ICF) syndrome types 3 and 4. Here, we demonstrate that the classical nonhomologous end joining (C-NHEJ) proteins Ku80 and Ku70, as well as HELLS, coimmunoprecipitated with CDCA7. The coimmunoprecipitation of the repair proteins was sensitive to nuclease treatment and an ICF3 mutation in CDCA7 that impairs its chromatin binding. The functional importance of these interactions was strongly suggested by the compromised C-NHEJ activity and significant delay in Ku80 accumulation at DNA damage sites in *CDCA7*- and *HELLS*-deficient HEK293 cells. Consistent with the repair defect, these cells displayed increased apoptosis, abnormal chromosome segregation, aneuploidy, centrosome amplification, and significant accumulation of $\gamma$H2AX signals. Although less prominent, cells with mutations in the other ICF genes *DNMT3B* and *ZBTB24* (responsible for ICF types 1 and 2, respectively) showed similar defects. Importantly, lymphoblastoid cells from ICF patients shared the same changes detected in the mutant HEK293 cells to varying degrees. Although the C-NHEJ defect alone did not cause CG hypomethylation, CDCA7 and HELLS are involved in maintaining CG methylation at centromeric and pericentromeric repeats. The defect in C-NHEJ may account for some common features of ICF cells, including centromeric instability, abnormal chromosome segregation, and apoptosis.
CDCA7 and HELLS mutations undermine nonhomologous end joining in centromeric instability syndrome

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Mutations in CDCA7 and HELLS that respectively encode a CXXC-type zinc finger protein and an SNF2 family chromatin remodeler cause immunodeficiency, centromeric instability, and facial anomalies (ICF) syndrome types 3 and 4. Here, we demonstrate that the classical nonhomologous end joining (C-NHEJ) proteins Ku80 and Ku70, as well as HELLS, coimmunoprecipitated with CDCA7. The coimmunoprecipitation of the repair proteins was sensitive to nuclease treatment and an ICF3 mutation in CDCA7 that impairs its chromatin binding. The functional importance of these interactions was strongly suggested by the compromised C-NHEJ activity and significant delay in Ku80 accumulation at DNA damage sites in CDCA7- and HELLS-deficient HEK293 cells. Consistent with the repair defect, these cells displayed increased apoptosis, abnormal chromosome segregation, aneuploidy, centrosome amplification, and significant accumulation of γH2AX signals. Although less prominent, cells with mutations in the other ICF genes DNMT3B and ZBTB24 (responsible for ICF types 1 and 2, respectively) showed similar defects. Importantly, lymphoblastoid cells from ICF patients shared the same changes detected in the mutant HEK293 cells to varying degrees. Although the C-NHEJ defect alone did not cause CG hypomethylation, CDCA7 and HELLS are involved in maintaining CG methylation at centromeric and pericentromeric repeats. The defect in C-NHEJ may account for some common features of ICF cells, including centromeric instability, abnormal chromosome segregation, and apoptosis.

Introduction

Immunodeficiency, centromeric instability, and facial anomalies (ICF) syndrome is a rare autosomal recessive disorder characterized by reduced immunoglobulin levels in the serum and recurrent infection (1). ICF patients possess naive B cells but lack mature B cells in the peripheral blood, the former of which may bear immune receptors with long complementarity-determining region 3’s (CDR3s) composed of full-length diversity (D) gene segments (2). Centromeric instability manifests as stretched heterochromatin, chromosome breaks, and multiradial configurations involving the centromeric regions of chromosomes 1, 9, and 16 in activated lymphocytes (3). The cytological defects are accompanied by CG hypomethylation in pericentromeric satellite-2 and -3 repeats of these chromosomes. Approximately half of ICF patients have mutations in the DNA methyltransferase 3B (DNMT3B) gene (ICF syndrome type 1: ICF1, OMIM #242860) (4–6). In ICF1 cells, CG hypomethylation is observed in pericentromeric repeats and subtelomeric regions, the latter of which are often abnormally short and vulnerable to DNA damage (7–11). In contrast, the remaining ICF patients show hypomethylation in pericentromeric repeats and centromeric α-satellite repeats (12), but not in subtelomeric regions (13), and are classified as type 2 (ICF2, OMIM #614069), type 3 (ICF3, OMIM #616910), type 4 (ICF4, OMIM #616911), and type X (ICFX). ICF2, ICF3, and ICF4 patients possess mutations in the zinc finger and BTR domain containing 24 (ZBTB24), cell division cycle associated 7 (CDCA7), and helicase lymphoid specific (HELLS) genes, respectively (14–17). The causative gene for ICFX remains unknown. A recent study also identified other regions that distinguish ICF1 from ICF2, 3, and 4 patients by CG methylation status (18).

Among the ICF2–4 genes, ZBTB24 encodes a protein with a BTR domain, an AT hook, and eight C2H2-type zinc finger motifs. CDCA7 encodes a protein with four CXXC-type zinc finger motifs, while HELLS (also known as LSH, PASG, or SMARCA6) encodes a protein with an SNF2 family ATPase domain. It has been reported that ZBTB24 acts as a transcriptional activator of CDCA7 (19). Furthermore, Xenopus Cdc2a7e, an egg-specific paralog of Cdc2a7, recruits Hells directly to chromatin and supports its nucleosome remodeling activity (20). These findings suggest that the 3 proteins work in the same biological pathway. Among these, HELLS, together with its homolog (DDM1) in Arabidopsis, is involved in DNA methylation, perhaps through its nucleosome remodeling activity that opens histone H1-containing heterochromatin (20–26). HELLS also promotes the efficient repair of DNA double-strand breaks (DSBs) (27). Perhaps because of this, murine embryonic fibroblasts with a Hells mutation display excessive
numbers of centrosomes and abnormal mitosis (28). Mouse mutants homozygous for a *Hells* deletion die soon after birth (29), and their hematopoietic cells poorly contribute to T and B cells in recipient mice (30). In contrast, the involvement of CDCA7 in ICF pathology and regulation of DNA methylation is poorly understood.

Here, we report that, in human embryonic kidney (HEK) 293T cells, CDCA7 interacts with HELLS, the classical nonhomologous end joining (C-NHEJ) proteins Ku80 (XRCC5 or Ku86) and Ku70 (XRCC6), and phosphorylated H2AX (γH2AX, a DSB marker) in an ICF mutation–sensitive manner. Various cytological and molecular changes that likely resulted from the defect in DNA repair were observed in *CDCA7* and *HELLS* mutant HEK293 cells and also in DNMT3B and ZBTB24 mutant cells. Furthermore, CDCA7 or HELLS deficiency caused a C-NHEJ defect and delay in Ku80 accumulation at DNA damage sites. Our results suggest that the defect in C-NHEJ accounts for some of the common features of ICF cells, including instability of satellite repeats, abnormal chromosome configuration, reduced proliferation rate, and apoptosis.

### Results

**HELLS and C-NHEJ proteins coimmunoprecipitate with CDCA7.** To understand the molecular function of CDCA7, we attempted to identify proteins that potentially interact with CDCA7 in HEK293T cells. To this end, we prepared expression vectors for FLAG-tagged WT CDCA7 and mutant (R274C) protein (FLAG-CDCA7 WT and R274C, respectively). This ICF3 mutation is located in the zinc finger domain (ref. 15 and Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/JCI99751DS1), and a corresponding amino acid substitution in *Xenopus* Cdca7e attenuates its DNA and chromatin binding (20). Endogenous proteins that coimmunoprecipitated with FLAG-CDCA7 WT and/or R274C proteins were identified by mass spectrometry. Table 1 provides a list of proteins that coimmunoprecipitated with FLAG-CDCA7 regardless of the mutation (peptide number ≥ 5, R274C/WT ≥ 0.6, n = 17). An extended list (peptide number ≥ 2, n = 36) is available in Supplemental Table 1. The list included HELLS and 14–3–3 proteins, which are the known interactors of CDCA7 (20, 31). These data support the validity of our experiment. Other notable proteins in the list were linker histones H1.4 and H1.3, as the homolog of HELLS in *Arabidopsis* (DDM1) is known to open H1-containing heterochromatin for DNA methylation (25).

Table 2 shows proteins that coimmunoprecipitated with FLAG-CDCA7 in an ICF3 mutation–sensitive manner (peptide number ≥ 5, R274C/WT < 0.6, n = 11). An extended list (peptide number ≥ 2, n = 50) is shown in Supplemental Table 2. Consistent with the reported mutation-sensitive interaction of *Xenopus* Cdca7e with nucleosomes (20), the list included core histones (H3.1, H4, and H2B1C). Intriguingly, the list also included Ku80 and PRKDC (the catalytic subunit of DNA-dependent protein kinase [DNA-PK]), which are involved in C-NHEJ, V(DJ) recombination, and immunoglobulin class switch recombination (32–35). Although the peptide number was below our cutoff level, Ku70, which forms a heterodimer with Ku80, and H2AX, of which the phosphorylated form (γH2AX) is a DSB marker, also coimmunoprecipitated with FLAG-CDCA7 in a mutation-sensitive manner. In addition, chromatin remodelers involved in DSB repair were included in the list. They included SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 5 (SMARCA5); SPT16 homolog, facilitates chromatin remodeling subunit (SUPT16H); and structure specific recognition protein 1 (SSRP1). The latter two form the facilitates chromatin transcription (FACT) complex (36, 37). These results provided a hint that CDCA7 might have a role in DNA repair, especially DSB repair.

We also determined proteins that potentially interact with FLAG-tagged WT and/or mutant (Q699R) HELLS proteins (FLAG-HELLS WT and Q699R). The mutation corresponded to the only amino acid substitution identified in ICF4 patients and is located in the helicase C-terminal domain (Supplemental Figure 1 and ref. 15). Only histone H2A1A coimmunoprecipitated with FLAG-HELLS (peptide number ≥ 5), and this interaction was mutation-sensitive (Supplemental Tables 3 and 4).

The coimmunoprecipitation of the above proteins was confirmed by Western blotting in the presence and absence of benzonase nuclease, which cleaves nucleosome-free DNA and RNA. Endogenous HELLS coimmunoprecipitated with both FLAG-CDCA7 WT and _R274C, regardless of the treatment (Figure 1). Likewise, CDCA7 coimmunoprecipitated with both FLAG-HELLS WT and Q699R, albeit with a lower efficiency, perhaps reflecting stoichiometry (Supplemental Figure 2A). In contrast, the coimmunoprecipitation of Ku80, Ku70, SMARCA5, SUPT16H, histone H3, and H2AX with FLAG-CDCA7 was sensitive to the R274C substitution, consistent with the mass spectrometry data (Figure 1 and Supplemental Table 2). Notably, Western blotting using a modification-specific antibody revealed the mutation-sensitive coimmunoprecipitation of γH2AX. Importantly, the coimmunoprecipitation of Ku proteins with FLAG-CDCA7 was sensitive to benzonase, but not RNase A, suggesting that the interaction involves DNA (Figure 1 and Supplemental Figure 2B). In summary, the findings indicated that CDCA7 interacts with HELLS, and
that this interaction is resistant to R274C substitution and nuclelease treatment. However, the interaction of CDCA7 with SMARCA5, SUPPT16H, histone H3, and γH2AX is clearly mediated by the zinc finger domain and may or may not involve DNA. In contrast, the interaction of CDCA7 with Ku proteins involves both the zinc finger domain and DNA.

To exclude the possibility that the observed interactions were due to CDCA7 overexpression, we prepared two HEK293 stable clones expressing FLAG-CDCA7 WT and R274C at near-endogenous levels and confirmed the commounoprecipitation of some of the proteins (Supplemental Figure 2C). Furthermore, we excluded the possibility of artificial interaction of abundant Ku proteins with CDCA7 via DNA fragments generated during the protein extraction procedure. We combined lysates prepared separately from FLAG-CDCA7 WT– and GFP-Ku80–expressing cells, performed immunoprecipitation with anti-FLAG antibody, and confirmed negligible commounoprecipitation (Supplemental Figure 2D). Finally, we examined whether the presence of DSBs had any effect on the interaction between CDCA7 and HELLs. The amount of HELLs that commounoprecipitated with FLAG-CDCA7 WT apparently increased upon bleomycin treatment (Supplemental Figure 2E). However, the level of HELLs protein, but not its mRNA, increased upon bleomycin treatment (Supplemental Figure 2E), likely accounting for the increased interaction. Interestingly, this upregulation was abrogated when CDCA7 was depleted (Supplemental Figure 2F).

**Mutations in ICF genes cause defects in cell proliferation and chromosome segregation.** We generated HEK293 cells carrying a mutation in each of the 4 causative genes by genome editing. We chose HEK293 cells because they express all ICF causative genes at relatively high levels. We obtained 2 null mutant clones for each ICF gene by targeting exons mutated in patients (15, 16) (Supplemental Table 5 and Supplemental Figure 1). Since HEK293 cells are hypotriploid (chromosome numbers 62–70) (38), some mutant cells had 3 mutated alleles. We also tried to derive Ku80 mutant cells and obtained only 1 hypomorphic clone (mut24) that possessed 2 alleles, each carrying a frameshift followed by a premature stop codon, with the third allele carrying amino acid substitutions at consecutive positions (K183E, G184R) (Supplemental Table 5 and Supplemental Figure 1). The predicted truncated CDCA7, HELLs, and Ku80 proteins were not detected by Western blotting and therefore seemed unstable (Figure 2A and Supplemental Figure 3). In many mutant lines, the mRNA level of the targeted gene decreased to below 60% of the WT level (Figure 2B), possibly as a result of nonsense-mediated mRNA decay (39). Consistent with the recent report that ZBTB24 is a transcriptional activator of CDCA7 (19), the levels of endogenous CDCA7 mRNA and protein decreased in ZBTB24 mutant cells (Figure 2, A and B, and Supplemental Figure 3), and were restored by the expression of WT ZBTB24 (Supplemental Figure 4, A and B).

During the establishment of the mutant cells, we recognized their poor proliferation. The doubling times of ZBTB24, CDCA7, HELLs, and Ku80 mutant cells were 1.3–1.4-fold longer than that of WT cells (Figure 3A). DNM3T3B mutant cells were less affected. Restoration of WT proteins in DNM3T3B and CDCA7 mutant cells, but not in HELLs or ZBTB24 mutant cells, recovered the proliferation rate (Supplemental Figure 4, A and C). This suggests that irreversible changes might have occurred in HELLs and ZBTB24 mutant cells (see later). In all mutant cells, the sub-G1 population (less than 3n), corresponding to the apoptotic fraction, and the aneuploid population with excessive DNA content (greater than 6n) increased significantly (Figure 3B). The existence of apoptotic cells and aneuploid cells was confirmed by a TUNEL assay and metaphase spreads (Figure 3C and Supplemental Figure 5A). The latter examination also revealed that the chromosomes were significantly stretched and fragile (Supplemental Figure 5A). Possible chromosome fusions were also detected (Supplemental Figure 5A).

In CDCA7, HELLs, and Ku80 mutant cells, the size of the nuclei and number of centrosomes were significantly increased, with frequent appearance of micronuclei and giant aggregated centrosomes.
The frequency of anaphase bridges was also increased (Figure 4C), suggesting that CDCA7, HELLS, and Ku80 are required for proper chromosome segregation. These results are consistent with the previously reported abnormal centromere number in HELLS mutant cells and increased chromosomal aberrations observed in Ku80 mutant cells (28, 40, 41). Although less frequent and prominent, DNMT3B and ZBTB24 mutant cells also showed similar aberrations (Figure 4, A–C).

Mutations in ICF genes cause accumulation of γH2AX. The phenotype of the mutant cells described above was consistent with the presence of a DNA repair defect. Strikingly, in the absence of DNA-damaging reagent, all mutant cells, including Ku80 mutant cells, accumulated γH2AX signals (Figure 5, A and B), a marker of DSB, particularly in the centromeric, pericentromeric, and telomeric regions (Supplemental Figure 6, A and B). This was observed in cells not undergoing apoptosis (see Figure 5 legend and ref. 42). If the telomeric regions did accumulate DSBs in Ku80 mutant cells, as suggested by the strong γH2AX signals, this might explain the reported role of this protein in telomere maintenance (43). Importantly, the γH2AX accumulation was reversed upon restoration of WT proteins in all mutant cells (Figure 5, A and B). The predominant γH2AX signals in satellite repeats suggest the importance of the ICF proteins in maintaining the integrity of such regions.

We next examined whether γH2AX accumulation is observed in lymphoblastoid cells from ICF patients. The patients included an ICF1 patient with heterozygous DNMT3B mutations (Q42X/R832Q) (P1), ICF2 siblings homozygous for a ZBTB24 mutation (H132Q fsX19/H132Q fsX19) (P1 and P2), an ICF3 patient homozygous for a CDCA7 mutation (G294V/G294V) (P3), and an ICF4 patient homozygous for a HELLS mutation (L801del/L801del) (P4) (ref. 15, 17, 44, 45, and Figure 5C). All these lymphoblastoid cells, especially those from the ICF3 patient, proliferated poorly and frequently died (Supplemental Figure 7, A–D). Like mutant HEK293 cells, lymphoblastoid cells from ICF2, ICF3, and ICF4 patients showed γH2AX accumulation (Figure 5, D and
E). Although less significant, lymphoblastoid cells from the ICF1 patient also accumulated γH2AX signals.

**CDCA7 and HELLS contribute to C-NHEJ.** The results so far suggest that ICF proteins may have a role in DSB repair. Considering the coimmunoprecipitation of Ku80, Ku70, and PRKDC with CDCA7 (Table 2 and Figure 1), C-NHEJ, the major DSB repair pathway, is likely affected. Thus, we examined the involvement of CDCA7 and HELLS in C-NHEJ. We established 3 independent HEK293 clones with stable integration of a C-NHEJ reporter possessing two I-SceI homing endonuclease recognition sites (pIRES-TK-GFP-dA3-1) (ref. 46 and Figure 6A, left). As expected, I-SceI expression decreased the uncut DNA and increased the joined DNA in all clones (Figure 6A, right). In this setup, successful C-NHEJ would excise the herpes simplex virus–thymidine kinase gene, leading to expression of enhanced green fluorescent protein (EGFP). We treated the cells with siRNAs against CDCA7 or HELLS (Figure 6B) and evaluated the C-NHEJ activity by counting EGFP-positive cells. In all clones, CDCA7 and HELLS knockdown respectively compromised C-NHEJ (Figure 6, C and D), indicating that CDCA7 and HELLS are positively involved in C-NHEJ at 3 independent genomic sites. Although the dominant repair pathway can vary during the cell cycle (47, 48), the cell cycle profiles were grossly normal in all knockdown cells except HELLS–double knockdown cells, which showed a slightly increased early S population (Figure 6E).

We then treated ICF mutant cells with an inhibitor of C-NHEJ (a DNA-PK inhibitor, NU7026) to examine the extent of the additional effect on cell viability. The reduction in viability in compar-
ison with untreated cells was not appreciably different in WT, ICF mutant, and Ku80 mutant cells (Supplemental Figure 8A), suggesting a compensation by the other DSB repair mechanisms including homologous recombination (HR). We also treated the cells with a poly(ADP-ribose) polymerase (PARP) 1/2 inhibitor (olaparib), which induces apoptosis in HR-deficient cells at a high frequency (49). The ICF mutant cells were insensitive to the inhibitor, as were WT and Ku80 mutant cells (Supplemental Figure 8B), suggesting that HR is preserved. In addition to the 2 inhibitors, we also treated the mutant cells with mitomycin C (MMC) and temozolomide (TMZ). MMC causes DNA interstrand cross-links, which can be repaired by the Fanconi anemia pathway, HR, nucleotide excision repair, and/or translesion synthesis (50). The ICF mutant cells were similarly sensitive to this compound compared with WT and Ku80 mutant cells (Supplemental Figure 8C). TMZ generates alkyl adducts to DNA, which are removed by O6-methylguanine methyl-
transf erase or base excision repair (51). The TMZ sensitivity of the ICF mutant cells was also similar to that of WT and Ku80 mutant cells (Supplemental Figure 8D). Taken together, the data indicate that C-NHEJ is affected in ICF mutant cells, but other DNA repair pathways, including HR, are largely intact.

CDCA7 and HELLS facilitate accumulation of Ku80 at DSBs. Because chromatin remodeling is required for DSBR repair (36, 37, 52–56), we hypothesized that the CDCA7/HELLS chromatin remodeling complex (15) might be involved in the recruitment of Ku80 to DSB sites. The recruitment of GFP-Ku80 was examined in CDCA7 and HELLS mutant cells by live-cell imaging after laser microirradiation (56). While we observed prompt and strong accumulation of GFP-Ku80 at DNA damage sites (presumed DSBs) within 10 seconds in WT cells, the accumulation was significantly delayed and attenuated in CDCA7 and HELLS mutant cells (Figure 7A–D). This suggests that CDCA7 and HELLS contribute to C-NHEJ by facilitating the accumulation of Ku80 at DSBs.

Defect in C-NHEJ alone does not induce CG hypomethylation of satellite repeats. To explore whether CG hypomethylation of satellite repeats in ICF cells involves defective C-NHEJ, we examined the CG methylation levels of centromeric satellite-2 repeats in ICF mutant cells with or without restoration of WT proteins. The restored mutant cells were designated rescue (Resc. plus clone number). Nuclei were stained with DAPI (blue). The exposure time was fixed for all images. Scale bars: 50 μm. (B) Percentage of cells with at least 10 γH2AX foci (mean ± SEM). The total cell number examined (n) is shown in parentheses. Apoptotic cells identified by DNA fragmentation were removed from the count. *P < 0.0017 (Mann-Whitney U test) was considered statistically significant at the 1% level after Bonferroni correction. (C) Family trees of ICF patients from whom the lymphoblastoid cells were derived (15, 17, 65). (D) Representative images from 5 images for each clone except for ICF3 (pc), of which only 2 images were available, showing accumulation of γH2AX signals (green) in lymphoblastoid cells from 2 healthy volunteers (HEV0190 and L1) and 5 ICF patients. Scale bars: 20 μm. (E) Percentage of cells with at least 10 γH2AX foci (mean ± SEM). The total cell number examined (n) is shown in parentheses. Statistical analysis was based on a comparison with control cells. *P < 0.0033 (Mann-Whitney U test) was considered statistically significant at the 5% level after Bonferroni correction. NA, not applicable.

Discussion

It was recently reported that CDCA7 knockdown impairs chromatin recruitment of HELLS in HeLa cells and that Xenopus Cdc7e stimulates the chromatin remodeling activity of Hells through direct interaction (20). In the present study, in addition to HELLS, core histones including γH2AX, the C-NHEJ proteins Ku80 and Ku70, and other chromatin-related proteins coimmunoprecipitated with CDCA7 in HEK293T cells. HEK293 cells carrying mutations in ICF genes displayed poor proliferation, abnormal chromosome segregation, increased apoptotic and aneuploid cells, and accumulation of γH2AX, all of which are attributable to impaired DSBR repair (58–60). The cellular defects were shared by lymphoblastoid cells derived from ICF patients to varying degrees. Importantly, reporter assays showed that CDCA7 depletion as well as HELLS depletion compromised the C-NHEJ activity of the cells. Furthermore, live-cell imaging after laser microirradiation revealed that accumulation of Ku80 at DSBs was significantly delayed and attenuated in CDCA7 and HELLS mutant cells, suggesting that these proteins promote C-NHEJ through facilitation of Ku80 recruitment.

The above findings indicate a general C-NHEJ defect in ICF mutant cells. The various repeat-associated defects observed in ICF mutant cells may reflect their exceptional susceptibility to DNA breaks and/or specific dependency on C-NHEJ for maintaining the integrity of the repeats. In the absence of Ku80, alternative repair pathways such as the HR pathway substitute for the defective C-NHEJ (61). Our experiments using small-molecule inhibitors suggest that HR is functional in ICF mutant cells. Since HR involves strand exchange between homologous sequences, predominant DSB repair by this mechanism could cause structural instability of satellite repeats, which is a hallmark of ICF cells (1, 3, 10, 11, 62). DNA replication stress may be one cause of DSBs in satellite repeats, as they are well-known hot spots (62, 63). Since Ku80 mutant cells as well as ICF mutant cells show anaphase chromosome bridges, the defect in C-NHEJ appears to cause chromosome missegregation likely due to HR-mediated DNA rearrangements at these repeats. We speculate that this chromosome segregation error further induces apoptosis and chromosome breaks.

A recent study showed that CDCA7 directly binds DNA and that this binding is disrupted by an ICF3 mutation within the zinc finger domain (R274C) (18). We found that the same mutation affects the coimmunoprecipitation of core histones including cell divisions (Figure 8, A and B). The reduction was progressive (compare data at 2 and 4 months) and was more drastic in satellite-2 than in α-satellite repeats. In contrast, the CG methylation levels of these repeats were not affected in Ku80 mutant cells, suggesting that a C-NHEJ defect alone is not sufficient to induce CG hypomethylation (Figure 8, A and B). The CG methylation level of α-satellite repeats was recovered by the expression of the corresponding WT proteins in ZB TB24, CDCA7, and HELLS mutant cells (Figure 8A). In contrast, the CG methylation level of satellite-2 repeats was not recovered in any mutant cells (Figure 8B). This suggests the difficulty in recovering a normal epigenetic state of the pericentromeric repeats. These findings suggest that the molecular basis of CG methylation regulation is different between α-satellite and satellite-2 repeats.
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[Figure 6: CDCA7 and HELLS are involved in C-NHEJ. (A) Schematic representation of the C-NHEJ assay (46) (left), and detection of uncut and joined DNA after 2 days of I-SceI expression in three HEK293 clones carrying a C-NHEJ reporter (pIRES-TK-EGFP-dA3-1) (right). PCR was performed using genomic DNA from cells with (+) or without (-) I-SceI expression. CMV, cytomegalovirus promoter/enhancer; TK, herpes simplex virus–thymidine kinase; IRES, internal ribosome entry site; pA or polyA, polyadenylation signal. (B) Representative blots from biological duplicate showing confirmation of CDCA7 and HELLS knockdown. The stable clones were treated with indicated siRNAs, cultured for 2 days, and subjected to Western blotting. (C) Representative data from biological triplicate and technical triplicate (n = 9) showing flow cytometric analysis of EGFP-positive cells. Two days after siRNA treatment, cells were transfected with pCBASceI, cultured for an additional 2 days, and subjected to the analysis. Percentage of EGFP-positive cells is indicated. SSC, side scatter. (D) Relative frequency of EGFP-positive cells in the knockdown cells shown in C (mean ± SEM, n = 9). The percentage of EGFP-positive cells in I-SceI– cells (background) was subtracted from that in I-SceI+ cells or I-SceI expressed cells. **P < 0.0017 (Mann-Whitney U test) was considered statistically significant at the 1% level after Bonferroni correction. (E) Cell cycle profiles of the knockdown cells. The stable clones were treated with a siRNA(s), cultured for 4 days, and subjected to flow cytometric analysis.]

γH2AX and C-NHEJ proteins. Thus, CDCA7 likely interacts via the zinc finger domain with nucleosomal DNA, which is resistant to benzonase. The coimmunoprecipitation of the C-NHEJ proteins was sensitive to benzonase (but not to RNase A), suggesting their direct association with DSB sites and indirect association with CDCA7 via nucleosome-free DNA. As described above, CDCA7 recruits HELLS to chromatin and stimulates the chromatin remodeling activity of HELLS through direct interaction (20). Therefore, it is tempting to speculate that the CDCA7/HELLS complex facilitates Ku80 recruitment to DSB sites through its chromatin remodeling activity, although how this occurs remains an open question.

The defect in the C-NHEJ pathway in Ku80 mutant cells was not sufficient to induce centromeric or pericentromeric CG hypomethylation, suggesting that the hypomethylation phenotype of ICF patients is not caused by the C-NHEJ defect. We observed that centromeric and pericentromeric repeats lose and gain CG methylation with different kinetics upon depletion and restoration.
of ICF proteins in HEK293 cells, respectively. Together with the fact that DNMT3B mutations (ICFI) affect only pericentromeric repeats (7–9, 12), this suggests that different mechanisms establish and/or maintain CG methylation in centromeric and pericentromeric repeats. The CDCA7/HELLS complex is perhaps a common component required for both repeats, and DNMT3B is a downstream effector specific to pericentromeric repeats. The nucleosome remodeling activity of the CDCA7/HELLS complex might also be required for recovering CG methylation in repaired DNA segments as part of the postrepair process (20–26).

Finally, it is known that B cells from ICF patients undergo apoptosis after in vitro activation (2) and that both T and B cells are significantly reduced in the peripheral blood of Hels mutant mice (30). Consistent with these reports, our mutant HEK293 cells and ICF lymphoblastoid cells showed poor proliferation and increased apoptosis. However, since C-NHEJ proteins are also involved in V(D)J and class switch recombination (33–35), and since impaired IgE class switch induction has been reported in a non-ICF1 patient (2), it would be interesting to investigate whether ICF3 and ICF4 immune cells have a defect in such cellular processes.

In conclusion, our results suggest that the defect in C-NHEJ may account for some of the common features of ICF cells, including instability of satellite repeats, abnormal chromosome configuration, poor proliferation, and apoptosis. Further studies are required to understand how the C-NHEJ defect is related to the CG hemihypomethylation and immunological defects of ICF syndrome.

Methods

Cells. A lymphoblastoid cell line from a healthy female (HEV0190) was purchased from the RIKEN Cell Bank (64), and a line from an ICF1 patient (PI) was obtained from a cell bank maintained in Saitama Children’s Medical Center (44). Details of the other lymphoblastoid cell lines from a healthy male (L1) and 4 ICF patients (pD, pV, pC, and pU) are described elsewhere (15, 17, 65). The lymphoblastoid cell lines were maintained in RPMI 1640 supplemented with 20% FBS and penicillin/streptomycin at 37°C in a 5% CO2 incubator. HEK293 and HEK293T cells were obtained from the American Type Culture Collection and maintained in DMEM supplemented with 10% FBS and penicillin/streptomycin at 37°C in a 5% CO2 incubator. HEK293T cells were used for transient expression of FLAG-CDCA7_WT, FLAG-CDCA7_R274C, FLAG-HELLS_WT, and FLAG-HELLS_Q699R. HEK293 cells were used to generate stable clones by transfection of plasmids and DNMT3B, ZBTB24, CDCA7, HELS, and Ku80 mutant cells using the CRISPR/Cas9 system.

Stable HEK293 clones with an approximately endogenous level of FLAG-CDCA7_WT and FLAG-CDCA7_R274C were established by transfection of the expression plasmid vector of each protein (p3xFLAG-CMV-10). After selection using Geneticin (600 μg/ml; Gibco) for a month, 3 clones were established (Figure 6A).

For derivation of DNMT3B, ZBTB24, CDCA7, HELS, and Ku80 mutant cells, a single guide RNA per each gene was designed using the CRISPR Design Tool (http://crispr.mit.edu/) (66). The sequences were: DNMT3B exon 6, 5′-GAGACGGCGGGCAACAGCAT-3′; ZBTB24 exon 4, 5′-GGATCAATGGCGGAATAT-3′; CDCA7 exon 7, 5′-TCAGAAGATGGTACAC-3′; HELS exon 19, 5′-GAACCCCGGCGAATCCGTC-3′; and Ku80 exon 6, 5′-GATGGCCCTTTCTGCTTATG-3′. The guide sequences were cloned into pX330-U6-Chimeric BB-CBh-hSpCas9 plasmid vectors (67) and cotransfected with the CRISPR Design Tool (http:/crispr.mit.edu/) (66). The mutant cells, a single guide RNA per each gene was designed by transfection of the expression plasmid vector of each protein (p3xFLAG-CMV-10). After selection using Geneticin (600 μg/ml), several clones were established by transfection with pIRES-TK-dA3-1 reporter plasmid and pIRES-TK-dA3-1 reporter plasmid were gifts from Niels Mailand (University of Copenhagen, Copenhagen, Denmark), Ayako Ui (Tokyo University of Technology, Tokyo, Japan), and Takashi Kohno (National Cancer Center Research Institute, Tokyo, Japan). pCBAsceI (68, 69) was purchased from Addgene (catalog 26477). The antibodies used in this study are summarized in Supplemental Table 6. We used 2 anti-Ku80 antibodies. One (Abcam, catalog ab79391) was for detection of both WT and mutant Ku80 (K183E/G184R), and the other (Abcam, catalog ab119935) was for detection of WT Ku80.

Cell viability assay using small-molecule compounds. WT or mutant HEK293 cells (1 × 10⁴) were seeded into 96-well plates and cultured in 50 μl DMEM supplemented with 10% FBS and penicillin/streptomycin overnight. Next day, 50 μl of the medium containing 2 times the final concentration of NU7026 (Wako, catalog 144-09651), olaparib (LKT Laboratories, catalog O4492), MMC (Nacalai Tesque, catalog 20898-21), or TMZ (Wako, catalog 206-19991) was gently added into each well and cultured for 48 hours. Then, 10 μl of Cell Count Reagent SF (Nacalai Tesque, catalog 07553) was added to each well and incubated for 1 hour, and the absorbances at 450 and 600 nm (for calibration) were measured using a Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific). Normalization of cell viability was done using the values obtained from untreated cells for each genotype. The assay was performed in biological triplicate and technical triplicate, and P values were determined by Welch’s t test.

Immunoprecipitation. HEK293T cells were harvested after 48 hours of transfection and lysed in 0.1% NP-40 lysis buffer (150 mM NaCl, 0.1% NP-40, and 50 mM Tris-HCl, pH 8.0). Cells were homogenized by sonication (until DNA fragments were <1.4 kb) and incubated

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on ice for 30 minutes. The supernatant was collected by centrifugation. Benzonase nuclease (Sigma-Aldrich), which digests all forms of DNA and RNA (except for DNA on mononucleosome) under various conditions (pH 6–10, 0°C–42°C), was added to the supernatant (≥250 U/sample) throughout the following steps (total incubation time ≥2 hours), if required. Precleaning was performed by addition of Protein A/G PLUS-Agarose (Santa Cruz Biotechnology) and normal mouse IgG (Santa Cruz Biotechnology) to the supernatant. After 1 hour of rotation at 4°C, the precleaned samples were collected and immunoprecipitation was performed using FLAG M2 affinity agarose gel (Sigma-Aldrich) for 1 hour at 4°C. After washing of the agarose with the lysis buffer 5 times, immunoprecipitated proteins were eluted using 3× FLAG peptides (Sigma-Aldrich) and subjected to Western blotting or mass spectrometry analysis. As a control, protein lysate from untransfected HEK293T cells was prepared and the same assay was performed.

Silver staining and mass spectrometry analysis. Eluted samples were dissolved in SDS sample buffer, fractionated by SDS-PAGE on an 8% gel, and stained with silver. Individual lanes of the stained gel were sliced into 13 pieces, and proteins within the pieces were subjected to in-gel digestion with trypsin. The resulting peptides were subjected to liquid chromatography–tandem mass spectrometry analysis with an Orbitrap Velos Pro ion-trap mass spectrometer (Thermo Fisher Scientific). The obtained data sets were analyzed using Mascot Daemon (Matrix Science). As a control, a protein lysate from untransfected HEK293T cells was prepared and the same assay was performed. Ribosome and proteasome components were removed from the analysis.

qPCR. Primers used for qPCR are summarized in Supplemental Table 6. PCR reactions were performed using a Thermal Cycler Dice Real Time System Single (TaKaRa Bio) according to the manufacturer’s protocol. Amplification conditions were 30 seconds at 95°C and then 40 cycles each consisting of 5 seconds at 95°C and 30 seconds at 60°C. The ACTB mRNA level was used for normalization. The mRNA abundance (ΔCt) of each gene was calculated by comparison with that of ACTB and shown as percentage relative expression level (2−ΔCt × 100).

Immunofluorescence. Immunofluorescence staining was performed as follows, except for metaphase chromosome spreads. Adherent cells were seeded in chamber slides 1 day before immunofluorescence, and lymphoblastoid cells were embedded in Smear Gel (GenoStaff) on a glass slide. The cells were fixed with 4% paraformaldehyde (PFA) for 30 minutes, permeabilized with 0.5% Triton X for 30 minutes, and blocked with Block Ace (DS Pharma Biomedical) for 1 hour at room temperature. Cells were incubated with the indicated antibodies for 1 hour at room temperature. After washing with PBS, cells were incubated with CF488 donkey anti-mouse IgG (H + L) antibody (Biotium, catalog 20014; 1:1,000) for 1 hour at room temperature. Nuclei were visualized using VECTASHIELD Antifade mounting medium containing DAPI (Vector Laboratories). Fluorescent images were taken using an LSM700 confocal laser scanning microscope (Carl Zeiss). The area of nuclei (μm²) and length of chromosomes (μm) were measured using the ZEN 2012 SP5 (black edition) software program (Carl Zeiss).

γH2AX, Giemsa, and DAPI staining of metaphase chromosome spreads. γH2AX staining of metaphase spreads was performed as described previously (11) with minor modifications. Briefly, cells were treated with KaryoMAX Colcemid (Thermo Fisher Scientific, catalog 15212012) in culture medium (final concentration, 0.04 μg/ml) for 50 minutes. Cells were then harvested and incubated in hypotonic buffer (0.2% KCl, 0.2% sodium citrate) for 30 minutes at room temperature. For Giemsa (Nacalai Tesque) or DAPI (Vector Laboratories) staining, cells were fixed with Carnoy’s solution, dropped on glass slides, dried on a heat block at 50°C, and stained. For γH2AX staining, unfixed cells were dropped on glass slides, fixed with 4% PFA for 30 minutes at room temperature, rinsed in water, and permeabilized with KCM buffer (120 mM KCl, 20 mM NaCl, 10 mM Tris, pH 7.5, 0.1% Triton X) for 10 minutes. The slides were blocked with 5% BSA (Sigma-Aldrich) for 1 hour at room temperature and incubated with anti-γH2AX mouse monoclonal antibody for 1 hour at 37°C. After washing of the slides with PBS, the cells were incubated with CF488 donkey anti-mouse IgG (H + L) antibody (Biotium, catalog 20014; 1:1,000) for 1 hour at 37°C. Chromosomes were stained with VECTASHIELD containing DAPI. Fluorescent images were taken using an LSM700 confocal laser scanning microscope.

TUNEL assay. Apoptotic cells were detected using the ApopTag kit (Merck) and fluorescence microscopy according to the manufacturer’s protocol.

Cell cycle analysis. Cells were grown in a 60-mm dish or 24-well plate, harvested, and treated with Cycle TEST PLUS (BD Biosciences) according to the manufacturer’s protocol. For flow cytometric analysis, FACScalibur (BD Biosciences) was used. The percentage of nuclei in G1, S, and G2/M phases of the cell cycle, and any sub-G1 population or polyploidy, were determined from 20,000 ungated cells.

C-NHEJ assay. Three HEK293 dA3-1 clones (nos. 5, 6, and 11) were transfected with a control siRNA or siRNAs against CDC7 or HLLS mRNA (final concentration 60 nM) using Lipofectamine RNAiMax (Invitrogen) and cultured for 2 days in puromycin-free media. The siRNAs (all from Sigma-Aldrich) included a universal negative control, SIC-001-25; CDC7, SAS_Hs01_00212366 (si-1) and SAS_Hs01_00212367 (si-2); and HLLS, SAS_Hs01_0026584 (si-1) and SAS_Hs01_0026585 (si-2). The cells were transfected with or without pCBASecl, together with siRNAs, or with pmxGFP (Lonza) alone, using Lipofectamine 2000 (Invitrogen), cultured for an additional 2 days, fixed with 3% formaldehyde, and subjected to flow cytometric analysis using FACSVerse (BD Biosciences). EGFP-positive cells were determined from 10,000 un gated cells. We used pmxGFP-transfected cells for the evaluation of the transfection efficiency, which always exceeded 90%. All assays were performed in biological triplicates, with each in technical triplicate. We subtracted the number of EGFP-positive cells transfected with siRNA(s) alone as
Laser microirradiation and live-cell imaging. We transfected WT, CDCA7 KO4, and HELLS KO11 cells with a GFP-Ku80 plasmid and established stable clones after Geneticin (Santa Cruz Biotechnology) selection for 2 weeks (600 μg/ml). Then the cells with stable expression of GFP-Ku80 were seeded in chambered coverglass (Lab-Tek II, Merck), cultured for 16 hours, and sensitized for DNA damage by treatment with 10 μg/ml Hoechst 33342 (PromoKine) for 10 minutes. Laser microirradiation was performed using an LSM700 confocal scanning a background, which were thought to be autofluorescent cells, from EGFP-positive cells transfected with the same siRNA(s) with I-SceI. PCR analysis was done with genomic DNA extracted from cells 2 days after I-SceI expression. The locations and sequences of primers used for the PCR were described in a previous report (ref. 46; Figure 6A, left, and Supplemental Table 6). The PCR amplification condition was 30 seconds at 98°C and then 28 cycles each consisting of 15 seconds at 98°C, 15 seconds at 56°C, and 30 seconds at 72°C.

Figure 8. The C-NHEJ defect alone does not cause CG hypomethylation at satellite repeats. (A and B) CG methylation (percent) at centromeric α-satellite (A) and pericentromeric satellite-2 repeats (B) in mutant cells. The methylation levels were measured by bisulfite sequencing (PCR products were cloned into TA vector and at least 10 clones were sequenced for each condition) at 2 and ≥4 months after the introduction of the mutation. Because the doubling time of the mutant cells was 25–30 hours (Figure 3A), 2 and ≥4 months roughly corresponded to 48–58 and ≥96–116 cell divisions, respectively. Restoration of the methylation levels in the “rescue” clones was evaluated at 6 weeks after the introduction of WT genes. Data are mean ± SEM. Statistical analysis was performed separately for the data obtained at 2 and ≥4 months and the data obtained after 6 weeks of WT restoration. For the former data sets, **P < 0.0007 (Mann-Whitney U test) was considered statistically significant at the 1% level after Bonferroni correction. For the latter data set, **P < 0.0004 (Mann-Whitney U test) was considered statistically significant at the 1% level after Bonferroni correction. The exact P values, which were significant (P < 0.05) before the correction, are shown for reference. See also Supplemental Figure 9.
laser microscope. At least 60 cells were microirradiated with a 405-nm pulse laser (the irradiation iteration fixed at 20 scans) for each genotype, and the kinetics of GFP-Ku80 accumulation was recorded every 0.5 seconds. The signal intensities (mean region of interest) in the damage region (region-1) and background region (region-2) adjacent to the damage region with the same size) were measured using the ZEN 2012 SP5 (black edition) software program (Carl Zeiss). Time required for accumulation of GFP-Ku80 (seconds) was determined by the time point at which the signal intensity in the damage region reached 1.2-fold that of the background. Fold change (region-1/region-2) was calculated every 0.5 seconds after laser microirradiation, and the maximum fold change during the first 30 seconds was determined.

**DNA methylation analysis.** Genomic DNA was subjected to bisulfite conversion as previously described (70). PCR was performed using primers shown in Supplemental Table 6 with ExTaQ Hot Start DNA Polymerase (TaKaRa Bio) for satellite-2 and with KAPA2G Robust HS RM with dye (KK5706; Nippon Genetics) for α-satellite. PCR products were electrophoresed, purified using a QiAquick Gel Extraction Kit (Qiagen), and cloned into T-vector pMD20 (TaKaRa Bio). More than 12 clones for each genotype or condition were sequenced using an Applied Biosystems 3730 or 3130xl genetic analyzer (Life Technologies). DNA methylation patterns were visualized using a quantification tool for methylation analysis (QUMA) (71). The bisulfite conversion rate was evaluated by the conversion rate at non-CG sites in the same PCR fragment, which always exceeded 99%.

**Statistics.** Mann-Whitney U test, Student’s t test (2-tailed), or Welch’s t test were performed for statistical analysis based on sample numbers and data distributions. Bonferroni correction was applied for multiple comparisons. Significant P values at the 5% and 1% levels for each analysis are described in figure legends.

**Study approval.** All clinical samples were obtained in an anonymized manner, and written informed consent was received from participants prior to inclusion in the study. The study was approved by the Kyushu University Institutional Review Board for Human Genome/Gene Research (no. 599-01) and the local ethics committee of Necker-Enfants Malades Hospital, Paris, France.

**Author contributions**
MU designed the research, conducted all experiments, analyzed data, and wrote the manuscript. GV and CF provided lymphoblastoid cell lines. HF and HS interpreted data and wrote the manuscript.

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